# Investigating the reversibility and tissue specificity of mitochondrial disorders

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## Abstract

Mitochondrial disorders comprise a large group of heterogeneous disorders which are characterized by impairments in the cellular energy production. One of the great challenges of mitochondrial disease is the variety of clinical features present in patients. Mitochondrial disorders affect more than one organ leading to complex multisystem dysfunctions. Tissues, in which the metabolic demand is higher, such as skeletal muscle, neurons, liver or heart are typically affected.

Mutations in both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) often lead to mitochondrial disorders. Although mtDNA encodes key proteins for the normal function of the mitochondrial respiratory chain enzymes, the vast majority of the essential components and proteins needed for the maintenance and replication of the mitochondrial DNA are encoded by the nDNA.

Exome sequencing in combination with bioinformatics tools has proven really effective in determining novel alterations in the genomic sequence. One aim of this thesis was to evaluate novel mutations from affected patients with combined respiratory deficiencies. As a result, mutations in *C12orf65* and in the novel disease gene *MiD49,* associated with mitochondrial disorders, are thoroughly presented.

Vitamin supplements, pharmacological agents and exercise therapy are common strategies used in patients suffering from mitochondrial disorders. It has been shown that in cell lines of patients suffering from two rare reversible infantile mitochondrial diseases (reversible infantile respiratory chain deficiency and reversible infantile hepatopathy due to TRMU deficiency) supplementation of L-cysteine resulted in an improvement in most respiratory chain complexes activities. During my PhD I studied and proved that L-cysteine supplementation was also beneficial in cells from patients suffering from common forms of mitochondrial disorders such as MELAS and MERRF as the supplementation resulted in improved mitochondrial respiratory chain function.

Finally, direct conversion of fibroblasts to neuronal progenitor cells was used to model mitochondrial disorders and study the tissue specificity. This project was very challenging due to the complex characteristics of mitochondrial biology.

In summary, this thesis reveals the description of novel genes and mutations associated with combined mitochondrial deficiencies. Furthermore, we detected a positive effect of L-cysteine on a subset of mitochondrial disorders, which can be the base of further therapy development.

# Acknowledgements

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## List of Abbreviations

**AAC1:** ADP/ATP carrier protein AAC1

**AAV:** Adeno-associated virus

**Acetyl-CoA:** Acetyl Coexnzyme A

**ACS system:** Alanine-Serine-Cysteine system

**AD:** Alzheimer ’s disease

**ATP:** Adenosine Triphospate

**CAC:** Carnitine-acylcarnitine

**CMT6:** Charcot-Marie Tooth disease type 6

**$CO_2$:** Carbon Dioxide

**CoQ:** Ubiquinone

**CPEO:** Chronic progressive External Ophthalmoplegia

**CR domain:** Codon Recognition domain

**DIC:** dicarboxylate carrier

**D-loop:** Displacement Loop

**EF-$G1_{mt}$:** Elongation factor G1

**EF-$Ts_{mt}$:** Elongation factor Ts

**EF-$Tu_{mt}$:** Elongation factor Tu

**ETC:** Electron Transport Chain

**$FADH_2$:** Flavin Adenine Dinucleotide

**Fe-S:** iron sulfur

**fMet:** formyl-methionine

**GDP:** Guanosine diphosphate

**GGQ motif:** Gly-Gly-Gln motif

**GS:** Glutathione Synthetase

**GSH:** Glutathione

**GTP:** Guanosine 5' triphosphate

**$IF2_{mt}$:** Mitochondrial elongation factor II

**$IF3_{mt}$:** Mitochondrial elongation factor III

**IMM:** Inner Mitochondrial Membrane

**KSS syndrome:** Kearns-Sayre syndrome

**Leu:** Leucine

**LHON:** Leber Hereditary Optic Atrophy

**LTBL:** Leukoencephalopathy with thalamus and brainstem involvement and high lactate

**Lys:** Lysine

**MELAS:** Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes

**MERRF:** Myoclonic epilepsy with ragged-red fibres

**mGSH:** mitochondrial Glutathione

**MNGIE:** Mitochondrial neurogastrointestinal encephalopathy

**mtDNA:** Mitochondrial DNA

**mTERF1:** Mitochondrial Termination Factor 1

**mtRF1a:** Mitochondrial translational factor 1a

**mtSSBP:** Mitochondrial single stranded DNA-binding protein

**NAC:** N-acetyl-cysteine

**NADH:** Nicotinamide Adenine Dinucleotide

**NCR:** Non-coding region

**OGC:** 2-oxyglutarate carrier

**OMM:** Outer Mitochondrial Membrane

**OXPHOS:** Oxidative Phosphorylation

**POLMRT:** Mitochondrial RNA polymerase

**PTH domain:** peptidyl tRNA hydrolase domain

**RFs:** Release Factors

**RIRCD:** Reversible Infantile Respiratory Chain Deficiency

**RNS:** Reactive Nitrogen Species

**ROS:** Reactive Oxygen Species

**RRF1mt:** Mitochondrial ribosome releasing factor 1

**RRF1mt:** Mitochondrial ribosome releasing factor 2

**rRNA:** ribosomal RNA

**SPG55:** Hereditary Spastic Paraplegia

**TALEN:** Transcription activator-like effector nucleases

**TEFM:** Mitochondrial transcription elongation factor

**TFAM:** Mitochondrial Transcription Factor A

**TFB2M:** Mitiochondrial transcription factor B2

**tRNA:** Transfer RNA

**ZFN:** Zinc Finger Nuclease

**γ-GCS:** γ-glutamylcysteine

# Chapter 1. Introduction

## 1.1 Mitochondrion

Mitochondria are essential for maintaining fundamental aspects of physiology such as the cellular energy balance, the modulation of calcium signalling, the cellular redox balance and the normal function of a number of significant biosynthetic pathways. Maintenance of a healthy mitochondrial population demands a complex system of quality control. Mechanisms impairing a system of the quality control lead to disordered cell function which manifests as disease. Moreover, mitochondria play a vital role in cell life and death as they regulate both apoptotic and necrotic cell death. Therefore, any defect in cell death might lead to inappropriate tissue growth and development of tumours. The last years, the centrality of mitochondrial dysfunction in a wide range of major human diseases is slowly becoming recognized emerging for novel therapeutic approaches for a large group of diseases (Duchen and Szabadkai, 2010).

### 1.1.1 *Origin and Structure*

The mitochondrion is a specialized organelle principally responsible for the production of cellular energy through oxidative phosphorylation (OXPHOS). Mitochondria were first identified in the 19$^{th}$ century and are present in almost all eukaryotic cells. In addition to energy production, mitochondria play vital role in cytosolic calcium-homeostasis (Pozzan and Rizzuto, 2000), in haem biosynthesis, in apoptosis (Newmeyer and Ferguson-Miller, 2003) and lastly in thermogenesis (Lowell and Spiegelman, 2000).

The principal theory illustrating the origin of the mitochondria is the endosymbiotic theory while the 'hydrogen hypothesis' is the most possible scenario of that theory. According to the 'hydrogen hypothesis', the host cell of the mitochondrial endosymbiosis was an anaerobic, strictly hydrogen-dependent and autotrophic archaebacterium whereas the symbiotic was a eubacterium, able to respire, generating molecular hydrogen as a waste product of anaerobic heterotrophic metabolism. The selective force that shaped the common ancestor of eukaryotic cells was the dependence of the host upon the hydrogen production by the symbiont (Martin and Müller, 1998). This partnership gave rise to the mitochondrion and the complex eukaryotic cell. Due to comparative mitochondrial genomics studies, it is believed that the

possible symbiotic eubacterium belonged to the class of α-Proteobacteria (subclass: Rickettsiales).

Mitochondria are usually described as inflexible, elongated cylinders with diameter of 0.5-1μm. However, live cell imaging techniques discovered that mitochondria are exceptionally mobile and plastic organelles.

Each mitochondrion is bordered by two highly specialised membranes termed outer and inner mitochondrial membrane (OMM and IMM respectively). Both membranes have different functions and separate the mitochondrion in two sections: the internal matrix and the intermembrane space (Figure 1.1).

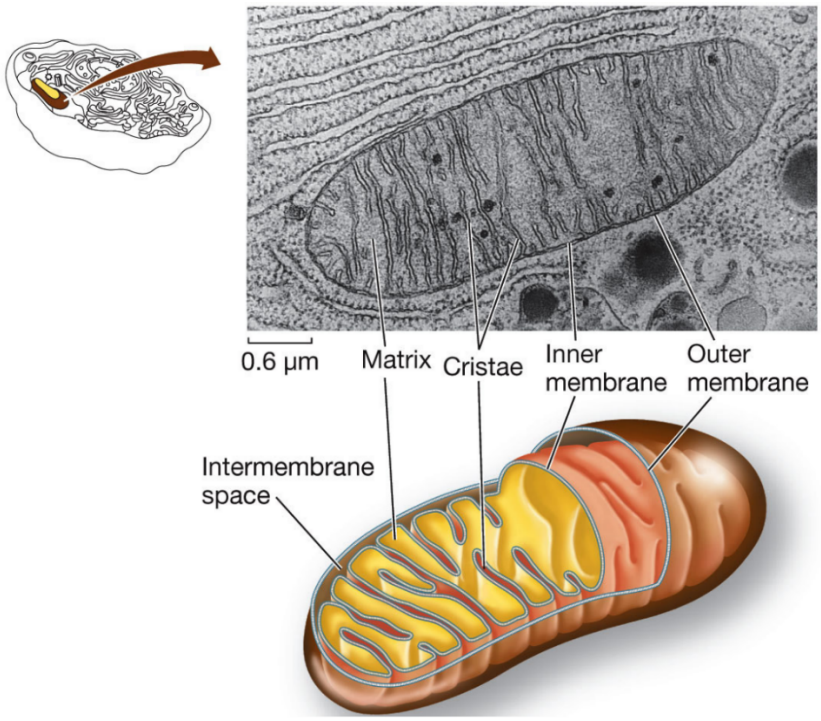



**Figure 1.1: Schematic diagram and electron micrograph of the mitochondria showing the matrix, cristae, inner membrane, outer membrane and intermembrane space. (Source: http://www.sinauer.com/)**

The outer mitochondrial membrane consists of a lipid bilayer characterised by a variety of proteins including a large number of copies of a transport protein, called porin. Porin has the ability to form large aqueous channels that allow the alley of all the molecules less than 5000 Daltons, including small proteins. Although the latter are present in the intermembrane space, only a few of them are allowed through the IMM. Therefore, the intermembrane space is

chemically equivalent to the cytosol in respect to the small molecules but the matrix contains only a few of them.

Similar to the OMM, IMM consists of a lipid bilayer characterised by high proportion of the double phospholipid cardiolipin. Cardiolipin, consists of four fatty acids, and makes the inner membrane highly selective and permeable only to $O_2$, $CO_2$ and $H_2O$ rather than ions and small molecules. Only the small molecules required by the mitochondrial enzymes located in the matrix are allowed to pass through the inner membrane via a variety of transport proteins (Patil and Greenberg, 2013). The IMM is extensively convoluted, forming a series of foldings, also called cristae, that project into the matrix. These foldings increase the area of the inner membrane and the number of the cristae is proportional to the demands of the ATP of the cell. For example, a cardiac muscle contains three times greater number of cristae compared to a liver cell due to higher ATP demands in cardiac cells (Bruce et al., 2002).

### 1.1.2 *Function*

One of the most prominent functions of mitochondria is the ATP production through respiration. Pyruvate molecules produced by glycolysis and fatty acids deriving from fats are actively transported to the inner mitochondrial membrane and converted to acetyl-CoA. The fatty acids are converted to acetyl-CoA through beta-oxidation while the pyruvate dehydrogenase complex is responsible for transforming the pyruvate to acetyl-CoA.

Following, the acetyl-CoA enters the mitochondrial matrix and is oxidized via the citric acid cycle. The citric acid cycle is the main metabolic centre of the cell and takes place in the mitochondrial matrix while glycolysis takes place in the cytosol (Bruce et al., 2002). It includes a series of reduction-oxidation (redox) reactions that result in the oxidation of an acetyl group ($CH_3CO$) to two molecules of $CO_2$. Also, high energy electrons carried by two activated carrier molecules called Nicotinamide Adenine Dinucleotide (NADH) and Flavin Adenine Dinucleotide ($FADH_2$), are generated through the citric acid cycle. Next, these two high energy electron carriers are transferred to the IMM where they enter the electron transport chain. The total yield of citric acid cycle per one molecule of glucose (2 pyruvate molecules) is 6 NADH, 2 $FADH_2$, and 2 ATP.

### 1.1.3 *Electron Transport Chain and Oxidative Phosphorylation (OXPHOS)*

The electron transport chain (ETC) is a series of protein complexes transferring electrons from electron donors to electron acceptors via redox reactions while this electron transfer is coupled with proton ($H^+$) transfer across a membrane. The electron transfer is called oxidative

phosphorylation (OXPHOS) and occurs in almost all aerobic organisms. The OXPHOS results in the formation of an additional 25 molecules of ATP per molecule of glucose, indicating its significant role in the energy production. The site of the ETC, where the OXPHOS takes place, is the inner mitochondrial membrane.

The ETC consists of four protein complexes (Complex I, II, III and IV) and the H+-ATP synthase, the principal enzyme responsible for the ATP synthesis.

Complex I, also known as NADH dehydrogenase, is composed of NADH dehydrogenase, a prosthetic group called Flavin Mononucleotide (FMN) and non-heme-iron proteins having at least one iron-sulfur centre. Complex I consists of 44 protein subunits (Vinothkumar et al., 2014). Two electrons from NADH are transferred to complex I then to FMN and through an iron-sulfur carrier to a lipid-soluble carrier termed ubiquinone (CoQ). Simultaneously, for each electron a hydrogen ion is pumped to the intermembrane space through complex I (Figure1.2).

Next, the electrons carried by CoQ are transferred to complex III. Complex III, known as CoQ-cytochrome reductase, consists of 11 protein subunits. The principal components of complex III are two heme proteins, cytochromes b and c1 and a non-heme-iron protein, known as the Rieske iron sulfur protein. Then, the electrons are transferred from complex III to complex IV via cytochrome c protein. As complex IV accepts one electron at a time from cytochrome c, one hydrogen ion is pumped through complex III as each electron is transferred.

Complex IV, also known as cytochrome c oxidase, is a large transmembrane protein consisting of 13 protein subunits and several metal prosthetic sites in mammals. The major role of complex IV is to transfer four electrons from cytochrome c to $O_2$ and produce two molecules of $H_2O$. In parallel, eight $H^+$ are translocated from the matrix to complex IV where four of them are used for the $H_2O$ production and the other four are pumped to the intermembrane space contributing to maintain the proton gradient across the membrane.

The flow of $H^+$ from the matrix to intermembrane space across the membrane results to:

1. Generation of a pH gradient across the inner mitochondrial membrane with the pH higher in the matrix than in the cytosol
2. Generation of a voltage gradient, termed membrane potential, across the inner mitochondrial membrane. As a result of the outflow of the positive ions, the voltage

gradient inside the inner mitochondrial membrane is negative whereas outside is positive.

That electrochemical proton gradient generated across the inner membrane is the driving force of the ATP synthesis. The ATP is synthesized at complex V, also known as ATP synthase. It consists of two regions; the $F_0$ portion, which is embedded within the inner mitochondrial membrane and the $F_1$ portion, which is outside the inner membrane and projects to the matrix. The overall reaction contributing towards ATP formation is:

$$ADP + P_i + \text{Energy (in the form of } H^+) \rightarrow ATP$$

It is worth mentioning that the transmembrane electrochemical gradient is not only used from the cell to drive the ATP synthesis but also to drive the active transport of selected metabolites across the mitochondrial inner membrane, including the maintenance of the cell's ATP pool highly charged.

Finally, complex II, also known as succinate dehydrogenase, consists of four protein subunits and is involved in both citric acid cycle and oxidative phosphorylation. Main role of complex II is the oxidation of succinate and as a result additional electrons are delivered into the CoQ. The main difference of complex II to complex I is that the transferring of electrons, produced by complex II, is not coupled to proton pumping from the matrix to the intermembrane space. Therefore, the electron transport pathway through complex II produces less ATP compared to complex I electron transport pathway.

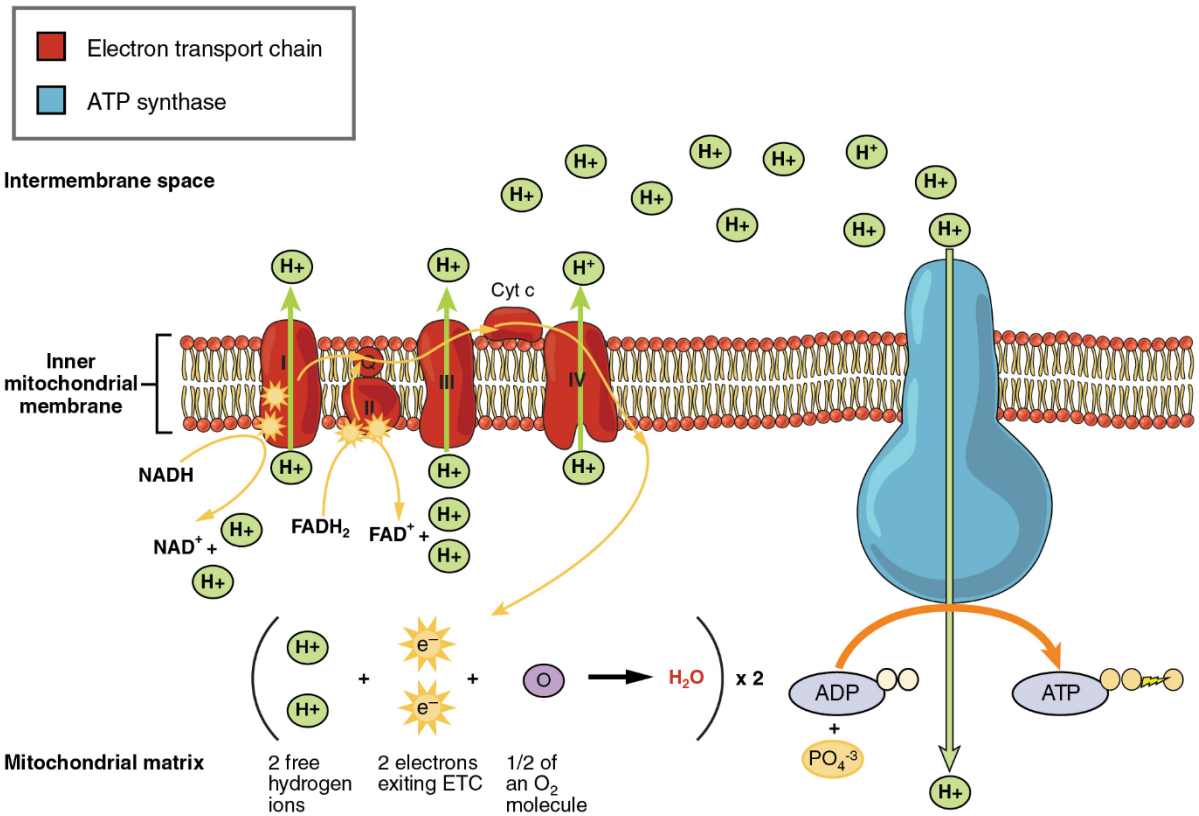



**Figure 1.2: OXPHOS system. NADH transfer hydrogen to Complex I where it binds to ubiquinone (UQ) and it is transferred to Complex III. Electrons are then passed from Complex III to cytochrome c. Cytochrome c is a mobile electron carrier and passes its electrons to Complex IV. Complex IV ultimately reduces molecular oxygen to from water while using the free energy of this process to translocate protons across the inner membrane. Complex V or ATP synthase is responsible for the production of the ATP. (Source: http://cnx.org)**

### 1.1.4 *Reactive Oxygen Species (ROS)*

During the process of oxidative phosphorylation 1-2% of all the electrons pass the respiratory chain and give rise to free radicals, the reactive oxygen species (ROS). ROS tend to damage biological macromolecules such as DNA, lipids and proteins (Raha and Robinson, 2000).

The $F_4S_4$ iron-sulfur clusters are mostly susceptible to superoxide. Therefore, the oxidation of one iron atom causes its release from the iron-sulfur cluster. As a result, complexes I and III and possibly complex II are affected due to the presence of important iron-sulfur clusters in these complexes.

Complexes I, II and III are mainly responsible for the generation of superoxide (Nicholls, 2002). Although inhibition of complexes I and II results in elevated ROS production in submitochondrial particles, it is believed that the major site of ROS production is complex I due to semiquinone (Raha and Robinson, 2000). The semiquione radical is a natural

intermediate and its direct oxidation by oxygen results in superoxide production. However, others believe that the electrons escape from one of the Fe-S clusters and results in the production of ROS.

It has been shown that cells are able to get rid of the ROS species (Halliwell, 1999) effectively. When the superoxide is generated, the cell can convert it to $H_2O_2$ by the enzyme superoxide dismutase ($MnSO_2$) which in turn is converted to $H_2O$ by catalase or glutathione peroxidase. According to previous studies, mice that lack the mitochondrial superoxide dismutase presented with dilated cardiomyopathy and neonatal lethality (Li et al., 1995).

Mice defective in exchanging ADP and ATP across the inner membrane due to lack of the heart isoform of the adenine translocator presented with mitochondrial cardiomyopathy with increased levels of ROS hydrogen peroxide in affected tissues and an increase of ROS detoxification enzymes. Based on these data, it is concluded that ROS production may impair the disease phenotype in the mitochondrial diseases resulting from defects in OXPHOS (Esposito et al., 1999).

## 1.2 Mitochondrial Genetics

### 1.2.1 *Mitochondrial Genome*

The mitochondrion is the only organelle containing extra-nuclear source of genetic material in eukaryotic cells, named mitochondrial DNA (mtDNA). Human mtDNA is a circular, double-stranded, 16 569 base pair molecule of DNA, which consists of 37 genes. The overall base composition of the mtDNA is 44% (G+C) but the two mtDNA strands have totally different composition. Heavy strand (H-strand) is guanine rich while light strand (L-strand) is cytosine-rich. In contrast to the presence of a single copy of nucleus in each cell, mtDNA is a multi-copy DNA and the number of copies in each cell depends on the energy requirements of each tissue (Taylor and Turnbull, 2005).

The mtDNA consists of 37 genes from which 13 encode subunits of the OXPHOS complexes, 2 genes encode ribosomal RNAs (12s and16s) and the remaining 22 genes encode mitochondrial tRNAs. Furthermore, 28 genes are encoded by the H-strand whereas the other 9 by the L-strand. The rest of the proteins needed for the replication, maintenance and metabolism of the mitochondria are encoded by the nuclear genome and are imported from the cytosol to the specific mitochondrial location (Mokranjac and Neupert, 2005). It is estimated that ~1500 proteins, necessary to the mitochondria, are transcribed from nuclear genes, translated in the cytosol and then delivered across the mitochondrial membrane.

Therefore, the mitochondria are also dependent upon the nuclear genome (Chinnery and Hudson, 2013, Stewart and Chinnery, 2015).

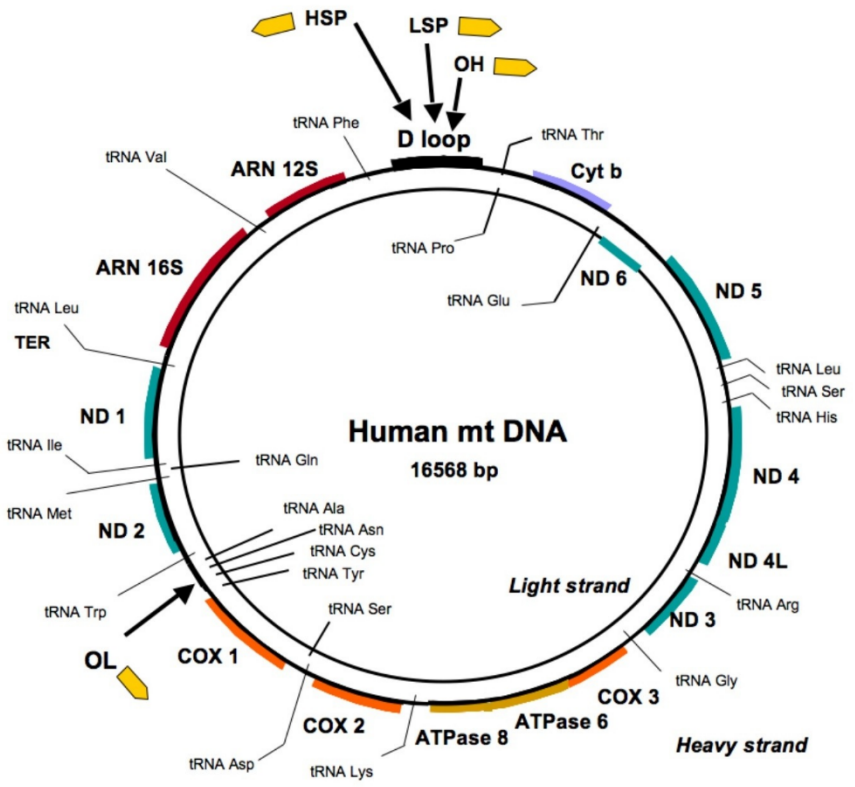



**Figure 1.3: A map of the human mitochondrial genome. The outer circle represents the heavy strand and the inner circle the light strand. The genes that encode the subunits of complex I are shown in blue; cytochrome c oxidase is shown in orange; cytochrome b of complex III is shown in purple; and the subunits of ATP synthase are shown in yellow. The two ribosomal RNAs are shown in red and the 22tRNAs are indicated by black lines. The displacement loop (D-loop), including the proposed origin of the H-strand replication (OH) and the heavy (HSP) and light (LSP) strand promoters, is represented in black. The origin of L-strand replication is shown as OL. (Source: http://mitoblog.org/).**

MtDNA is characterized by the absence of intronic regions as between the genes there are none or just a few non-coding bases which are tightly packed. It contains a significantly variable non-coding region, named displacement loop (D-loop), which contains the initiation site of mtDNA replication (Andrews et al., 1999). A few genes might also lack the termination codon. In this case, UAA codons are introduced at the post-transcriptional level.

The mitochondrial genetic code consists of 60 codons, one less than the nuclear genetic code. Four out of the 60 codons are stop codons; UAA, AUG, AGA and AGG. The first two codons serve as stop codons in the nuclear genetic code as well but the latter (AGA and AGG) specify arginine in the nuclear genetic code. Also, in mitochondria the nuclear stop codon

UGA encodes tryptophan and AUA encodes methionine rather than isoleucine. As it was mentioned before, there are only 22 different types of mitochondrial tRNAs that able to interpret 60 codons. The *third-base wobble* characteristic allows 8 out of the 22 tRNAs to recognise families of four codons differing only at the third base. The remaining 14 tRNAs recognise pair codons that share the first two base position and carry either a purine or a pyrimidine at the third base. Hence, 22 tRNAs are able to interpret 60 codons (Strachan and Read, 2010).

| ENCODING OF OXPHOS COMPONENTS | | | |
|---|---|---|---|
| **Components of OXPHOS** | | | |
| | **Mitochondrial DNA encoded subunits** | **Nuclear DNA encoded subunits** | **Assembly proteins** |
| **Complex I** | 7 | ~39 | ~11 |
| **Complex II** | 0 | 4 | ~2 |
| **Complex III** | 1 | 10 | ~9 |
| **Complex IV** | 3 | 10 | ~30 |
| **Complex V** | 2 | ~14 | ~3 |

**Table 1.1: Mitochondrial and nuclear encoded subunits of the mitochondrial respiratory chain complexes (Source: (Schon et al., 2012)**

### 1.2.2 *Mitochondrial DNA structure*

Initially, it was believed that the mitochondrial DNA was covered with histones in a similar way to nuclear DNA. However, later studies showed that approximately 5-7 molecules are compacted into protein structures called nucleoids. The nucleoids are nucleoprotein complexes around 70nm in size (Nass, 1969, Iborra et al., 2004).

These structures are mainly consisted of TFAM protein (mitochondrial transcription factor A), mtSSBP (mitochondrial single-stranded DNA-binding protein) and a variety of other

mitochondrial metabolic proteins such as: mtDNA polymerase (POLG), mtRNA polymerase, suv3-like helicase and DEAD protein box 28 (Wang and Bogenhagen, 2006).

More recent studies suggested that each nucleoid consists of one molecule of mtDNA and TFAM. According to Kukat et al, TFAM plays a vital role in packaging and organising the mtDNA in nucleoids (Kukat et al., 2011).

### 1.2.3 *Mitochondrial DNA inheritance - Heteroplasmy*

The mitochondrial DNA is strictly maternally inherited (Giles et al., 1980). To our best knowledge, only one case of paternal inheritance in humans has been recorded to date (Heckerling, 2002). However, paternal mitochondrial inheritance of mtDNA is common in other animals.

According to studies, three different explanations have been suggested for the lack of paternal transmission in humans. Firstly, the increased number of mtDNA copies contained in the unfertilised egg compared to only 100 copies present in the sperm results in a 'dilution effect' of the paternal mtDNA. Secondly, the ubiquitination of the sperm mtDNA during the formation of the mammalian zygote and lastly the exclusion of the paternal alleles also known as 'mtDNA bottleneck' theory (Chinnery and Hudson, 2013). Recent studies on extreme depth next generation sequencing of mtDNA have not shown any paternal transmission in humans (Pyle et al., 2015).

As it was discussed previously, cells usually contain more than one copy of mtDNA molecules and the condition where the sequence of the different copies is identical is called homoplasmy. The mutation rate of the mtDNA is 10-20 times higher compared to nuclear DNA due to either lack of protective histones or inefficient mtDNA repair or close proximity to ROS product of the OXPHOS system (Smits et al., 2010, Chinnery and Hudson, 2013). As a result, frequently mutated copies of mtDNA co-exist with normal copies in the same cell. This co-existence of normal and mutated copies of mtDNA is termed as heteroplasmy (Wallace, 1999) (Figure 1.4). The relative amount of the mutated mtDNA copies compared to the normal ones should reach a certain threshold to affect the function of the respiratory chain. This critical threshold differs from mutation to mutation and also between tissues and organs. Therefore, as it will be discussed later, mitochondrial disorders caused by mtDNA mutation are characterised by tissue selectivity and clinical heterogeneity (Macmillan et al., 1993). Moreover, due to mitotic segregation the distribution of normal and mutated mtDNA copies

is random;  hence, the mutation loads in patients' cells and tissues usually vary during their life (Smits et al., 2010).

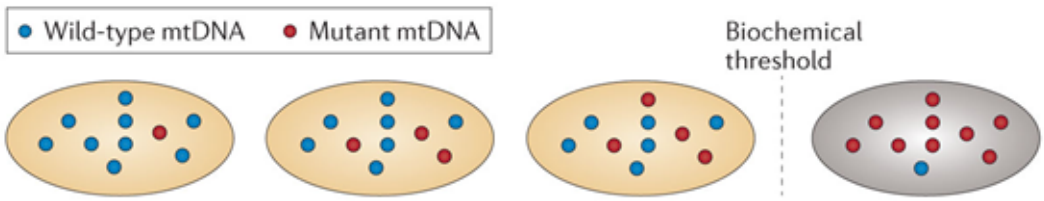



**Figure 1.4: mtDNA heteroplasmy and the threshold effect (Source: (Stewart and Chinnery, 2015))**

### 1.2.4 *Mitochondrial DNA maintenance, replication and transcription*

The mtDNA replication occurs independently to the nuclear DNA cell cycle and replication and also occurs in non-dividing cells such as the skeletal muscle fibers and central neurons (Bogenhagen and Clayton, 1977). To date, two models of mtDNA replication in mammalian cells have been proposed (Smits et al., 2010, Pearce et al., 2013). The first model suggests that the two strands of the mtDNA are synthesized simultaneously, as it happens in the nuclear DNA. On the contrary, the second model suggests a delay between the synthesis of the two strands. The latter model, displays the lagging strand template, which is either coated with protein or hybridized to RNA (Pearce et al., 2013).

The main factors involved in the mtDNA replication are: a DNA polymerase (polymerase γ, POLG), a DNA helicase (Twinkle) and the binding protein mtSSBP.  Polymerase γ is a heterotrimer enzyme consisting of a catalytic subunit (POLG) and two identical accessory subunits (POLG2). POLG has the ability of proof-reading while the subunits bind the DNA and increase the processivity of POLG. The DNA helicase Twinkle unwinds the double-stranded mtDNA from 5' to 3'. Lastly, the binding protein mtSSBP has been suggested to stabilize the integrity of single-stranded regions of DNA at the replication sites and stimulate the activity of Twinkle and POLG (Smits et al., 2010). Also, RNase MRP (RNase mitochondrial RNA processing endonuclease), endonuclease G, RNAse H1and DNA ligase III are crucial for the mtDNA replication. RNase MRP and endonuclease G have been suggested to process the precursor RNA primers to H-strand for the initiation of the replication while RNase H1 is responsible for removing the RNA primers. Regarding the mitochondrial DNA ligase III, is involved in both replication and repair of the mitochondrial

genome. To date, the exact mechanism of the mtDNA replication has not been fully understood (Smits et al., 2010).

The mitochondrial genome contains three promoters; two of them are located in the heavy strand (HSP1 and HSP2) while the third one is located in the light strand (LSP). LSP and HSP1 are embedded in the NCR region whereas HSP2 has been recently mapped on the mitochondrial gene located next to the NCR region, encoding the $tRNA^{Phe}$ (Zollo et al., 2012).

All promoters produce polycistronic transcripts and as it has been shown the products of HSP1 and HSP2 partly overlap. In particular, HSP1 transcript is responsible for producing rRNAs for mitochondrial ribosomes (mitoribosomes). The mitochondrial termination factor, mTERF1 prevents the transcription of HSP1 beyond the rRNA genes. On the other hand, the HSP2 transcript produces 10 mRNAs and 14 tRNAs (Bonawitz et al., 2006). Finally, the LSP promoter produces a transcript which is equal to the two-thirds of the mitochondrial genome in length and encoded the protein ND6 (subunit of complex I), the remaining 8 tRNAs and the necessary RNA primers for the mtDNA replication. Only a few nuclear-encoded proteins are involved in the mitochondrial transcription and these are: the mitochondrial RNA polymerase (POLRMT) and its accessory subunit (TEFM) (Minczuk et al., 2011), TFAM and the transcription factor TFB2M.

As mentioned above, mtDNA is packaged in nucleoids, a process mediated by the nucleoid's protein components. Studies have shown that TFAM apart from being a key regulator factor for mitochondrial transcription, is also a crucial factor for mtDNA packaging (Maniura-Weber et al., 2004). In addition to that, it seems that all the nucleoid's components function as mediators of factors necessary for replication and transcription (Gilkerson et al., 2013).

In the next step, the ribonucleases RNase P and Z cleave the tRNAs at their 5' and 3' termini respectively (Shutt and Shadel, 2010). Following, the mRNAs are polyadenylated and associated with a range of factors such as LRPPRC (a PPR-containing protein), that directs them to the mitochondrial ribosome for translation.

### 1.2.5 *Mitochondrial translation*

The four major steps of the mitochondrial translation are: initiation, elongation, termination and ribosome recycling.

The 13 proteins encoded by the mitochondrial genome are translated from 9 monocistronic and 2 dicistronic mRNAs. Both dicistronic mRNAs contain overlapping reading frames. The

start codons of the mitochondrial translation, AUG and AUA, are located at the 5' end of the mRNAs (apart from the dicistronic mRNAs) and direct the insertion of the formylmethionine (fMet) or methionine at the initiation or during the chain elongation respectively.

The mitochondrial elongation factors 2 ($IF2_{mt}$) and 3 ($IF3_{mt}$) are necessary for assembling an initiation complex on 55S ribosomes with fMet-tRNA at the start codon of a mitochondrial mRNA.

The initiation factor $IF3_{mt}$ is suggested to dissociate the 55S ribosomes by loosening the interaction of the two subunits (28S and 39S). Therefore, 39S subunit is released and a 28S: $IF3_{mt}$ complex is formatted. Also, the initiation factor $IF2_{mt}$ binds a GTP molecule although the time point when this takes place is not clearly known. Following, the mRNA enters the 28S subunit by a protein-rich entrance (Koc and Spremulli, 2002) (Christian and Spremulli, 2009).

The movement of the mRNA is paused when 17 nucleotides of the mRNA have entered the ribosome. During this phase, the 28S subunit inspects the codon at the 5' end of the mRNA while the $IF2_{mt}$:GTP promotes the binding of the fMet-tRNA to the ribosome. As long as there is a start codon at the P site, a stable initiation complex between the fMet-tRNA and the 5' start codon is formatted. In any other case, the mRNA leaves the small subunit and is dissociated. Next, the 28S initiation complex is joined by the large subunit while the $IF2_{mt}$ hydrolyzes GTP to GDP. As a result, the initiation factors are released and the mitochondrial translation is ready to enter the elongation step.

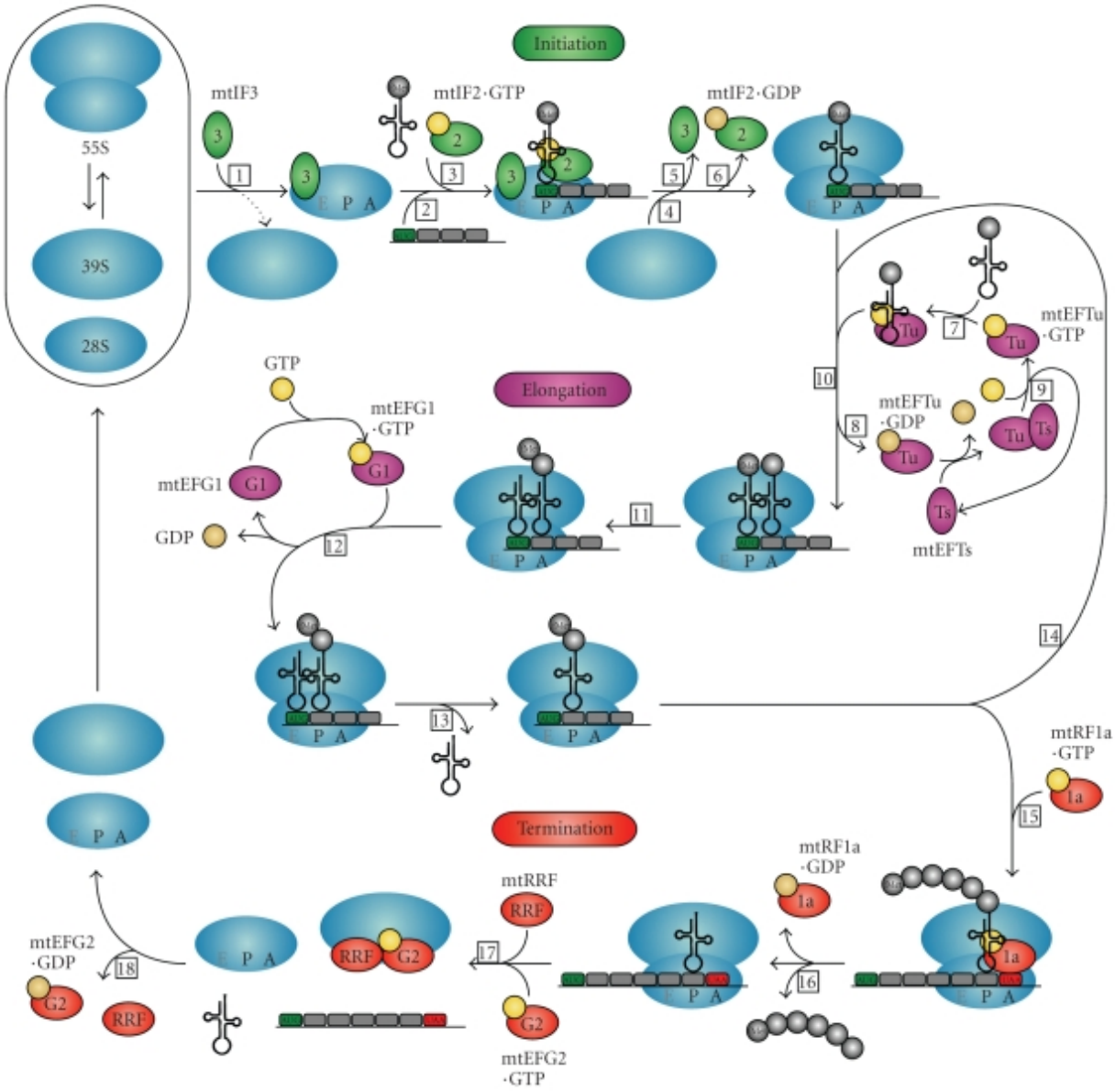



**Figure 1.5: Schematic diagram of the mitochondrial protein synthesis machinery (Source: (Smits et al., 2010)**

The elongation step of the polypeptide chain in mitochondria is similar to the elongation step in prokaryotes (Christian and Spremulli, 2009).Initially, the ternary complex EF-$Tu_{mt}$•GTP•aa-tRNA is formatted by the active form of the elongation factor Tu (EF-$Tu_{mt}$) binding an aa-tRNA (Nagao et al., 2007). The complex enters the A-site of the ribosome and is selected only if cognate codon-anticodon interactions can take place. When the complex is selected, the GTP is hydrolysed and the EF-$Tu_{mt}$•GDP is released. Next, the elongation factor Ts formats an intermediate EF-$Tu_{mt}$•EF-$Ts_{mt}$ complex promoting the exchange of GDP for GTP.

The peptide bond formation is catalysed by the ribosome resulting in a deacylated tRNA in the P-site and a peptidyl-tRNA one residue longer in the A-site. The translocation step is catalysed by the mitochondrial elongation factor G1(EF-$G1_{mt}$). The EF-$G1_{mt}$ removes the deacylated tRNA from the P-site and moves the peptidyl-tRNA from the A-site to the P-site.

The next step of the mitochondrial translation is the termination and recycling step. As it was discussed previously, the codons UAA and UAG serve as stop codons in the mammalian mitochondria. When either of the stop codons appears in the A-site of the ribosome, are recognised by the release factor mtRF1a. Then, mtRF1a binds to the ribosome in the presence of GTP. The latter, promotes the hydrolysis of the peptidyl-tRNA bond by the peptidyl-transferase centre on the 39S subunit and the release of the completed polypeptide. Following, the factor $RRF1_{mt}$ (ribosome releasing factor) and $RRF2_{mt}$ (also known as EF-$G2_{mt}$) bind to the A-site of the ribosome promoting the dissociation of the ribosomal subunit and the release of the deacylated tRNA and mRNA (Chrzanowska-Lightowlers et al., 2011). A new round of protein synthesis initiates when these factors are released from the ribosome.

## 1.3 Mitochondrial Disorders

The synchronized co-operation between the nuclear and the mitochondrial DNA is necessary for the normal function of the mitochondria. Therefore, mitochondrial dysfunction leads to a mitochondrial disease (Chinnery and Hudson, 2013).

The first description of patients carrying pathogenic mutations in their mtDNA dates back to 1988 (Holt et al., 1988, Wallace et al., 1988). Holt et al studied 25 patients with mitochondrial myopathy 9 of which were found to have two populations of muscle mtDNA. One of the mtDNA populations had deletions of up to 7 kilobases in length. Therefore, it was showed for the first time that mtDNA heteroplasmy occurs in human and that defects of the mitochondrial genome may be associated with human disease (Holt et al., 1988). In the same year, Wallace et al identified the first mutation in the *NADH* dehydrogenase subunit 4 gene correlated with the LHON disease (Wallace et al., 1988). In later years, nuclear DNA mutations were identified to cause mitochondrial disorders (Koopman et al., 2012, Gorman et al., 2015).

According to a study published in 2015 by Gorman et al, the prevalence of affected patients or at risk of developing mitochondrial disorder is 1:4300 of the population. Regarding the north of England, 23% of the affected adults carried mutations in a known or presumed nuclear gene while 40% carried mtDNA point mutations causing Leber hereditary optic neuropathy (LHON) (Gorman et al., 2015).

One of the great challenges of the mitochondrial disease is the variety of clinical features present in patients. Mitochondrial disorders affect more than one organ leading to complex multisystem dysfunctions. Tissues, in which the metabolic demand is higher, such as skeletal muscle, the central nervous system or heart, are typically affected (Chinnery and Hudson, 2013).

Mitochondrial disorders may manifest throughout any decade of life and are characterised by tissue specificity associated with certain mitochondrial genotypes. Also, patients carrying identical mutations might have different clinical presentations reflecting the genetic heterogeneity of the diseases. For example, in patients suffering from Leigh or Alper's syndrome, the main systems affected are the central nervous system and the liver. However, involvement of others organs such as cardiac or skeletal muscle has been described in some patients (Lightowlers et al., 2015) (Figure 1.6).

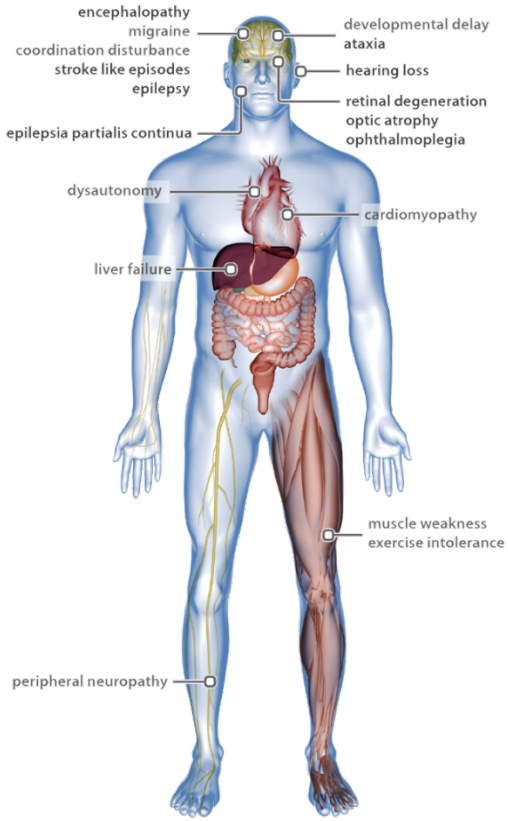



**Figure 1.6: Schematic diagram of the possible symptoms present in patients affected by a mitochondrial disease (Source: www.khondrion.com)**

### 1.3.1 *Mitochondrial encoded mutations*

A common cause of mitochondrial disorders is the single-large-scale deletion of the mtDNA. To date, more than 120 different mtDNA deletions have been recorded associated with

mitochondrial diseases (Brandon et al., 2005). The mtDNA deletions are usually sporadic, not transmitted to the offspring and often caused by inefficient mtDNA repair mechanisms (Krishnan et al., 2008, Chinnery et al., 2004). Usually the depleted regions of the mtDNA contain tandem repeat sequences (Schon et al., 1989). Every mtDNA deletion recorded to date is heteroplasmic.

The three main clinical syndromes caused by mtDNA deletions are Pearson's syndrome, Kearns-Sayre syndrome (KSS) and chronic progressive external ophthalmoplegia (CPEO). Pearson's syndrome is a severe disorder arising in infancy characterized by sideroblastic anaemia with pancytopenia and exocrine pancreatic failure. Kearns-Sayre syndrome is characterized by a wide clinical spectrum including retinitis pigmentosa, progressive external ophthalmoplegia, cardiomyopathy, deafness, short stature and a range of neurological symptoms. Finally, CPEO is commonly presented in adults leading to progressive paralysis of the eye which results in ptosis and impaired eye movement (Greaves et al., 2012).

Another cause of mitochondrial disorders is point mutations located within genes encoding rRNAs and tRNAs. This group of mutations is usually maternally inherited. Mutations located in protein-encoding genes are thought to affect specific respiratory chain complexes whereas mutations in RNA genes result in an overall impairment of the mitochondrial proteins synthesis machinery. The bulk of the point mutations associated with mitochondrial disorders are found within mt-tRNA genes (Chinnery and Hudson, 2013).

The first mitochondrial disease correlated to a mtDNA point mutation is LHON (Leber hereditary optic neuropathy) (Wallace et al., 1988). The three most common mtDNA mutations (typically homoplasmic) causing LHON are m.3460G>A, m,11778G>A and 14484T>C (Carelli et al., 1997), all of them affecting mtDNA genes encoding Complex I subunits. LHON is maternally inherited and typically presented with bilateral, painless, sub-acute visual failure in young adult males. However, the homoplasmic presence of aforementioned mtDNA mutations does not always lead to visual impairment, resembling the clinical heterogeneity of the disease. Recent studies have suggested that mtDNA variants or environmental factors may act as modulating factors of LHON expression (Chinnery and Hudson, 2013). It has been shown that there are certain polymorphic changes recurring more frequently in LHON than in control populations. These mutations are recognised as haplogroup-specific variants and thus weighted for their contribution to LHON pathogenesis. Furthermore, LHON disease is tightly linked with gender difference. It is often reported that about 50% of males will be affected but only about 10% of females. Finally, other factors

factors such as smoking, alcohol abuse, the antibiotic ethambutol and the environmental noxes have been correlated to the manifestation of the disease (Maresca et al., 2014)

Leigh syndrome is a neurodegenerative disorder presented with a variety of symptoms including neurological features, such as developmental delay, hypotonia, peripheral neuropathy, optic atrophy and extraneurologic manifestations like hypertrophic cardiomyopathy, liver failure and renal tubulopathy. The onset of the disease is typically between 3-12 months of life but it might manifest anytime from the neonatal period to the adulthood (Rahman et al., 1996). The genetic cause of Leigh syndrome can either be a mtDNA mutation (i.e. m.8993T>G/C, (Ciafaloni et al., 1993)) or mutations in various nuclear genes (i.e. *SURF1*, (Wedatilake et al., 2013)).

One of the most frequent maternally inherited mitochondrial disorders caused by mtDNA point mutation is the mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) which was first described in 1984 (Pavlakis et al., 1984).

MELAS syndrome is a multisystemic disease presented with a variety of symptoms such as: stroke-like episodes, lactic acidosis, epilepsy, myopathy, recurrent headaches, hearing impairment, diabetes and short stature. The onset of the disease in 65-76% of the cases is before the age of 20 years. However, 5-8% developed the disease before the age of 2 years while 1-6% after the age of 40 years (El-Hattab et al., 2015).

More than 90% of patients diagnosed with MELAS syndrome present stroke-like episodes, dementia, epilepsy, lactic academia, ragged red fibres and exercise intolerance. Other common symptoms among MELAS patients are hemiparesis, recurrent headaches, muscle weakness, peripheral neuropathy and learning disabilities. Less than 50% of the affected have developed diabetes ataxia, depression, anxiety, optic atrophy, pigmentary retinopathy, motor development delay and cardiomyopathy (El-Hattab et al., 2015).

The mutations m.3243A>G and m.3271T>C in the gene MT-TL1 encoding the mt $tRNA^{Leu(UUR)}$are the most common mutations associated with MELAS disease. The mutations result in the absence of the post transcriptional taurinomethylation of the uridine at the wobble position of the $tRNA^{Leu(UUR)}$ anticodon leading to reduced translation of the specific tRNA (Umeda et al., 2005). The main complexes affected are complexes I and IV (Suzuki and Nagao, 2011). Cybrid studies have shown that these mutations lead to decreased mitochondrial translation and respiratory activity (Hayashi et al., 1993).

Another frequent maternally inherited mitochondrial disorder is Myoclonic Epilepsy with ragged red fibers (MERRF). MERRF syndrome is a multisystem disorder initially presented with myoclonus followed by epilepsy, ataxia, weakness and dementia. The onset of the disease is usually during childhood, after normal early development.

The mutations m.8344A>G and m.8356T>C are the most common mutations associated with MERRF disease. The mutations are located in the mtDNA gene *MT-TK* encoding the $tRNA^{Lys}$. The presence of the mutations results in the absence of the taurnimethylation and 2-thiolation of the uridine at the wobble position of the anti-codon for the $tRNA^{Lys}$, leading to decreased translation of the specific tRNA. It has been shown that $rho^0$ cells harboring either the m.8344A>G or m.8356T>C pathogenic variants were characterized by reduced protein synthesis and oxygen consumption (Masucci et al., 1995).

| Gene product | Number of mutations | Main disorder | |
|---|---|---|---|
| **rRNAs** | | | |
| **12S rRNA** | 5 | Deafness | Isolated RC defect |
| **16S rRNA** | 1 | Atypical MELAS | Isolated RC defect |
| **tRNAs** | | | |
| **$tRNA^{Ala}$** | 3 | Myopathy | Combined RC defect |
| **$tRNA^{Arg}$** | 2 | Various | Combined RC defect |
| **$tRNA^{Asn}$** | 5 | Myopathy | Combined RC defect |
| **$tRNA^{Asp}$** | 2 | Various | Combined RC defect |
| **$tRNA^{Cys}$** | 3 | Various | Combined RC defect |
| **$tRNA^{Gln}$** | 3 | Various | Combined RC defect |
| **$tRNA^{Glu}$** | 7 | Reversible respiratory chain deficiency | Combined RC defect |
| **$tRNA^{Gly}$** | 3 | Various | Combined RC |

| | | | |
|---|---|---|---|
| | | | defect |
| **tRNA$^{His}$** | 4 | Various | Combined RC defect |
| **tRNA$^{Ile}$** | 14 | PEO | Combined RC defect |
| **tRNA$^{Leu(CUN)}$** | 8 | Myopathy | Combined RC defect |
| **tRNA$^{Leu(UUR)}$** | 23 | MELAS | Combined RC defect |
| **tRNA$^{Lys}$** | 14 | MERRF | Combined RC defect |
| **tRNA$^{Met}$** | 2 | Various | Combined RC defect |
| **tRNA$^{Phe}$** | 14 | Myopathy | Combined RC defect |
| **tRNA$^{Pro}$** | 5 | Multisystem | Combined RC defect |
| **tRNA$^{Ser(AGY)}$** | 4 | Myopathy | Combined RC defect |
| **tRNA$^{Ser(UCN)}$** | 12 | Myopathy;Deafness | Combined RC defect |
| **tRNA$^{Thr}$** | 2 | Various | Combined RC defect |
| **tRNA$^{Trp}$** | 12 | Encephalomyopathy | Combined RC defect |
| **tRNA$^{Tyr}$** | 4 | Myopathy | Combined RC defect |
| **tRNA$^{Val}$** | 6 | Multisystem | Combined RC defect |
| **Polypeptides** | | | |
| **ATP synthase 6** | 13 | NARP or MILS | Isolated RC defect |
| **ATP synthase 8** | 2 | Various | Isolated RC defect |
| **COX I** | 10 | Various | Isolated RC defect |

| | | | |
|---|---|---|---|
| **COX II** | 8 | Various | Isolated RC defect |
| **COX III** | 6 | Myopathy | Isolated RC defect |
| **Cytochrome *b*** | 21 | Sporadic Myopathy | Isolated RC defect |
| **ND1** | 16 | MELAS;LHON | Isolated RC defect |
| **ND2** | 3 | Various | Isolated RC defect |
| **ND3** | 5 | Leigh's syndrome | Isolated RC defect |
| **ND4** | 5 | LHON | Isolated RC defect |
| **ND4L** | 1 | LHON | Isolated RC defect |
| **ND5** | 12 | MELAs | Isolated RC defect |
| **ND6** | 11 | LHON | Isolated RC defect |

**Table 1.2: mtDNA mutations in human primary respiratory chain disorders (Source: (Schon et al., 2012)**

### 1.3.2 *Nuclear encoded mutations*

As discussed previously, mtDNA encodes 37 genes required for the normal function of the mitochondrion and the remaining proteins are encoded by the nuclear DNA. Therefore, mitochondria are dependent upon the nuclear DNA which encodes enzymes essential for the mtDNA replication, repair, transcription and translation. Mutations in nuclear encoded genes have been associated with a variety of mitochondrial disorders (Chinnery and Hudson, 2013). The number of disease-causing molecular alterations in nuclear genes is growing exponentially and mutations in these genes underlie the vast majority of respiratory chain (RC) defects in children.

Nuclear-mitochondrial diseases can be categorised into four different groups:

1. Disorders resulting from mutations in nuclear-encoded subunits or assembly factors of the OXPHOS system
2. Disorders with defective mtDNA stability and maintenance
3. Disorders resulting from mutations in genes encoding translational elongation factors or mitochondrial ribosomal proteins
4. Disorders resulting from mutations in genes controlling the mitochondrial network dynamics (Chinnery and Hudson, 2013, Boczonadi and Horvath, 2014)

The vast majority of mitochondrial respiratory chain proteins are encoded by nuclear genes. Mutations in a variety of nuclear genes encoding respiratory chain subunits have been found in patients with mitochondrial cytopathies (DiMauro and Hirano, 2009) and the bulk of these mutations occur in nuclear genes encoding complex I subunits (Distelmaier et al., 2009).

Complex I deficiency caused by mutations in nuclear genes is associated with a wide spectrum of clinical phenotypes varying from lethal neonatal diseases to adult onset neurodegenerative disorders (Loeffen et al., 2000, Lebre et al., 2011). At least 44subunits of complex I are encoded by the nDNA while pathogenic mutations have been identified in 14 of the structural subunits (Chinnery and Hudson, 2013). Leigh or Leigh-like syndrome is associated with pathogenic mutations in several complex I subunit genes [*NDUFS1* (Bénit et al., 2001), *NDUFS3* (Haack et al., 2012, Bénit et al., 2004), *NDUFS4* (van den Heuvel et al., 1998), *NDUFS7* (Smeitink and van den Heuvel, 1999), *NDUFS8* (Loeffen et al., 1998), *NDUFV1* (Bénit et al., 2001, Schuelke et al., 1999), *NDUFA10* (Hoefs et al., 2011), *NDUFB3* (Haack et al., 2012) and *NDUFA2* (Hoefs et al., 2008)]. Pathogenic mutations in *NDUFS2* (Loeffen et al., 2001), *NDUFS6* (Kirby et al., 2004), *NDUFV2* (Bénit et al., 2003), *NDUFA1* (Chinnery and Hudson, 2013), *NDUFA11* (Berger et al., 2008) and *ACAD9* (Haack et al., 2010) typically manifest as hypertrophic cardiomyopathy and encephalopathy. Nevertheless, mutations identified in assembly factors of complex I may manifest as Leigh syndrome (*NDUFAF2* (Calvo et al., 2010) and *NDUFAF5* (Gerards et al., 2010)), encephalopathy (*NDUFAF4* (Saada et al., 2008)) and cardioencephalopathy (*NDUFAF1* (Dunning et al., 2007).

Complex II is entirely encoded by the nDNA and mutations in *SDHB*, *SDHC* and *SDHD* have been associated with patients suffering from pheochromocytoma and paraganglioma (Baysal, 2002). However, in rare cases Leigh syndrome has been associated with mutations in *SHDA* and also other structural complex II subunit genes (Alston et al., 2012, Chinnery and Hudson, 2013). It has been suggested that mutations in these genes lead to accumulation of succinate

and reactive oxygen species that eventually results in overexpression of hypoxia inducible factor I with ensuing the formation of these tumors (Kantorovich et al., 2010). Regarding assembly factors of complex II, mutations in the *SDHAF1* gene have been detected in patients presenting isolated complex II deficiency and infantile leukoencephalopathy (Ghezzi et al., 2009). Additionally, paraganglioma has also been associated with mutations in *SDH5*, a gene necessary for the flavination of the SDH1 subunit (Hao et al., 2009).

Regarding complex III, only mutations in two nuclear encoded subunits (*UQCRB* and *UQCRQ*) have been associated with hypoglycaemia and lactic acidosis and severe psychomotor retardation combined with various neurological symptoms respectively (Haut et al., 2003, Barel et al., 2008) to date. However, a newly identified mutation in *TTC19* has been associated with progressive neurodegenerative disorder in late infancy (Ghezzi et al., 2011). The gene *TTC19* encodes a complex III structural subunit. In humans, mutations in the gene B*CS1L*, encoding an assembly factor of complex III, have been associated with different phenotypes including neonatal proximal tubulopathy, hepatic involvement, encephalopathy and Bjornstad syndrome (de Lonlay et al., 2001, Hinson et al., 2007).

To date, mutations in three different structural subunits of complex IV (*COX6BI*, *COX7B* and *COX4I2*) have been associated with extremely rare mitochondrial disorders presented as severe, typically fatal, infantile diseases. Siblings from a consanguineous Saudi Arabian family carrying a homozygous mutation in *COX6B1* presented with gait instabilities, visual disturbances, progressive neurological deterioration and leukodystrophy (Massa et al., 2008). A single mutation in the structural subunit gene *COX4I2* has been reported in patients with exocrine pancreatic insufficiency, dyserythropoietic anaemia and calvarial hyperostasis (Shteyer et al., 2009) while mutations in *COX7B* are atypically associated with facial dysmorphisms and congenital abnormalities (Zvulunov et al., 1998). On the other hand, mutations in a variety of nuclear genes encoding assembly factors of complex IV are frequent causes of human diseases resulting in complex IV deficiency. Mutations in *SURF1* ( cytochrome c oxidase assembly factor) have been frequently associated with Leigh syndrome and COX deficiency (Sue et al., 2000). Mutations in *SCO1* and *SCO2*, which are required for the mitochondrial copper transport acting on subunit II of COX, are disease-causing (Valnot et al., 2000a, Papadopoulou et al., 1999). Furthermore, mutations in *COX10* and *COX14* have been associated with leukodystrophy and Leigh syndrome respectively (Valnot et al., 2000b, Antonicka et al., 2003b, Oquendo et al., 2004). Mutations in the gene called *LRPPRC*, encoding a protein which is involved in the stability of subunits I and III of COX (Xu et al., 2004) have been reported in patients presenting the French-Canadian type of Leigh syndrome.

Additionally, mutations in *COA5* (cytochrome c oxidase assembly factor 5) are associated with neonatal hypertrophic cardiomyopathy (Huigsloot et al., 2011), mutations in *TACO1* (translational activator of cytochrome c oxidase I) are associated with late-onset Leigh syndrome (Seeger et al., 2010) and finally mutations in *FASTKD2* (FAST kinase domains 2) with cytochrome c oxidase defective encephalomyopathy (Ghezzi et al., 2008).

Regarding complex V, only mutations in the gene *ATP5E* encoding a structural subunit if complex V have been associated with complex V deficiency (Mayr et al., 2010). However, mutations in the assembly factors of complex V encoded by the genes *ATPAF2* (Chinnery and Hudson, 2013), *ATP12* (De Meirleir et al., 2004) and *TMEM70* (Cízková et al., 2008, Shchelochkov et al., 2010) have been associated with complex V deficiency, myopathy and neurological features.

Defects in proteins involved in the mitochondrial replication or in the dNTP synthesis and as a result the mtDNA copy number is affected. These defects lead to either early-onset autosomal recessive conditions (Spinazzola and Zeviani, 2005) or to multiple DNA deletions inherited as autosomal dominant or recessive phenotypes of progressive external ophthalmoplegia and additional neurological symptoms.

The gene encoding the enzyme polγ, *POLG*, is frequently mutated and disorders caused by mutations in *POLG* are characterised by a wide spectrum of clinical phenotypes (Rötig and Poulton, 2009). For example, a few mutations in *POLG* have been associated with autosomal dominant progressive external ophthalmoplegia (adPEO) with multiple mtDNA deletions (Van Goethem et al., 2001). On the other hand, recessive mutations in the same gene have been reported in patients with an early-onset mtDNA depletion syndrome, named Alpers-Huttenlocher syndrome (Naviaux et al., 1999).The gene *POLG2* encodes the accessory subunit of POLG, which is a 55kDa protein and increases the affinity of the enzyme polγ to the DNA. Although mutations in POLG2 gene are rarely described, a few of them have been linked to mitochondrial diseases. Two mutations (c.1352G>A and c.1207-1208ins24) have been identified in patients characterized by late-onset autosomal dominant progressive external ophthalmoplegia with multiple DNA deletions in muscle. Another study revealed 8 heterozygous mutations in POLG2, 7 of which were novel (G103S, L153V, P205R, R369G, D386E, S423Y, and L475DfsX2), in a cohort of 112 patients suspected for POLG mutations (Copeland, 2014). Mutations in an *C10orf2* gene result in adPEo associated with multiple mtDNA deletions. The gene *C10orf2* encodes the mitochondrial protein Twinkle, an mtDNA replicative helicase bound to mtDNA in mitochondrial nucleoids (Spelbrink et al., 2001).

Nevertheless, mutations in *ANT1* (adenine nucleotide translocase type 1) impair the nucleotide balance and eventually the mtDNA replication resulting in adPEO (Agostino et al., 2003).

The first step of the deoxypurine salvage pathway is catalysed by the deoxyguanosinase kinase, which is encoded by the gene *DGUOK*. Mutations in this gene are typically presented as neonatal-onset of liver failure associated with neurological dysfunction (Spinazzola and Zeviani, 2009). Mutations in *MPV17*, which encodes a mitochondrial inner membrane protein of unknown function, have been associated with mtDNA depletion. Diseases caused by mutations in *MPV17* present a wide clinical phenotypic spectrum (El-Hattab et al., 2010). Others genes that have been associated with impaired mtDNA copy number and mitochondrial disorders are *TK2* (thymidine kinase 2) (Saada et al., 2001, Mancuso et al., 2002, Oskoui et al., 2006), *RRM2B* (ribonucleotide reductase regulatory TP53 inducible subunit M2B) (Bourdon et al., 2007, Shaibani et al., 2009), *SUCLA2* (succinate-CoA ligase ADP-forming beta subunit) (Van Hove et al., 2010, Morava et al., 2009), *SUCLG1* (succinate-CoA ligase alpha subunit) (Van Hove et al., 2010, Randolph et al., 2011) and *TP* (thymidine phosphorylase) (Nishino et al., 1999, Martí et al., 2003, Szigeti et al., 2004).

Defects in nuclear genes encoding proteins involved in the mitochondrial protein synthesis machinery lead to diseases characterized by neurological features associated with combined respiratory defects (Nogueira et al., 2011) (Boczonadi and Horvath, 2014).

One typical example of those nuclear genes is *PUS1*, which encodes an enzyme that converts uridine into pseudouridine, necessary for posttranscriptional modification of the tRNAs. Mutations in *PUS1* lead to impaired pseudouridylation and are associated with the rare MLASA (myopathy, lactic acidosis and sideroblastic anaemia) syndrome (Bykhovskaya et al., 2004). Furthermore, mutations in *TRMU* (tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase) also affect the 2-thiolation of the wobble position of the tRNAs for Lys, Gln and Glu leading to impaired mitochondrial synthesis. Patients carrying mutations in *TRMU* present combined respiratory chain defects and impaired mitochondrial translation resulting in infantile acute liver failure (Zeharia et al., 2009). Nonetheless, molecular defects in genes encoding mitochondrial elongation factors have also been associated with mitochondrial diseases. For example, patients with mutations in genes *TUFM* (Tu translation elongation factor), *TSFM* (Ts translation elongation factor) and *GFM1*(G elongation factor) illustrate a severe phenotype, typically with lethal outcome, associated with combines respiratory chain deficiency (Valente et al., 2007, Smeitink et al., 2006).

Regarding the mitochondrial ribosomal protein, only mutations in two genes (*MRPS16* and *MRPS22*) have been associated with hypertrophic cardiomyopathy and neonatal lactic acidosis (Miller et al., 2004, Saada et al., 2007). Mutations in these genes lead to impaired assembly of the small mitoribosomal subunit and eventually decreased levels of 12S rRNA levels.

Mutations in the mitochondrial inner membrane transporter SLC25A19 have been associated with Amish microcephaly (Siu et al., 2010) while the homozygous mutations p.Gly72Glu in SLC25A3 was present in siblings presenting lactic acidosis, hypertrophic cardiomyopathy and muscular hypotonia, who died in the first year of life (Mayr et al., 2007). Furthermore, mutations in *SLC5A4* are linked to AAC1 deficiency and adPEO, mutations in *SLC5A20* with CAC deficiency, mutations in *SLC5A15* with HHH syndrome and mutations in *SLC5A22* linked to neonatal myoclonic epilepsy (Palmieri, 2008).

Finally, another group of mitochondrial diseases is caused by molecular defects in genes encoding mitochondrial aminoacyl-transfer RNA synthetases. Mutations in *RARS2* (arginyl-tRNA synthetase 2) and *DARS2* (aspartyl-tRNA synthetase 2) have been associated with severe encephalopathy and pontocerebellar hypoplasia and leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation respectively (Edvardson et al., 2007, Lin et al., 2010), mutations in *HARS2* and *LARS2* with Perrault syndrome (Pierce et al., 2011, Pierce et al., 2013), mutations in *EARS2* with leukoencephalopathy with thalamus and brainstream involvement and high lactate (LTBL) (Steenweg et al., 2012), mutations in *YARS2* have been associated with the MLASA syndrome (Riley et al., 2010), mutations in *AARS2* with hypertrophic cardiomyopathy (Götz et al., 2011)and mutations in *FARS2* with Alper's syndrome, encephalomyopathy, epilepsy and lactic acidosis (Elo et al., 2012).

Mitochondrial fission and fusion occur constantly and play a vital role in the normal function of the mitochondria. Therefore, molecular defects disturbing the mitochondrial dynamics are likely to be disease-causing. Mutations in *OPA1* lead to autosomal dominant atrophy (Amati-Bonneau et al., 2009) while mutations in *MFN2* have been associated with Charcot-Marie-Tooth disease (Züchner et al., 2004). Furthermore, only one reported mutation in *Drp1* resulted in defective mitochondrial and peroxisomal fission leading to abnormal brain development, optic atrophy, hyperplasia with lactic academia and severe microcephaly (Waterham et al., 2007). Moreover, mutations in *KIF5A* (kinesin family member 5A) have been associated with dominant hereditary spastic paraplegia due to impaired mitochondrial mobility (Fichera et al., 2004) while patients with mutations in *GDAP1* (ganglioside induced

differentiation associated protein 1) presented with autosomal recessive, early-onset type of either demyelinating or axonal neuropathy (Niemann et al., 2005).

In conclusion, the advent of new technologies such as next generation sequencing has shed light on the genetic and clinical heterogeneity of mitochondrial cytopathies. The increased awareness of the large number of nuclear genes associated with mitochondrial cytopathies will improve the understanding of the disorders and help us develop sufficient therapeutic strategies.

| | Gene | Protein (function) | |
|---|---|---|---|
| **MtDNA replication** | **POLG** | Polymerase γ catalytic subunit | Combined RC defect |
| | **POLG2** | Polymerase γ accessory subunit | Combined RC defect |
| | **C10orf2** | Twinkle (mtDNA disease) | Combined RC defect |
| **Nucleotide synthesis and transport** | **DGUOK** | Deoxyguanosine kinase | Combined RC defect |
| | **TK2** | Thymidine kinase | Combined RC defect |
| | **TYMP** | Endothelia cell growth factor 1 (thymidine phosphorylase) | Combined RC defect |
| | **SLC25A4** | Adenine nucleotide translocator 1 | Combined RC defect |
| | **SLC25A3** | Solute carrier family 25 member 3 (phosphate transporter) | Combined RC defect |
| | **SUCLG1** | Succinate-CoA ligase α-subunit | Combined RC defect |
| | **SUCLA2** | Succinate-CoA ligase β-subunit | Combined RC defect |
| | **RRM2B** | Ribonucleotide reductase M2 B | Combined RC defect |
| | **MPV17** | Mt inner membrane protein | Combined RC defect |
| **Mt protein import** | **TIMM8A** | Translocase of innet mt membrane 8 homolog A (small TIM complex subunit) | Combined RC defect |
| | **DNAJC19** | DnaJ homolog, subfamily C, member 19 (TIM23 complex subunit) | Combined RC defect |
| **Mt membrane biogenesis and maintance** | **TAZ** | Tafazzin (cardiolipin metabolism) | Combined RC defect |
| | **OPA1** | Optic atrophy 1 (mt fusion) | Combined RC defect |

| | | | |
|---|---|---|---|
| | **MFN2** | Mitofusin 2(mt fusion) | Combined RC defect |
| | **DNM1L** | Dynamin 1-like (mt and peroximal fission) | Combined RC defect |
| **Mt protein processing and quality control** | **SPG7** | Spastic paraplegia 7 or paraplegin (m-AAA protease subunit) | Combined RC defect |

**Table 1.3: Nuclear encoded genes implicated in mitochondrial disorders**

### 1.3.3 *Treatment of mitochondrial disorders*

Developing successful treatments for mitochondrial disorders has proved to be challenging. One of the main challenges is the complex genetic and phenotypic heterogeneity. Individuals carrying the same mtDNA or nDNA defect might present different clinical manifestation due to tissue specificity. Therefore, it is very difficult to collect large groups of patients to conduct statistically valid, randomised, double-blinded, placebo-controlled clinical trials. Normally, clinical trials are conducted on patients' groups carrying the same genetic defect, characterized by the same clinical features and biochemical findings and lastly the participants are at the same disease progression stage. Furthermore, lack of natural history on affected individuals makes efficacy assessment and treatment challenging. Finally, it is of great importance to choose the correct outcome measures during a clinical trial (Kanabus et al., 2014).

Despite many years of research on mitochondria, there is no established treatment for mitochondrial disorders to date. The results from the different pharmacological treatments and reports that have been used in individuals with mitochondrial disorders are variable. These treatments include antioxidants (coenzyme Q10, idebenone, vitamin C, vitamin E, cysteine, N-acetyl cysteine and menadione), agents improving lactic acidosis (dichloroacetate and dimethylglycine), agents regulating secondary chemical deficiencies caused by mitochondrial disorders (carnitine, creatinine). Respiratory chain co-factors (nicotinamide, thiamine, riboflavin, succinate and coenzyme Q10) and lastly hormones (growth hormone and corticosteroids) (Chinnery and Turnbull, 2001, Pfeffer et al., 2012).

According to a Cochrane systematic review published in 2012 only 12 out of 1335 abstracts reviewed met the Cochrane inclusion criteria for well-conducted unbiased clinical trials (Pfeffer et al., 2012). Five studies investigated the efficacy of dicloroacetate (DCA) and four studies the efficacy of creatine either alone or as a cocktail with CoQ10 and lipoic acid. Moreover, single studies investigated the effectiveness of CoQ10, dimethylglycine and whey-

based cysteine supplementation. However, the aforementioned clinical trials were characterised by limited number of patients and often by genetic heterogeneity between the selected individuals (Kanabus et al., 2014).

Three studies out of 12 did not show any effect on the patients (Klopstock et al., 2000, Kornblum et al., 2005, Liet et al., 2003) while one study regarding the efficacy of DCA was terminated due to side effects (Kaufmann et al., 2006). On the contrary, supplementation with CoQ10 led to increased serum Q10 levels and decreased serum lactate after 1 min of cycle ergometry without any other outcome measures to change significantly (Glover et al., 2010). Furthemore, combined supplementation with creatine, CoQ10 and lipoic acid led to significant decreased of plasma lactate levels (Rodriguez et al., 2007). Lastly, supplementation with whey-based cysteine did not show any significant improvement (Mancuso et al., 2010).

A few mitochondrial disorders are responsive to specific therapies. For example, early supplementation with CoQ10 to patients with defects in CoQ10 biosynthesis is related to clinical outcome. However, not all patients responded clinically to the supplementation (Kanabus et al., 2014). Moreover, a subset of individuals suffering from Leigh syndrome due to biotinidase deficiency and from biotin thiamine responsive nasal ganglia disease responded to biotin treatment. Also, riboflavin supplementation was beneficial to patients with riboflavin transporter disorders in the Brown Vialetto Van Laere Spectrum and with mutations in ACAD9 gene (Scholte et al., 1995, Gerards et al., 2011).

Oral administration of idebenone (a short-chain of benzoquinone) in combination with vitamin supplementation ($B_{12}$ and C) resulted in accelerated visual recovery and improvement of final visual outcome in patients with LHON (Mashima et al., 2000, Carelli et al., 2001). However, in two cases the individuals did not show any visual benefit (Barnils et al., 2007). Therefore, a Phase II double blind randomized placebo controlled trial was conducted were 85 affected individuals were enrolled (Klopstock et al., 2013). The follow up study showed beneficial effects of 6 months of treatment with idebenone even after the discontinuation of the active medication (Klopstock et al., 2013). The European Medicines Agency (EMA) approved Raxone (active substance: idebenone) under exceptional circumstances. According to EMA, the company that markets Raxone should conduct further studies on the long-term effects and safety of the medication (www.ema.europa.eu/).

Regarding amino-acid supplementations, three different amino-acids (L-arginine, citrulline and taurine) have been proposed as potential therapeutic targets in MELAS syndrome. Stroke,

which is one of the major symptoms of MELAS syndrome, are thought to result from vascular endothelial dysfunction. Therefore, observation of low levels of citrulline in some affected patients led to the hypothesis that the disturbed nitric oxide homeostasis might be partly responsible for the pathogenesis of MELAS syndrome (Naini et al., 2005). Open-label studies examining the effect of the supplementation with arginine of patients presenting with MELAS syndrome demonstrated reduced frequency and severity of stroke-like episodes in affected patients (Koga et al., 2005, Koga et al., 2006, Koga et al., 2010). Recent studies have suggested that citrulline might be even more effective than arginine in MELAS syndrome (El-Hattab et al., 2012).

Moreover, an open-label study in which patients suffering from MELAS syndrome followed a high-dose oral taurine administration over a 9-year period, demonstrated amelioration of epilepsy and prevention of strokes (Rikimaru et al., 2012).

Constant exercise is linked to mitochondrial proliferation as an adaptive mechanism of the cell to the increased energy needs. Similarly, elevated mitochondrial biogenesis is also observed in many mitochondrial disorders possible as a compensatory mechanism due to the need for higher energy metabolism. Therefore, it has been suggested that exercise might be beneficial for individuals suffering from mitochondrial disorders and consequently from exercise intolerance. It is still unclear whether exercise promotes proliferation of wild-type of both mutant and wild-type mitochondria (Taivassalo et al., 2001, Taivassalo et al., 2006). Increased levels of only normal mitochondria would result in increased levels of ATP production whereas increased levels of wild-type and mutant mitochondria could possibly trigger the ROS production. Preliminary studies have showed that exercise results in increased levels of total mtDNA content but the ratio of wild-type/mutant remains steady (Murphy et al., 2008).

Recently novel approaches have been suggested for treatment of mitochondrial disorders. The Zinc Finger Nucleases (ZFN) and Transcription Activator Like Effector Nucleases (TALEN) successfully target a subset of pathogenic mtDNA mutations and lead to degradation of the molecule (Nightingale et al., 2016). However, the lack of the restriction sites derived from mutations, the specificity of restriction endonuclease targeting and lastly the efficiency of targeting recombinant proteins into cell and mitochondria are still challenging and need further investigation. Another way of manipulating the mtDNA is the use of peptide nucleic acids which have the ability to selectively bind and induce direct mtDNA strand degradation (Mukherjee et al., 2008, Kyriakouli et al., 2008). Manipulation of

tRNA synthetases has proved successful for stabilizing the mt-tRNA molecules (Rorbach et al., 2008) and recently it was demonstrated that overexpression of human non-cognate mitochondrial leucyl tRNA synthetase partially rescued the biochemical dysfunction due to mtDNA defects (Perli et al., 2014, Hornig-Do et al., 2014). Lastly, gene transfer using adeno-associated viral vectors (AAV) can be used to replenish the impaired expression due to pathogenic mutations in nDNA or mtDNA. Typical examples of AAV are the AAV-*ETH1* and AAV-*ND4* (Nightingale et al., 2016). The main challenges with the use of AAV are the misexpression of the target-gene in the cell, the efficient delivery to the desired cell population and the packaging of constructs as adenoviruses are not able to carry constructs larger than 5kb.

Additionally, the protein replacement or the removal of accumulated toxic metabolites via systemic protein delivery has been explored as a potential treatment in mitochondrial neurogastrointestinal encephalopathy (MNGIE) but there is still no convincing evidence of sustained clinical benefit (Hussein, 2013). Systemic injection of TFAM in LHON cybrids led to increased respiratory chain protein levels and enhanced cellular respiration (Iyer et al., 2009). However, mice with overexpressing TFAM demonstrated increased mtDNA copy number accompanied with mtDNA deletions and respiratory chain deficiency (Ylikallio et al., 2010). Therefore, it is of great importance to be cautious when interpreting results from different cellular and animal models as they might be misleading.

In recent years, the stimulation of mitochondrial biogenesis has become attractive therapeutic target of mitochondrial disorders. The mitochondrial proliferation is coordinated by the transcriptional co-activator PPAR-γ 1α (PGC-1α) though a complex signalling cascade. A few studies have shown that the drugs Bezafibrate (Yatsuga and Suomalainen, 2012), Resveratrol (Csiszar et al., 2009) and AICAR (Viscomi et al., 2011) have beneficial effects on either cell or animal models with mitochondrial diseases. All of them target and try to regulate the signalling cascade of the mitochondrial proliferation. However, it is still not clear whether proliferation of both mutated and normal mtDNA copies is beneficial to the cell.

Finally, according to preclinical and increasing clinical evidence stem cell therapies can be used in several neurological disorders with mitochondrial dysfunction such as Parkinson's disease. Patients with MNGIE underwent haemo/peritoneal dialysis or platelet transfusions and as a result the levels of circulating toxic thymidine (Hussein, 2013).

## 1.4 Mitochondrial Dynamics

Mitochondria are flexible, plastic organelles that have the ability to move along cytoskeletal tracks and fuse and divide. Hence, they can build interconnected networks (Bereiter-Hahn, 1990). The activities of fusion and fission are simultaneously antagonistic and balanced. Both of them assist the cell to adapt to different physiological conditions. Increased activity of fusion results in large mitochondrial networks, required by metabolic active cells. On the other hand, increased fission is characterised by numerous mitochondrial fragments characteristic of a less active cells.

### 1.4.1 *Mitochondrial Fusion*

Mitochondrial fusion is a conserved process along all the eukaryotic cells containing mitochondria.

Mitofusin 1(*Mfn1*) and Mitofusin 2 (*Mfn2*) are two of the three core proteins actively involved in the mitochondrial fusion in mammals, mediating the outer mitochondrial membrane fusion. Both of them are large GTPases, located in the outer mitochondrial membrane and characterised by two transmembrane regions, a short loop in the intermembrane space while major parts of them are projecting to the cytosol (Rojo et al., 2002, Westermann, 2010). Also, both proteins contain heptad repeats, which mediate the tethering between neighbouring mitochondria (Koshiba et al., 2004, Santel et al., 2003). Overexpression of either Mfn1 or Mfn2 results in abnormal mitochondrial morphology illustrating the significance of those proteins (Legros et al., 2002). The expression pattern of those proteins differs between different mammalian tissues but this is possibly related to other functions of those proteins. For example, Mfn2 is involved in $Ca^{2+}$ signalling and is highly expressed in cardiac tissue and skeletal muscle. Mutations in Mfn2 are correlated to Charcot-Marie-Tooth disease whereas mutations in Mfn1 do not lead to the same disease, indicating the diversity of their functions (Züchner et al., 2004, Polke et al., 2011).

The third core component of the mitochondrial fusion machinery is the optic atrophy protein 1 (OPA1). OPA1 is located in the inner mitochondrial membrane and is responsible for the inner mitochondrial membrane fusion. It has 8 isoforms generated by alternative splicing and alternative processing at two cleavage sites. The OPA1 cleaving results in a long (l-OPA1) and short form of OPA1 (s-OPA1), both necessary for the mitochondrial fusion (Song et al., 2007). A range of proteases have been suggested to take part in the alternative processing of OPA1 such as: the rhomboid-related protease presenilins-associated rhomboid –like (PARL),

AAA proteases located in the matrix and the intermembrane space and the inner membrane peptidase OMA1 (Westermann, 2010). Mutations in OPA1 have been correlated to autosomal dominant optic atrophy (Delettre et al., 2000, Alexander et al., 2000, Burté et al., 2015) showing the significance of OPA1 expression in the optic nerve. Apart from its role in mitochondrial fusion, OPA1 plays a role in the diameter regulation of cristae junctions during apoptosis (Westermann, 2010).

The first necessary step is the close contact of the mitochondria. Once the two organelles are close enough, Mfn1 and Mfn2 form homotypic or heterotypic complex that mediates the outer mitochondrial membrane fusion. Next, OPA1 promotes the inner mitochondrial fusion which is dependent upon the inner membrane potential (Figure 1.7). Live cell imaging experiments have shown that merging of the membranes, intermembrane space and matrix happen almost simultaneously (Karbowski et al., 2004).

According to studies, a fusion event takes place every 5-20 min per mitochondrion and only around 20% of the closed-contact mitochondria manage to fuse (Twig et al., 2008). Many theories have been proposed regarding the selection of mitochondrial partners and the activation switch of the mitochondrial fusion in a cell. Recent studies suggest that the key role of the mitochondrial fusion is to marry the state of the cell with the mitochondrial function. Hence, the 'cytosolic milieu can inhibit or activate mitochondrial fusion' (Pernas and Scorrano, 2016).

Membranes of the endoplasmic reticulum (ER), which are closely opposed to mitochondria, are known as mitochondria-associated membranes (MAMs). It has been shown that following $Ca^{2+}$ release from the ER, mitochondria are exposed to higher $Ca^{2+}$ concentrations that the cytosol (Rizzuto et al., 1998). Therefore, it was suggested that there is a physical, proteinaceous linkage between these two organelles. According to Rizzuto et al, the total surface area of mitochondria juxtaposed to the ER is around 5-20% (Rizzuto et al., 1998). The MAMs seem to be a highly flexible and mercurial collection of proteins which are able to recruit a variety of signalling components according to the cell's needs (van Vliet et al., 2014).One of the best studied MAM-resident proteins is Mfn2. Knockdown of *Mfn2* in murine fibroblasts caused disruption of the ER-mitochondrial contact sited and led to morphological changes in both ER and mitochondria. Furthermore, absence of Mfn2 was associated with defective mitochondrial $Ca^{2+}$uptake outlining the necessity of ER-mitochondrial contact sites for the calcium homeostasis (de Brito and Scorrano, 2008).

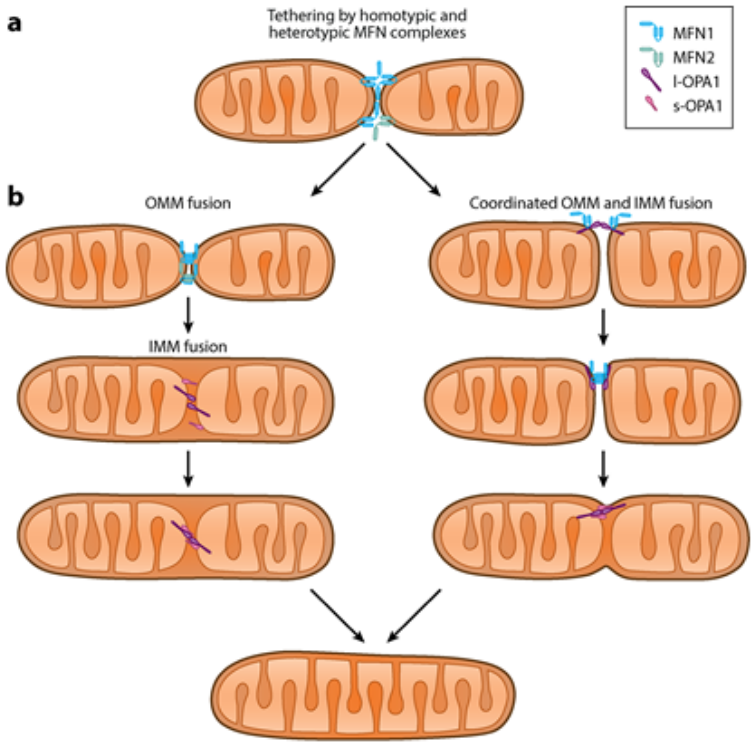



**Figure 1.7: a) Schematic representation of tethering between two mitochondria by homotypic or heterotypic interactions, b) (Left) Independent OMM and IMM fusion, (Right) Simultaneous OMM and IMM fusion (Source: (Pernas and Scorrano, 2016))**

### 1.4.2 *Mitochondrial Fission*

The opposite procedure of mitochondrial fusion is mitochondrial fission. Mitochondrial fission is also involved in apoptosis.

Mitochondrial fission consists of three key steps. Labelling of the fission site, the aggregation of the cytosolic dynamin-related protein 1 (Drp1) dimers and oligomers into a spiral-shape around the fission sites and thirdly the GTP hydrolysis and Drp1 helix compression that divides the mitochondrion (Figure 1.8) (Chan, 2012, van der Bliek et al., 2013).

Drp1 is a conserved protein containing an N-terminal GTPase, a dynamin-like middle domain and a C-terminal GTPase effector involved in self-assembly. Drp1 protein exists in the cytosol as dimers or tetramers and is recruited to the mitochondrial surface by certain receptors. In human it has been identified an alternative brain-specific splice variant characterised by an insertion between the middle domain and the GTPase effector domain (Westermann, 2010). MEFs containing null alleles of Drp1 are characterised by elongated

mitochondria and Drp1 knockout mouse presents embryonic lethality (Ishihara et al., 2009, Osellame et al., 2016).

According to studies, the mitochondrial fission sites are co-located with the contact sites of mitochondria with the endoplasmic reticulum (ER) (Friedman et al., 2011). Actin polymerization at ER-mitochondria contact sites by a protein called formin 2 (INF2) and recruitment of myosin II are necessary steps to an efficient fission event (Korobova et al., 2013, Korobova et al., 2014).

Second step is the recruitment of Drp1 to the mitochondrial surface by specific receptors. To date, four adaptors have been described to recruit Drp1: fission 1(Fis1); mitochondrial fission factor (Mff) and the mitochondrial dynamin proteins MiD49 and MiD51 with 49kDa and 51kDa molecular weight respectively. Recent studies showed that Fis1 is not in close proximity with Drp1 as the other adaptors making the role of Fis1 in mitochondrial fission a bit controversial in contrast to Mff that is possibly the main adaptor of Drp1 (Otera et al., 2010). However, Fis1 and Mff can also recruit Drp1 to the peroxisomes (Koch et al., 2005). On the other hand, the MiD proteins, discovered in 2011, are specific only to Drp1 recruitment to the mitochondria (Palmer et al., 2011). Studies have shown that constantly increased levels of cytosolic $Ca^{2+}$ might regulate activation of Drp1and consequently mitochondrial fission (Cereghetti et al., 2008). Since Drp1 is recruited from the cytosol, is polymerized into spirals around the constriction sites and the compression starts. Recent studies have shown that loss of one of the mitochondrial fission adaptors does not affect the levels of the other adaptors, the mitochondrial morphology or the levels of mitochondrial Drp1. On the other hand, deletion of multiple adaptors led to increased mitochondrial connectivity and reduced Drp1 association with the mitochondrial outer membrane. Furthermore, loss of the adaptors in MEFs presented apoptotic resistance as they retained cytochrome c possibly due to impaired formation of Drp1 oligomers (Osellame et al., 2016).

The division of the matrix, outer and inner mitochondrial membrane is not fully understood yet. Also, it is not known if the constriction of the mitochondrial membranes occurs simultaneously or in distinct steps. The final result is the production of one or more daughter mitochondria (Pernas and Scorrano, 2016).

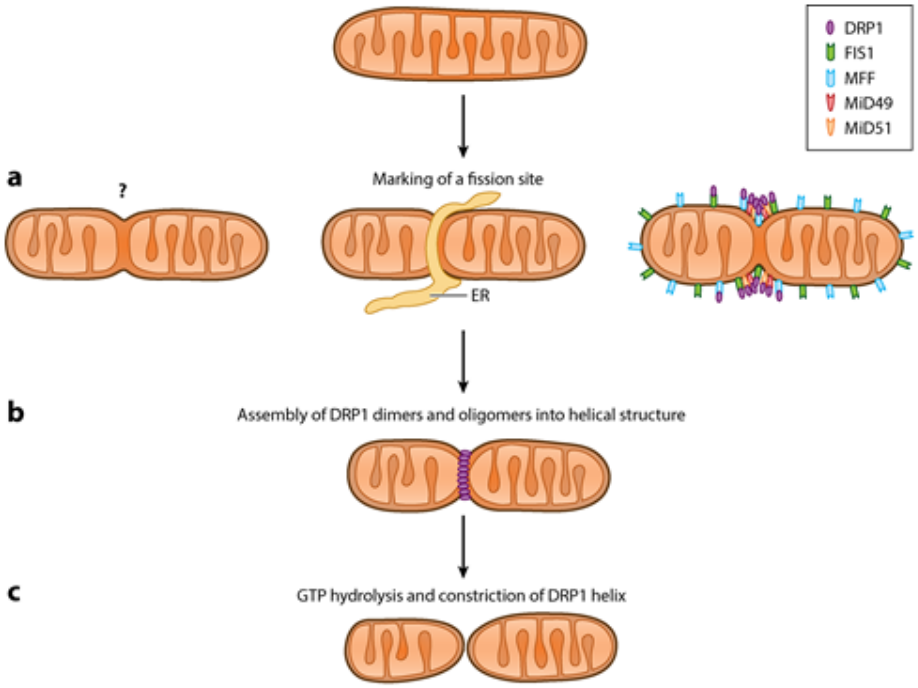



**Figure 1.8: Mitochondrial fission. a) Recruitment of DRP1, b) Helical structure of DRP1, c) GTP hydrolysis and division of OMM and IMM generating two daughter mitochondria (Source: (Pernas and Scorrano, 2016)**

The tightly balanced mitochondrial fusion and fission determine number, morphology and activity of these multifunctional organelles. Fusion and fission modulate multiple mitochondrial functions, ranging from energy and reactive oxygen species production to $Ca^{2+}$ homeostasis and cell death. Mitochondrial fusion produces interconnected mitochondrial network and is essential for the maintenance and inheritance of mtDNA, the transmission of membrane potential and Ca2+ signaling along the mitochondrial network (Westermann, 2010). The opposing process, mitochondrial fission, leads to smaller, more discrete organelles and plays important roles in mitochondrial partitioning during mitosis, cytoskeleton-mediated trafficking to energy-demanding intracellular compartments and in selective autophagic removal of damaged mitochondria by the process called mitophagy (Archer, 2013).

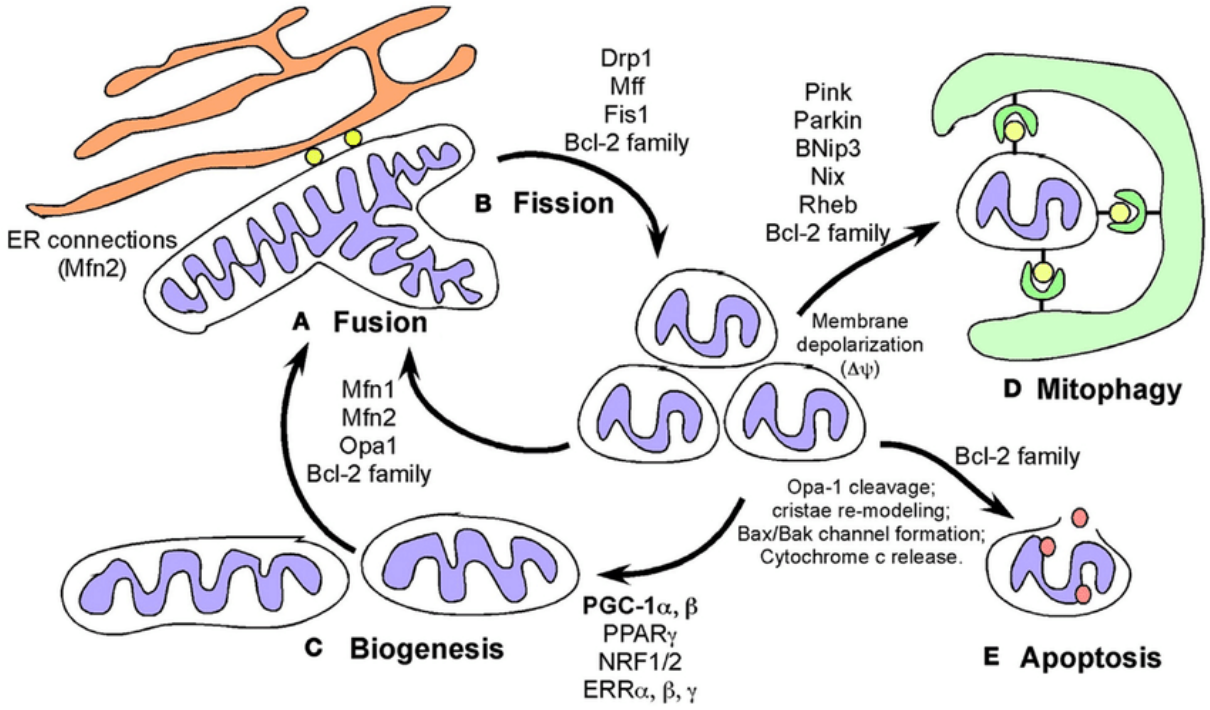



**Figure 1.9: Roles of mitochondrial dynamics**

In conclusion, mitochondrial play a vital role in the cell and any mutation affecting their normal function may lead to mitochondrial disorder. Mitochondrial disorders comprise a large group of heterogeneous diseases characterised by clinical heterogeneity and tissue specificity. These characteristics complicate the development of an efficient treatment. The improved understanding of the exact molecular pathomechanism of each mitochondrial disease and the role of tissue specificity will help us to develop effective treatments.

# Chapter 2. Aims

## 2.1 Overview

Mitochondria are necessary organelles in the eukaryotic cells, whose primary function is the ATP production through the oxidative phosphorylation system. Although mtDNA encodes key proteins for the proper function of the mitochondria, the vast majority of the essential respiratory chain components and proteins needed for the maintenance and replication of the mitochondrial are encoded by the nDNA. Mutations in both mtDNA and nDNA often lead to mitochondrial disorders. Mitochondrial disorders comprise a large group of heterogeneous disorders which are characterized by impairments in the cellular energy production. Patients suffering from mitochondrial disorders usually display multi-systems disorders, with high demand oxygen tissues being affected the most.

1. It has previously been shown that supplementation of L-cysteine in cell lines of patients suffering from reversible infantile respiratory deficiency (RIRCD) and reversible infantile hepatopathy (TRMU deficiency) showed an improvement in most respiratory chain complexes activities. As a result, L-cysteine may have broader effect in the extended group of mitochondrial disorders. Therefore, I studied the effect of *in vitro* supplementation with L-cysteine and N-acetyl-cysteine in patient cell lines, with the aim to explore the potential of those substrates as treatment for a certain group of mitochondrial disorders. In these studies, the applied fibroblasts cell lines will be isolated from patients suffering from multiple types of mitochondrial disorders.

2. Secondly, to investigate the reason behind the tissue specific presentation of some mitochondrial translation deficiencies my research will focus on the effect of mitochondrial translational deficiencies in neuronal cell types. Aim of this part of the research is to examine the tissue specificity of the mitochondrial translational deficiencies in neurons generated by direct conversion of fibroblasts to neuron progenitor cells.

3. Final aim of my research is to identify novel mutations in the nuclear genome which may have an effect in the process of mitochondrial translation. I will evaluate variants resulting from exome sequencing which may play potential role in mitochondrial translation deficiencies in patients who suffer from mitochondrial disorders.

# Chapter 3. Materials and Methods

## 3.1 Cell Culture

All cell culture work was performed in a sterile environment ensured by a biological class II airflow safety cabinet (HeraSafe; Thermo-Scientific, Hampshire, UK). All cell culture flasks were incubated in a humidified incubator at 37$^{O}$C with 5% carbon dioxide ($CO_2$) throughout the experiment.

### 3.1.1 *Fibroblasts and myoblasts maintenance*

Fibroblast and myoblasts cell cultures were obtained from the Medical Research Council (MRC) BioBank, Centre for Neuromuscular Diseases, Newcastle. Informed consent was obtained from all subjects. Fibroblast cells were grown in high glucose Dulbeccos modified Eagle's medium (DMEM, Gibco, Life-Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Dorset UK). When experiments allowed, cells were supplemented with 100U/mL penicillin (Sigma-Aldrich, Dorset UK) and 100mg/mL streptomycin (Sigma-Aldrich, Dorset UK). Myoblasts were cultured in Skeletal Muscle cell growth medium (ready to use) (Promocell, Germany) supplemented with the 1x Skeletal Muscle cell growth supplement mix (Promocell, Germany), 10% fetal bovine serum and 2mM L-glutamine (Gibco, Life-Technologies, Paisley, UK).

Cells were cultured in filter cap cell culture flasks (Greiner Bio-one, Stonehouse, UK) with either 25cm$^2$ or 75 cm$^2$ growth area.

Growth media has been changed every two to three days. Briefly, the growth medium was aspirated and the cells were washed with 1x Phosphate Buffered Saline (PBS, Oxoid, Thermo-Scientific, Hampshire, UK). Next, the appropriate volume of fresh growth media was added to the flask.

### 3.1.2 *Cell sub culturing*

When cell cultures reached 70-80% of confluency, cells were divided in two-three new flasks. Growth media was aspirated and cells were washed with 1x PBS. Afterwards, cells were treated with 1x Trypsin-EDTA (Life-Technologies, Paisley, UK) and incubated for 5 minutes at 37$^{o}$C. Subsequently, cells were transferred to a 15mL tube and centrifuged for 5 minutes at 318g (Heraeus™ Megafuge™ 16R, Thermo Scientific, Hampshire, UK). The supernatant was aspirated and the obtained cell pellet was resuspended in fresh growth media. The cells were

divided equally into new cell culture flasks and then the flasks were replaced in the cell culture humidifier.

### 3.1.3 *Cell counting*

Cell counting was performed with a haemocytometer (Improved Neubauer; Hawksley, Lancing, UK) and Trypan Blue Staining (Sigma-Aldrich, Dorset, UK). Cells within the cell pellet were resuspended in 1mL of growth medium. Afterwards, 10uL of the cell suspension were mixed gently with 10uL of Trypan Blue Staining and 10uL were transferred on the haemocytometer. A glass cover was placed over the counting chamber and cells were viewed and counted under the 10x objective of the microscope.

Subsequently, the number of the cells was counted within each 16 square corner of the grid (Fig. 3.1). The haemocytometer is designed so that the number of cells in one set of 16 corner squares is equivalent to the number of cells in $10^4$/mL. Therefore, to obtain the count, the total number of the cells from the corners was summed, divided with four and then multiplied by two, to adjust the dilution in Trypan Blue.

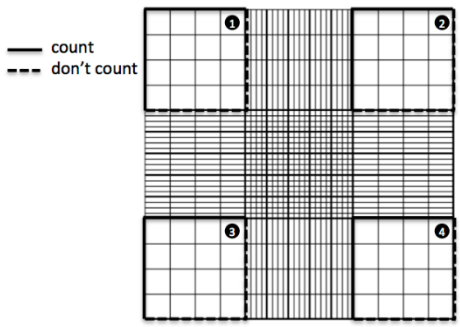



**Figure 3.1: Grid pattern of an improved Neubauer cell counting chamber**

### 3.1.4 *Preservation of the cells*

Cells required for long-term use were stored in cryovials and frozen in liquid nitrogen. After splitting cells, the obtained cell pellets were resuspended in 1mL of filter sterilized freezing medium (90% FBS, 10% Dimethyl sulfoxide (DMSO, Sigma-Aldrich, Dorset, UK). Afterwards, the resuspended cells were transferred to 2mL cryovials (Greiner Bio-one, Stonehouse, UK) and stored short term at -80$^{o}$C, and for longer term in liquid nitrogen.

To retrieve cells from liquid nitrogen, they were thawed quickly at 37$^{o}$C in a temperature controlled water bath (Grant Bath JB AQUA 18 PLUS; Thermo-Scientific, Hampshire, UK), then transferred to a 15mL tube containing 4mL of growth medium and subsequently centrifuged for 5 minutes at 318g. Afterwards, the supernatant was aspirated, the cell pellet was resuspended in 5mL of growth medium, and the resuspended cells were transferred to T-25 cell cultured flask which was then replaced in the cell culture humidifier.

### 3.1.5 *Mycoplasma detection*

All cultured cell lines were tested regularly for mycoplasma infection using a luminescent detection kit (Lonza, UK) according to manufacturer's instructions. Initially, 100uL of cell supernatant were transferred to a 1.5mL Eppendorf tube and 100uL of re-constituted MycoAlert™ reagent added to the sample followed by 5 minutes incubation in RT. At the end of the incubation, a luminescent reading was taken (reading A). Afterwards, 100uL of the MycoAlert™ substrate were added to the sample and the mix incubated for 10 minutes in room temperature (RT). Then, a second reading was taken (reading B) and the ratio of reading B to A was calculated. The sample was characterised as 'mycoplasma negative' if the ratio was below 0.9 and as 'mycoplasma positive' if greater than 1.2. If the ratio was calculated between 1-1.2 the sample was re-tested.

All mycoplasma positive samples were destroyed by 2ml of 2% (w/v) Virkon® (Du Point, Hertfordshire, UK).

### 3.1.6 *L-cysteine and N-acetyl cysteine (NAC) supplementation*

L-cysteine (Sigma-Aldrich, Dorset, UK) and NAC (Sigma-Aldrich, Dorset, UK) solutions (50mM stock solutions) were prepared fresh before each use, and the appropriate volume was added to each flask. During the supplementation with L-cysteine or NAC, fibroblasts were grown in DMEM supplemented with 10% FBS without antibiotics. The growth medium was renewed every 48 hours as described previously.

## 3.2 Purifications

### 3.2.1 *DNA purification from cultured cells*

DNeasy Blood & Tissue Kit (Qiagen, Manchester, UK) was used to isolate DNA from cultured cells according to manufacturer's instructions.

Cells were initially treated with trypsin (as described previously), placed in a 1.5mL Eppendorf tube and pelleted after 5 minute's centrifugation at 300g. The supernatant was discarded and the obtained cell pellet was resuspended in 1x PBS to a final volume of 200uL. Afterwards, 20uL of proteinase K (provided with the kit) were added to the resuspended cells followed by 10 minutes incubation at 56$^{o}$C.

Secondly, 200uL of ethanol (96-100%) (Sigma-Aldrich, Dorset, UK) were added to the sample and the solution pulse-vortexed for 15 seconds. The homogenised mixture was transferred to a QIAamp Mini spin column (provided with the kit), placed in a 2mL collection tube (provided with the kit), and centrifuged for one minute at 6000g. The filtrate was discarded and 500uL of Buffer AW1 (provided with the kit) were added to the column followed by one minute centrifugation at 6000g. Subsequently, 500uL of Buffer AW2 (provided with the kit) were added to the spin column followed by a full speed centrifugation for 3 minutes. The filtrate was discarded; the column was placed in a new 2mL collection tube (provided with the kit) and centrifuged at full speed for one minute. Finally, the column spin was placed in a clean 1.5mL Eppendorf tube and 100uL of Buffer AE were added. The column spin was incubated in RT for one minute and then, centrifuged at 6000g for one minute. The yield and purity of the purified DNA were determined by NanoDrop 2000 UV-Vis (Thermo-Scientific, Hampshire, UK). The elute was stored at -20$^{o}$C until required.

### 3.2.2 *DNA purification from whole blood*

Nucleon Blood Non-Chloroform Kit (Gen-Probe Life Sciences Ltd., Manchester, UK) was used to isolate DNA from whole blood according to manufacturer's instructions.

Five mL of whole blood were transferred to a 15mL tube and 2 mL of Reagent A were added. Next, the solution was inverted 4 times at RT and centrifuged at 3500g for 5 minutes. The supernatant was discarded and the remaining pellet was resuspended in 5 mL of Reagent A (provided with the kit). The solution was vortexed and centrifuged at 3500g for 5 minutes.

The supernatant was discarded and the remaining cell pellet consisting of blood cells was resuspended in 1mL of Reagent B (provided with the kit) and vortexed. Afterwards, 350uL of Reagent C (provided with the kit) were added and the solution was mixed by inverting at least 7 times. Next, 300uL of Nucleon Resin (provided with the kit) were added drop-wise to the top of the sample and the solution was centrifuged at 3500g for 4 minutes.

After the centrifugation, the supernatant was transferred to a clean 15mL tube and one volume of 100% isopropanol (Sigma-Aldrich, Dorset UK) was added. The tube was inverted several

times until the DNA precipitation was visible. Following, the solution was centrifuged at 4000g for 5 minutes and the supernatant was discarded. Finally, the remaining cell pellet was resuspended in 1mL of 70% ethanol and centrifuged at 4000g for 5 minutes. The supernatant was discarded and the pellet was left to air dry. The yield and purity of the purified DNA were determined by NanoDrop 2000 UV-Vis (Thermo-Scientific, Hampshire, UK). The elute was stored at -20$^{o}$C until required.

### 3.2.3 *RNA purification from cultured cells*

RNeasy Mini Kit (Qiagen, Manchester, UK) was used to isolate RNA from cultured cells according to manufacturer's instructions.

Cells were pelleted as described before and 350uL of Buffer RLT (provided with the kit) were added to the cell pellet. After vortexing thoroughly, the lysate was transferred directly into a QIAshredder spin column (provided with the kit) placed into a 2mL collection tube (provided with the kit) and centrifuged at full speed for 2 minutes.

Next, one volume of 70% ethanol was added to the homogenized lysate and mixed well by pipetting. Afterwards, 700uL of the sample were transferred into a RNeasy spin column (provided with the kit) placed into a 2mL collection tube (provided with the kit) and centrifuged at 8000g for 15 seconds and the filtrate was discarded. Then, 700uL of Buffer RW1 (provided with the kit) were added to the spin column followed by a 15 seconds centrifugation at 8000g. The filtrate was discarded and 500uL of Buffer RPE (provided with the kit) were added to the spin column. Subsequently the column was centrifuged again for 15 seconds at 8000g and 500uL of Buffer RPE were added to the spin column followed by 2 minutes' centrifugation at 8000g.

Finally, the RNeasy spin column was placed in a new 1.5mL collection tube (provided with the kit) and 30uL of RNase-free water (provided with the kit) was added directly to the spin column. The spin column was centrifuged for one minute at 8000g.

The RNA yield and purity of the purified RNA were measured with NanoDrop 2000 UV-Vis. The elute was stored at -20$^{o}$C until required.

### 3.2.4 *RNA purification from whole blood*

Whole blood samples were obtained from controls and patients after informed consent was obtained, and stored in PAXgene Blood RNA Tube (IVD) (Qiagen, Manchester, UK). For long-term storage, blood samples were stored at -80$^{o}$C.

Initially, 2mL of whole blood were transferred to a 15mL tube and centrifuged for 10 minutes at 3500g. The supernatant was discarded and 5mL of DEPC water were added to the obtained pellet. The pellet was resuspended and centrifuged for 10 minutes at 3500g. Afterwards, the supernatant was discarded and the remaining cell pellet was homogenously resuspended in 600uL of Buffer RLT (provided with the kit). One volume of 70% ethanol was added and the lysate was mixed by pipetting.

The following steps followed are described in section 3.2.2.

### 3.2.5 *DNA extraction for agarose gel*

QIAquick Gel Extraction Kit was used to purify DNA products from agarose gel according to manufacturer's instructions.

The required DNA fragment was excised with a scalpel from the agarose gel, placed into a 1.5mL cleaned Eppendorf tube and weighted on a HR-150AZ fine balance (A&D Company). Three volumes of Buffer QG (provided with the kit) were added per one volume of gel.

The Eppendorf tube was incubated for 10 minutes at 50$^{o}$C and vortexed every 3 minutes until the gel slices were dissolved completely. One volume of isopropanol was added to the Eppendorf tube followed by mixing. The sample was placed into a QIAquick spin column (provided with the kit), placed into a 2mL collection tube (provided with the kit) and centrifuged for one minute at 17.900g. The filtrate was discarded and 500uL of Buffer QG were added to the spin column followed by one minute centrifugation at 17.900g.

To wash bound DNA on the filter, 750uL of Buffer PE were added to the spin column and centrifuged for one minute at 17.900g. Next, the QIAquick spin column was placed into a clean 1.5mL micro centrifuge tube and 30uL of Buffer EB (provided with kit) were added. The spin column was incubated in RT for one minute and afterwards centrifuged for one minute at 17.900g. The yield and purity of the eluted DNA were quantified with NanoDrop 2000 UV-Vis.

### 3.2.6 *Total protein extraction from frozen muscle tissue*

The frozen muscle tissue samples were thawed in a Petri dish filled with dry ice and segmented. The segmented pieces were transferred to a 1.5mL Eppendorf tube, placed on wet ice, and weighted on a fine balance. Following, they were washed four times with cold PBS to remove any remaining blood. Afterwards, the segmented pieces were transferred into a glass Elvehjem potter and a proportion of ten volumes to the weight of the tissue of lysis buffer (Table 3.1) were added. The homogenization was performed by a motor-driven Teflon pestle with eight up and down strokes at 600rpm (rpm: rounds per minute). Next, the homogenate was transferred into a 1.5 mL Eppendorf tube and incubated on wet ice for 15 minutes. Then, the lysate was centrifuged for 15 minutes at 4$^{o}$C at 10000g. The supernatant was transferred to a new 1.5mL Eppendorf tube and the protein quantification was measured by Bradford assay (3.3.1). The extracted protein was stored at -80$^{o}$C until required.

| **Lysis Buffer** |
|---|
| 50mM Tris-HCl pH 7.5 |
| 130mM NaCl |
| 2mM $MgCl_2$ |
| 1% Triton X-100 |
| 1 tablet Protease Inhibitor per 10 mL |

**Table 3.1: Composition of lysis buffer used for total protein extraction from muscle tissue, fibroblasts and myoblasts**

## 3.3 Quantifications

### 3.3.1 *Protein quantification*

Protein quantification was carried out using a colorimetric method (Bradford, 1976) . To quantify the concentration of protein, a standard curve was used. Hence, serial dilutions of a known concentration protein named Bovine serum albumin (BSA, Sigma-Aldrich, Dorset UK) were prepared (Table 3.4) and the absorbance measured at 595nm by a MultiScan Ascent plate reader (Thermo-Scientific, Hampshire, UK).

| Column | Protein Concentration (mg/mL) | Ratio of 1 mg/mL BSA solution (uL) to sterile water (uL) |
|---|---|---|
| **A** | 0.0 | 0/100 |
| **B** | 0.05 | 5/95 |
| **C** | 0.1 | 10/90 |
| **D** | 0.2 | 20/80 |
| **E** | 0.3 | 30/70 |
| **F** | 0.4 | 40/60 |
| **G** | 0.5 | 50/50 |

**Table 3.2: Serial dilutions used for the standard curve**

The obtained values were used to generate a calibration curve (Fig. 3.2), from which the experimental protein samples were extrapolated.

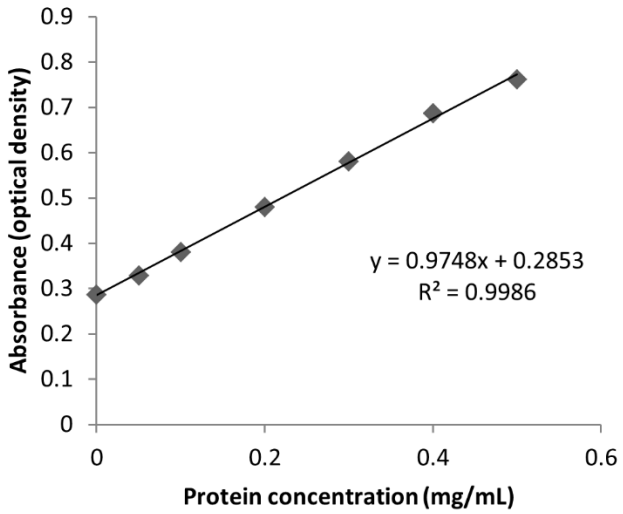



**Figure 3.2: Representative example of standard curve after the measurement of the absorbance of the serial dilutions.**

### 3.3.2 *Heteroplasmy measurement*

To quantify the levels of heteroplasmy in cells lines carrying the mitochondrial mutations m.3243A>G and m.8344A>G a pyrosequencing assay was designed. A specifically designed pair of primers (one of them biotinylated to allow for specific isolation of product) generates ~200bp amplicon and then a third sequencing primer are used to amplify the 8-10 base-paired of interest and quantify a mitochondrial heteroplasmic mutation within a sample. The following technique was performed using the PyroMark™ Q24 (Qiagen, Manchester, UK) using shallow 24 well sequencing plates (Qiagen, Manchester, UK) and 0.2mL sterile 8 strip PCR tubes (Greiner Bio-one, Stonehouse, UK).

The primers used to detect the m.3243A>G and m.8344A>G mutations have been previously described by White H.E. et al (White et al., 2005) (Table 3.5). The assay method was designed using the PyroMark™ Q24 software (Qiagen, Manchester, UK).

| | **m.3243A>G (MELAS)** | **m.8344A>G (MERRF)** |
|---|---|---|
| **Forward Primer** | CCTCCCTGTACGAAAGGACA | CATGCCCATCGTCCTAGAAT |
| **Reverse Primer** | Biotin-TGGCCATGGGTATGTTGTTA | Biotin-TTTTATGGGCTTTGGTGAGG |
| **Sequencing Primer** | GGTTTGTTAAGATGGCAG | TAAGTTAAAGATTAAGAGA |
| **Sequence to analyse** | (**A/G**) GCCCGGTAATC | (**A/G**) CCAACACCT |
| **Dispensation order** | CA**G**CGTAT | T**AG**CACAC |

**Table 3.3: Pyrosequencing primers used for determining the heteroplasmy rate of the mtDNA mutations m.3243A>G and m.8344A>G.**

Each sample was amplified as described in section 3.5 (Annealing temperature: 60$^{o}$C) and the amplicons electrophoresed and visualised as described in section 3.7. Afterwards, 10uL of PCR product were transferred to a new sterile PCR tube (STARLAB, Milton Keynes, UK) and 2uL sepharose beads (GE Life science, Amersham Place, UK), 40uL binding buffer (Qiagen, Manchester, UK) and 28uL autoclaved PCR-grade deionised water were added to each sample. The PCR tubes were transferred to a BioShake thermoshaker (Quantifoil Instruments GmbH, Jena, Germany) and agitated for 10 minutes at 2000rpm. Meanwhile, the

vacuum workstation was prepared according to manufacturer's instructions and five separate troughs were filled with 50mL of 70% ethanol, 40mL of denaturation solution, 50mL 1x wash buffer, 50mL high-purity water and 70mL high-purity water respectively. The filter probes on the vacuum tool were washed thoroughly by high-purity water according to the instructions. Furthermore, the sequencing primer was diluted to 0.3uM in annealing buffer and 25uL of that mixture transferred to a PyroMark Q24 sequencing plate and subsequently the sequencing plate was placed on the specific site of the working station.

Once the samples were agitated, they were transferred to the deep plate support wells located in the front on the workstation. Then, by switching on the vacuum tool, the liquid from the PCR tubes was aspirated, and the captured beads on the filter probes of the vacuum tool were processed through ethanol for 5 seconds, denaturation buffer for 5 seconds and the wash buffer for 10 seconds. Afterwards, the vacuum tool was held vertically for 5 seconds to allow the filter probes to be drained before being switched off. Next, the filter probes were placed into the annealing buffer mixture containing 0.3um of sequencing primer, and the vacuum tool was gently shaken to release the beads into the mixture. Finally, the vacuum tool was switched on and processed through high-purity water for 10 seconds, before a final cleanse through 70mL of high purity water, switched off, and placed in the parking position.

The sequencing plate was then heated for 2 minutes at 80$^{o}$C on a digital dry water bath heat block (Benchmark Scientific, New Jersey, U.S.A), allowed to cool to room temperature over 5 minutes, and then placed into the pyrosequencing machine. The sequencing cartridge was then loaded with the pre-calculated volumes (according to the number of reactions) of enzyme mixture, substrate mixture and dNTPs, and placed in the dispensing unit in the machine.

Once the assay was finished, the data were analysed using the PyroMark™ Q24 software. The sequencing cartridge was cleaned using deionised water and the sequencing reaction plate was disposed of.

### 3.3.3 *Copy number quantification*

Quantitative PCR is a reliable and reproducible method to measure the mitochondrial DNA amplified in real time during the PCR process. The template of genes (Table 3.6) used for standard curves were amplified using the standard PCR procedure explained in section 3.5. The products were then separated by agarose gel and extracted from the agarose gel as described in section 3.2.4. The number of template copies per uL for each template was calculated using the following equation:

$$Copies\ per\ uL = \frac{\text{Concentration}}{\text{molecular weight}} * K$$

where concentration = DNA in ng/μL (expressed as $10^9$); molecular weight = template length (bp) x 2 x 330; and K = Avogadro's constant (6.022x1023 mol-1).

Serial dilutions of the template DNA in the range of $10^8$-$10^2$copies/uL enabled the generation of standard curves. The standard curves were used to ensure a linear curve in each reaction ($R^2$ between 98%-102%) with amplification efficiency within the optimal range (90-110%). The reactions were performed in a 96-well plate (Bio-Rad, Hertfordshire, UK) with a final volume 20uL and sealed by microplate plate sealers. 1x iTaq™ Universal Probes Supermix (Bio-Rad), 0.3uM forward and reverse primers, 0.2uM *ND1*-HEX and *β2M*-FAM probes and PCR-grade autoclaved sterile deionised water (to make up to 20uL reaction). The starting concentration of DNA samples was 10ng/uL. Negative controls and melting curve analysis of the amplified DNA product were used to confirm the absence of DNA contamination. The cycling conditions consisted of initial denaturation at 95$^o$C for 3 minutes, followed by 40 cycles of denaturation at 95$^o$C for 10 seconds, with annealing an extension at 62.5$^o$C for 1 minute. The relative mtDNA copy number per cell was calculated using the ΔCt data by the following equation:

ΔCt = Ct *MT-ND1* – Ct *β2M*

Relative mtDNA copy number per cell = $2(2^{-\Delta Ct})$

| Gene | Forward Primer | Reverse primer | $T_m$ ($^oC$) | Product size (bp) |
|---|---|---|---|---|
| **Template** | | | | |
| **β2M** | CGCAATCTCCAGTGACAGAA | GCAGAATAGGCTGCTGTTCC | 60 | 1092 |
| **MT-ND1** | CAGCCGCTATTAAAGGTTCG | AGAGTGCGTCATATGTTGTTC | 60 | 1040 |
| **qPCR** | | | | |
| **β2M** | CACTGAAAAAGATGAGTATGCC | AACATTCCCTGACAATCCC | 62.5 | 231 |
| **MT-ND1** | ACGCCATAAAACTCTTCACCAAAG | GGGTTCATAGTAGAAGAGCGATGG | 62.5 | 111 |

**Table 3.4: Primers sequence of template genes used for generating the standard curves**

## 3.4 Levels of protein expression

### 3.4.1 *Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

Cells were pelleted as described in section 3.1.2. The obtained cell pellets were subsequently resuspended in 50uL of lysis buffer (Table 3.1), vortexed for 30 seconds every 5 minutes (3 times) and subsequently centrifuged at 16.000g for 5 minutes. Protein quantity within the remaining supernatant containing the cellular extracts was measured by Bradford assay.

NuPAGE™ Novex™ 4-12% Bis-Tris Protein Gels and NuPAGE® MES SDS Running Buffer (Thermo Fisher Scientific, UK) were utilised for the pre-cast gel and running buffer. The sample preparation was performed according to manufacturer's instructions.

| **Reagent** | **Reduced Sample** |
|---|---|
| **Sample** | XuL |
| **NuPAGE® LDS Sample Buffer** | 2.5uL |
| **NuPAGE® Sample Reducing Agent (10x)** | 1uL |
| **Deionized Water** | up to 6.5uL |
| **Total Volume** | 10uL |

**Table 3.5: Composition of protein samples**

Protein samples with a final concentration of 20ug/ml were loaded to each well after being heated at 70$^{o}$C for 10 minutes. SeeBlue Plus2 Pre-Stained (Thermo Fisher Scientific, UK) and biotinylated ladder (CST) allowed the correct estimation of the molecular weight of each protein.

Electrophoresis was performed using the XCell SureLock™ Mini-Cell Electrophoresis System. The Upper and Lower buffer chambers were filled with 1x NuPAGE® MES SDS Running Buffer (Thermo Fisher Scientific, UK) and 500uL of NuPAGE® Antioxidant (Thermo Fisher Scientific, UK) in the upper buffer chamber.

Following electrophoresis, the separated proteins were transferred onto PVDF membrane using iBlot® 2 Dry Blotting System (Thermo Fisher Scientific, UK) according to manufacturer's instructions.

The PVDF membrane was incubated for 1 hour at RT in 5% blocking buffer (5% non-fat milk in Tween Tris-buffered Saline (TTBS)) before adding the primary antibody. The dilution factor of the primary antibody and the incubation time differed for each antibody used. Next, the membrane was washed 3 times for 10 minutes with TTBS and incubated for 1 hour at RT with horseradish peroxidase-conjugated secondary antibody. After washing with TTBS, the membrane was incubated for 5 minutes with Pierce™ ECL Western Blotting Substrate (ThermoFisher Scientific). The visualization of the proteins was performed with Amersham Imager 600 (General Electric). Details of primary and secondary antibodies can be found in Table 3.9.

ImageJ software was used to analyse the relative densities of different protein bands. The relative expression of protein in each sample was then compared to GAPDH, a ubiquitously expressed protein, which was used as a loading control.

### 3.4.2 *Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE)*

Blue native Polyacrylamide Gel allows us to separate intact mitochondrial complexes by electrophoresis based upon how much Coomassie dye was bound to the holoenzymes, which is proportional to their size. The steps followed have previously been described by Leary and Sasarman *et al*. (Leary and Sasarman, 2009).

Fibroblasts from a T75 cell culture flask were trypsinised as described previously, washed once with 1x PBS and cell pelleted for 5 minutes at 319g in a 1.5mL Eppendorf tube. The obtained cell pellet was resuspended in cold PBS with protease inhibitors (1 tablet per 10mL, cOmplete™ Mini EDTA-free Protease Inhibitor Cocktail). Next, 10uL of the resuspended cells were diluted in 90uL of $dH_2O$ and sonicated for 4 seconds (Misonix Sonicator S3000 Liquid Processor). Next, the protein quantity of the diluted samples was measured by the Bradford assay.

Cells were lysed by adding digitonin (Sigma-Aldrich, Dorset UK, 4mg/mL in cold PBS mixed with the protease inhibitor) with a final concentration of digitonin 2mg/mL and final concentration of cells at 2.5mg/mL. The ratio between digitonin and protein was equal to 0.8. The fibroblasts were incubated for 10 minutes on wet ice and afterwards 1x

PBS was added (up to 1.5mL) to dilute digitonin, and the samples were centrifuged at 10000g at 4$^{o}$C for 10 minutes.

Once the supernatant was removed, the obtained cell pellet was resuspended in Blue native sample buffer [0.5 mL 3x gel buffer (1.5 M aminocaproic acid (Sigma-Aldrich, Dorset, UK), 150 mM Bis-tris (Sigma-Aldrich, Dorset, UK), pH 7.0), 0.5 mL 2M aminocaproic acid (Sigma-Aldrich, Dorset UK), and 4 mL 500 mM EDTA] and lauryl malthoside was added to a final concentration 1%. The samples were vortexed, incubated on ice for 15 minutes, and centrifuged at 20000g at 4$^{o}$C for 20 minutes at the end. The supernatant was kept and the protein quantified by the Bradford assay. The samples were stored at -80$^{o}$C until required.

NativePAGE™ Novex™ 3-12% Bis-Tris Protein Gels, NativePAGE™ Running Buffer and NativePAGE™ Cathode Buffer Additive (Thermo Fisher Scientific, UK) were utilised for the pre-cast gel and running buffer. SBG loading buffer (with volume equal to half of the volume of the lauryl malthoside added before), was added to the samples. NativeMark™ Unstained Protein Standard allowed us to estimate correctly each of the mitochondrial complex.

The upper buffer chamber was filled with 1x NativePAGE™ Running Buffer mixed with NativePAGE™ Cathode Buffer Additive (5% v/v) whereas the lower buffer chamber was filled with 1x NativePAGE™ Running Buffer. After 30 minutes of electrophoresis at 120V, the running buffer in the upper buffer chamber was replaced with 2.5% v/v NativePAGE™ Cathode Buffer Additive and the electrophoresis was set at 180V for the remainder of the duration.

Following the electrophoresis, the separated proteins were transferred onto a PVDF membrane as described previously, and the membrane destained by destaining solution (Table 3.8). The following steps are described in section 3.4.1.

The antibodies used for detecting the mitochondrial complexes are listed in Table 3.9.

ImageJ software was used to analyse the relative densities of different protein bands. The relative expression of protein in each sample was then compared to expression levels of SDHA, a ubiquitously expressed protein in mitochondria used as a loading control.

| Tween Tris-buffered Saline (TTBS) | De-staining solution |
|---|---|
| 20mM Tris-HCl pH 7.0 | 30% methanol |
| 29.2g NaCl | 10% acetic acid |
| 0.1% Tween 20 | up to 1L |
| up to 1L | |

**Table 3.6: Composition of TTBS and De-staining solution**

| Antibody | Company | Dilution | Molecular Weight (kDa) | Recommendations |
|---|---|---|---|---|
| **Anti-NDUFA9 (ab14713)** | Abcam | 1:500 | 36 | 4°C O/N |
| **Anti-SDHA antibody** | Abcam | 1:5000 | 70 | 2hrs RT |
| **Anti-Ubiquinol-Cytochrome C Reductase Core Protein I antibody (ab110252)** | Abcam | 1:1000 | 53 | 2hrs RT |
| **Anti-COX4 + COX4L2 antibody (ab110261)** | Abcam | 1:1000 | 20 | 4°C O/N |
| **Anti-ATP5A antibody (ab14748)** | Abcam | 1:4000 | 53 | 2hrs RT |
| **Total OXPHOS Rodent WB Antibody Cocktail (ab110413)** | Abcam | 1:500 | Multiple | 4°C O/N |
| **Total OXPHOS Blue Native WB Antibody** | Abcam | 1:250 | Multiple | 4°C O/N |

| | | | | |
|---|---|---|---|---|
| **Cocktail (ab110412)** | | | | |
| **Anti-VDAC1 (ab14734)** | Abcam | 1:1000 | 37 | 4°C O/N |
| **Anti-Cytochrome C antibody (ab13575)** | Abcam | 1:500 | 12 | 4°C O/N |
| **Anti-OPA1(ab119685)** | Abcam | 1:1000 | 112 | 4°C O/N |
| **Anti-Mitofusin 2 (ab56889)** | Abcam | 1:500 | 86 | 4°C O/N |
| **Anti-DRP1 (ab56788)** | Abcam | 1:1000 | 82 | 4°C O/N |
| **Anti-SMCR7L (MiD51) (ab89944)** | Abcam | 1:1000 | 51 | 4°C O/N |
| **Anti-SMCR7 (MiD49) (16413-1-AP)** | Proteintech | 1:500 | 49 | 4°C O/N |
| **Anti-SMCR7 antibody - N-terminal (MiD49) (ab182535)** | Abcam | 1:500 | 49 | 4°C O/N |
| **Anti-SMCR7 antibody (MiD49)** | Novus Biologicals | 1:250 | 49 | 4°C O/N |

| | | | | |
|---|---|---|---|---|
| **Anti-PGC1 (ab54481)** | Abcam | 1:250 | 105 | 4$^{o}$C O/N |
| **Anti-GAPDH (sc-25778)** | Santa Cruz | 1:4000 | 37 | 4oC O/N |
| **Anti-Rabbit HRP (P0399)** | DAKO | 1:2500 | - | 1hr RT |
| **Anti-Mouse HRP (P0260)** | DAKO | 1:2500 | - | 1hr RT |

**Table 3.7: List of used antibodies**

## 3.5 Polymerase Chain Reaction (PCR)

All PCR reactions were performed using a hot start *taq* DNA polymerase and amplified using a Veriti® thermocycler (Applied Biosystems, Life-Technologies, Paisley, UK). Table 3.10 and 3.11 describe the reaction and cycling conditions for the two different enzymes used respectively. The primers (Integrated DNA Technologies, United States) were designed with Primer3 (http://primer3.ut.ee/).

| **IMMOLASE™** | | **MyTaq™ HS DNA Polymerase** | |
|---|---|---|---|
| **Reagents** | **Final Concentration (per 25uL reaction)** | **Reagents** | **Final Concentration (per 25uL reaction)** |
| **10x Immobuffer** | 1x Immobuffer | **5x MyTaq Reaction Buffer** | 1x MyTaq Reaction Buffer |
| **dNTP mix** | 2mM | **Primers (Rev and Fw)** | 0.4uM |
| **Primers (Rev and Fw)** | 0.25uM | **Autoclaved PCR-grade deionised water** | up to 25uL |
| **$MgCl_2$** | 4mM | **Enzyme** | 1U/25uL reaction |
| **Enzyme** | 1U/25uL reaction | **DNA** | 50ng/uL |
| **Autoclaved PCR-grade deionised water** | up to 25uL | | |
| **DNA** | 50ng/uL | | |

**Table 3.8: Composition of a single PCR reaction with Immolase and MyTaq polymerases**

| IMMOLASE™ | | MyTaq™ HS DNA Polymerase | | |
|---|---|---|---|---|
| **Activation** | 95°C for 10 minutes | **Initial Denaturation** | 95°C for 1 minute | |
| **Denaturation** | 95°C for 1 minute | **Denaturation** | 95°C for 15 seconds | 30-40 cycles |
| **Annealing** | 1 minute | **Annealing** | 15 seconds | |
| **Extension** | 30 seconds/Kb | **Extension** | 72°C for 10 seconds | |
| **Final Extension** | 72°C for 10 minutes | **Final Extension** | 72°C for 10 minutes | |

**Table 3.9: Cycling conditions for PCR reactions with Immolase and MyTaq polymerase.**

## 3.6 Reverse Transcription PCR

High Capacity cDNA reverse transcription (Applied Biosystems, Life-Technologies, Paisley, UK) and OneStep RT-PCR kits (Qiagen, Manchester, UK) were used to produce single stranded cDNA from RNA according to manufacturer's instructions. OneStep RT-PCR kit allows both reverse transcription and specific amplification whereas High Capacity cDNA reverse transcription allows only reverse transcription of the total RNA using random primers. The starting concentration of RNA used for reverse transcription was 2mg/mL.

The preparation of the RT master mixes was held on ice and the reactions performed on a Veriti® thermocycler according to manufacturer's instructions.

The cDNA samples were stored at -20°C until required.

## 3.7 Agarose Gel Electrophoresis

The PCR amplicons were separated with agarose gel electrophoresis. The visualisation of the DNA fragments was utilised by ethidium bromide. A 2% (w/v) agarose gel was used to resolve the products: In 100mL of 1x TAE buffer (Applichem) 2g of agarose (Bioline) were dissolved in a flask and heated for 2 minutes in an 800W microwave oven (Sony, Tokyo, Japan). Afterwards, 40uL of ethidium bromide added. The solution was then poured in a flat, horizontal gel casting tray with a comb, and allowed to solidify at room temperature. In each well, 5uL of PCR product mixed with 5uL of Orange G solution (50% $dH_2O$, 50% glycerol [v/v], and a few grains of orange G to colour the solution) were loaded and electrophoresed for 45 minutes at 65V. The gel was imaged using a GelDocIt transilluminator gel imaging system (UVP, California, U.S.A)

## 3.8 Sanger Sequencing

The first step of sample preparation before sequencing involves cleaning the PCR amplicons with ExoFAP treatment. Exonuclease I (Exo I, Thermo-Scientific, Hampshire, UK) is responsible for the digestion of the single stranded PCR primers into dNTPs and FastAP (Thermo-Scientific, Hampshire, UK);a thermosensitive alkaline phosphatase,catalyses the release of 5' and 3'-phospate groups from DNA, RNA and nucleotides.

Therefore, 10U of Exo I and 1U of FastAP were added to 3/5 uL of the PCR reaction on a MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems, Life-Technologies, Paisley, UK) and the mixture was incubated initially for 15 minutes at 37$^{o}$C before 15 minutes at 85$^{o}$C.

Next, the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo-Scientific, Hampshire, UK) was used according to the manufacturer's instruction (Table 3.12) and the samples were amplified on a Veriti® thermocycler (Applied Biosystems, Life-Technologies, Paisley, UK).

| Reagent | Concentration | Cycling Conditions | | |
|---|---|---|---|---|
| **BigDye® Terminator Reaction Mix 2.5x** | 0.125x | **Initial Denaturation** | 96°C for 1 minute | |
| **BigDye Sequencing Buffer 5x** | 0.5x | **Denaturation** | 96°C for 10 seconds | 25 cycles |
| **Primer (10uM)** | 0.5uM | **Annealing** | 50°C for 5 seconds | |
| **Template** | 1-20ng | **Extension** | 60°C for 4 minutes | |
| **Deionized water** | Up to 20uL | **Final Extension** | 4°C for ∞ | |

**Table 3.10: Composition and cycling condition of Big Dye reaction**

The final step was Ethanol/EDTA precipitation to remove any unincorporated dye terminators from the sequencing reaction. Hence, after the completion of the Big Dye Termination reaction the 96 well plate was removed from the thermal cycler and in each well 2uL of EDTA (125mM, Sigma-Aldrich, Dorset UK) , 2uL of Sodium acetate solution (3M, Sigma-Aldrich, Dorset UK) and 70uL of 100% ethanol were added. Afterwards, the plate was sealed with a plate sealer, mixed by inverting several times and incubated at RT for 15 minutes. Next, the plate was centrifuged at 2000g for 30 minutes and subsequently the plate was inverted and span up to 100g. Then, 70uL of 70% ethanol were added to the each well and the plate was centrifuged at 1650g for 15 minutes. Subsequently, the plate after the centrifugation was inverted and span up to 100g. Finally, the plate was allowed to air dry in the dark for 10 minutes.

The plate containing the cell pellets was either stored at -20$^{o}$C or the pellet in each well was resuspended in 10uL of highly deionized formamide (Hi-Di™ Formamide, Thermo-Scientific, Hampshire, UK)

Before processing the plate on the Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, Life-Technologies, Paisley, UK) it was incubated for 2 minutes at 95$^{o}$C.

The sequencing results were analysed by SeqScape (Thermo-Scientific, Hampshire, UK).

## 3.9 Oxygen Consumption

Oxygen consumption was measured in adherent fibroblasts and myoblasts with a XF96 Extracellular Flux Analyzer (Seahorse Bioscience Billerica, MA, USA) as described before (Invernizzi et al., 2012).

Each cell line was seeded in 12 wells of a XF96-well cell culture mircoplate (Seahorse Bioscience) at a density 30x10$^{3}$ cells/well (20x10$^{3}$ cells/well for iNPCs) in 80uL of DMEM and incubated for 24hrs at 37oC in 5% $CO_2$ atmosphere. After replacing the growth medium with 180uL of bicarbonate-free DMEM (prewarmed at 37$^{o}$C), cells were preincubated for 30 minutes before starting the assay procedure. Oxygen consumption rate (OCR), leaking respiration (LR), maximal capacity respiration (MCR) and not electron transport chain respiration (NMR) were determined by adding 1 μM oligomycin (Sigma-Aldrich, Dorset UK) (LR), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (Sigma-Aldrich, Dorset UK) (MCR: 2 injections of 0.5 μM and 1 μM respectively) and 1μM Rotenone/antimycin (Sigma-Aldrich, Dorset UK) (NMR), respectively.

The data were corrected by the NMR and expressed as pmol of oxygen/min/mg of protein. The quantity of protein was measured by Bradford assay(Bradford, 1976).

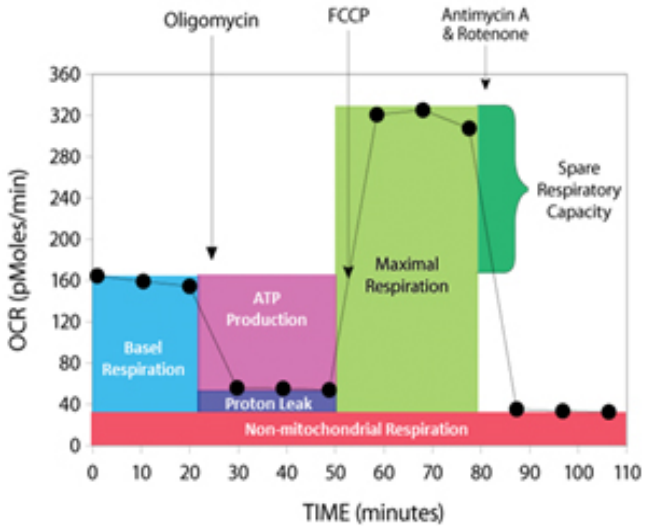



**Figure 3.3: Representative graph of oxygen consumption measurement with a XF96 Extracellular Flux Analyzer**

## 3.10 Direct conversion of human skin fibroblasts to tripotent iNPCs

Direct reprogramming of adult human fibroblasts to induced neuronal progenitor cells (iNPC) was conducted as described previously (Meyer et al., 2014). The day before transduction 100.000-150.000 cells were seeded on a 6-well cell culture plate. The growth medium used for the fibroblasts was DMEM (DMEM, Gibco, Life-Technologies, Paisley, UK) enriched with high concentrations of glucose (4.5g/L D-Glucose), GlutaMAX supplement and sodium pyruvate (110mg/L). The growth medium was supplemented with 10% of FBS, 100U/mL penicillin and 100mg/mL streptomycin.

The next day the cells were transduced with four retroviral vectors carrying the four reprogramming factors (Oct3/4, Klf4, Sox2 and c-Myc). From each virus, the appropriate amount of MOI (Multiplicity Of Infection) was used in a total volume of 800uL in each well before overnight incubattion at 37$^{O}$C.

The next day cells were washed three times with PBS and fresh fibroblast growth medium added to the well followed by an overnight rest.

Next, after 24 hours, the fibroblast growth medium was replaced by conversion medium (Table 3.13). Approximately a week after the transduction, morphological changes could be detected. The cells from the flat fibroblastic shape became smaller with distinct extensions. Additionally, cells started to form sphere-like structures that could be picked and cultured further as monolayers.

At this point, or when cells became dense they were split, seeded on fibronectin coated well, and the conversion medium was replaced by neuronal progenitor cell medium (NPC growth medium) (Table 3.13).

| Conversion Growth Medium | NPC Growth Medium |
|---|---|
| DMEM/F-12 plus 10% FBS | DMEM/F-12 plus 10% FBS |
| 1% N2 (Life-Technologies, Paisley, UK) | 1% N2 (Life-Technologies, Paisley, UK) |
| 1% B27 (Life-Technologies, Paisley, UK) | 1% B27 (Life-Technologies, Paisley, UK) |
| 20ug/mL FGF2 (PeproTech, US) | 40ug/mL FGF2 (PeproTech, US) |
| 20ug/mL EGF(PeproTech, US) | |
| 5ug/mL Heparin (Sigma-Aldrich, Dorset UK) | |

**Table 3.11: Composition of growth mediums used during the direct conversion**

### 3.10.1 *iNPC Subculturing*

When the cell culture reached 70-80% of confluency in the 6-well cell culture plate, cells were split. The growth medium was aspirated and cells were washed with PBS. Afterwards, the cells were treated with 1x Accutase (Thermo-Scientific, Hampshire, UK) and incubated for 3 minutes at 37$^{o}$C. Next, the cells were transferred to a 15mL tube and centrifuged for 4 minutes at 200g. The supernatant was aspirated and the obtained cell pellet resuspended in fresh NPC growth medium. Cells were divided equally into new wells coated with fibronectin (EMD Millipore, UK) and the cell culture plate replaced in the cell culture humidifier.

The cells were frozen in freezing medium containing 10% of DMSO per 1mL of iNPCs growth medium.

## 3.11 Statistical Analysis

Data are presented as ± standard deviation using two-way ANOVA test on Sigma plot (version 11.0) and paired t-test. A p-value of ≤0.05 was considered significant.

# Chapter 4. Studying the effect of L-cysteine and N-acetylcysteine in mitochondrial translation diseases

## 4.1 Overview

### 4.1.1 *Post-transcriptional modifications of mt-tRNAs*

The human mtDNA encodes 37 genes: 13 for the essential subunits of the complexes I, III, IV and V, 22 for tRNAs and two for mt-rRNAs. These 13 proteins are translated by the mitochondrial protein synthesis machinery, which consists of mitochondrial ribosomes, mt-tRNAs and several transcription factors. Many essential components of the mitochondrial protein synthesis machinery such as ribosomal proteins, translational factors, aminoacyl-tRNA synthetases and various factors required for the mitochondrial protein synthesis are encoded in the nucleus and transported into the mitochondria.

The mammalian mt-tRNAs have three types of unusual secondary structures (Figure 4.1). The bulk of the tRNAs are characterised by a highly conserved cloverleaf structure (Type 0). The secondary structure of the tRNA resembles a D loop, a T loop, an anticodon loop, an extra loop and acceptor stem (Figure 4.1). In 1980, a unique tRNA, in humans and bovine mitochondria, the $tRNA^{Ser(AGY)}$, was revealed through mtDNA sequencing (Anderson et al., 1981, Anderson et al., 1982). The mammalian mt- $tRNA^{Ser(AGY)}$ lacks the entire D-loop (Type III). Later studies revealed that the $tRNA^{Ser(UCN)}$ also has a non-canonical cloverleaf structure (Type I). Finally, a few mt-tRNAs such as $tRNA^{Phe}$ and $tRNA^{Asp}$ have been classified as Type II tRNAs as they lack the canonical D-loop/T-loop interaction. The various structural motifs of mt-tRNAs have been suggested to be a compensatory mechanism for the deleterious effects caused by the evolutionary pressure due to size reduction of the mtDNA sequence occurred in mammals (Suzuki and Nagao, 2011).

All the tRNAs are characterised by modified nucleosides which are introduced post-transcriptionally (Cantara et al., 2011). These modifications offer stability to the tRNAs and are required for their proper function. After the update of the RNA modification pathways database in 2008 (Czerwoniec et al., 2009), 119 different post-transcriptional modifications in RNA were revealed. The bulk of these modifications were present in mt-tRNAs.

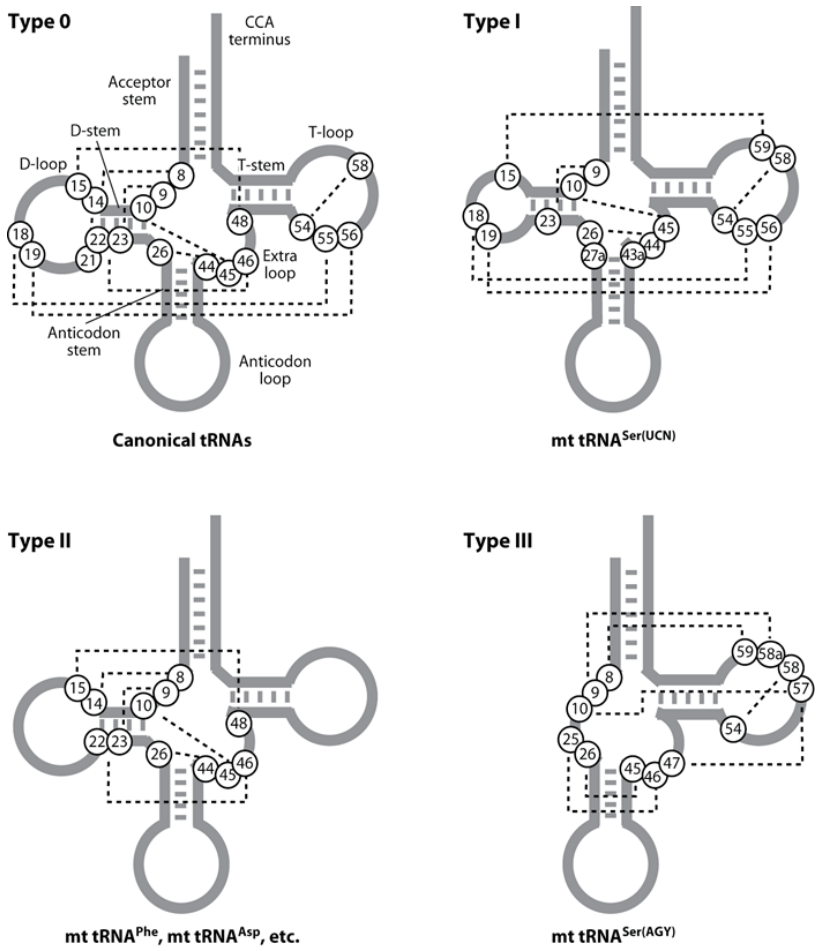



**Figure 4.1:Schematic secondary structures of human mitochondrial tRNAs. Canonical tRNA is represented as Type 0. Three types of mt-tRNAs are shown: types I, II and III. Circled numbers represent the nucleotide positions according to the tRNAdb numbering system (Jühling et al., 2009). Tertiary interactions between nucleobases are indicated by dotted lines. (Suzuki and Nagao, 2011)**

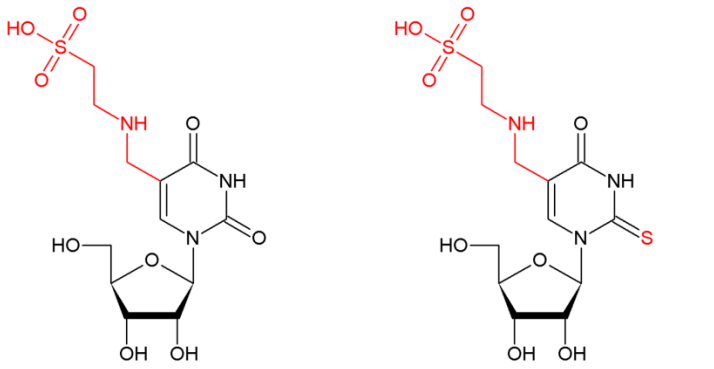
The mammalian mitochondrial decoding system consists of 60-sense codons that are classified to eight different family boxes and are deciphered by 22 species of mitochondrial tRNAs. Each of eight family codon boxes in mitochondria is decoded by a single tRNA. This unique characteristic qualifies the mammalian mitochondrial decoding system to be the smallest set of tRNAs necessary to translate all sense codons among different species (Suzuki, 2014). Therefore, post-transcriptional modifications at the wobble position of the mt-tRNAs play a critical role in the correct recognition between mt-tRNAs and codons.

Each tRNA responsible for a family box, is frequently characterized by unmodified uridine (U34) at the wobble position (first position) of the anticodon expanding the codon recognition capabilities of each tRNA. According to Crick's wobble rule, U34 can recognise only A and G at the third position of a codon. However, in a few decoding systems, including the

mitochondrial decoding system, U34 is able to recognise any of the four base pairs, a phenomenon called 'four-way wobble rule' (Suzuki and Nagao, 2011, Powell et al., 2015).

In mitochondria, the unmodified U34 is only identified in tRNAs responsible for family box codons in which at least one G or C is present at the first or second letter of codons (Suzuki, 2014). Also, the tRNAs responsible for two-codon sets ending in purines (NNR; N=any four nucleotides, R=A or G) are characterized by modified uridines ($xm^5s^2U$-type) at their wobble position. It has been suggested that the $xm^5s^2U$-type modification strengthens the recognition of NNR codons and prevents the misrecognition of codon ending in pyrimidines (NNY; Y= U and C) by restricting the conformational stability of the U34 (Suzuki, 2014).

For example, in the tRNAs of Lys, Glu and Gln U34 is modified at carbons 2 and 5. Carbon 2 is modified exclusively through thiolation ($s^2$), whereas carbon 5 can be methyl modified in various ways along different species. In the mammalian mitochondrial tRNAs taurinomethyl ($\tau m^5$) is identified. In this case, the $xm^5s^2U$-type modification offers rigidity to the U34 wobble base of tRNAs Lys, Glu and Gln (Sasarman et al., 2011).




5-Taurinomethyl uridine ($\tau m^5$)

5-Taurinomethyl 2-thiouridine ($\tau m^5s^2$)

**Figure 4.2: Chemical structures of 5-taurinomethyluridine ($\tau m^5$) and 5-Taurinomehyl 2-thiouridine ($\tau m^5s^2$)**

Absence of post-transcriptional modifications at the wobble positions of mammalian mitochondrial tRNAs for Leu and Lys has been linked to mitochondrial MERRF and MELAS respectively (Kirino and Suzuki, 2005). In MELAS, the pathogenic mutations m.3243A>G

and m.3271T>C of tRNA$^{Leu}$ result in the absence of τm$^5$U modification, leading to reduced translation of UUG codons and consequently lower expression of the UUG-rich protein ND6 (Kirino et al., 2004). Similarly, the mutation m.8344A>G in the mt-tRNA$^{Lys}$ leads to absence of τm$^5$s$^2$U modification and is characterized by severe translation deficiency of both types of AAR codon (Yasukawa et al., 2001). It is hypothesized that the presence of each mutation possibly disturbs the recognition sites of the enzyme responsible for the modifications (Kirino and Suzuki, 2005, Kirino et al., 2006). As mentioned earlier, the unmodified U34 can recognize any of the four bases as long as the codon-anticodon interaction is stabilized by one or two GC pairing at the first two base pairings. However, the cognate codons of the tRNA$^{Leu(UUR)}$ and tRNA$^{Lys}$ do not contain any G or C at the first or second position and the mutant tRNA with the unmodified uridine is not able to decipher the cognate codon effectively (Suzuki, 2014).

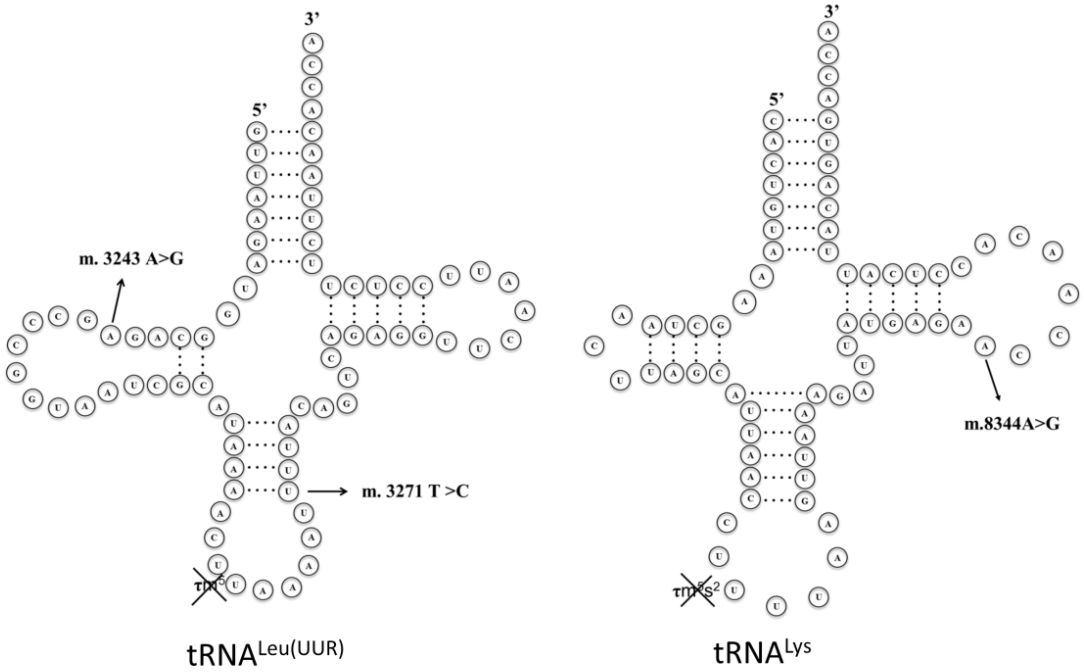



**Figure 4.3: Cloverleaf structures of human mt-tRNA$^{Leu(UUR)}$ and mt-tRNA$^{Lys}$ with the following modifies nucleosides: 5-taurinomethyluridine and 5-taurinomethyl 2-thiouridine. The pathogenic mutations of MELAS and MERRF are indicated.**

The identification of the enzymatic activity responsible for the τm$^5$s$^2$U modification in humans is still not fully understood. In E. coli, MnmE and GidA catalyse the incorporation of a carboxymethylaminomethyl at position 5 of the wobble uridine and the yeast homologs are MSS1 and MTO1. The human proteins GTPBP3 (MSS1) and MTO1, both localized in

mitochondria, were able to reverse the mitochondrial phenotype in corresponding yeast deletion strains (Li and Guan, 2002, Li et al., 2002) indicating that they are responsible for the taurinomethyl modification at carbon 5 of U34. It is hypothesized that GTPBP3 and MTO1 use taurine for the mt-tRNA modification in a similar way to which *E. coli* use glycine. However, to date there is no evidence to support this theory (Suzuki, 2014). Mutations in these genes are associated with combined respiratory chain deficiency resulting in hypertrophic cardiomyopathy and lactic acidosis (Ghezzi et al., 2012, Baruffini et al., 2013, Kopajtich et al., 2014).

The responsible enzyme for thiolation ($s^2$) of the carbon 2 of U34 is a mitochondrial specific thiouridylase called MTU1, also known as *TRMU*. It has been suggested that the initial step of 2-thiolation might rely on the cysteine desulfurase NFS1, a component of the iron sulfur cluster assembly machinery for supplying the sulfur atom (Nakai et al., 2004). Afterwards, the sulfur from the cysteine is transferred to still unknown sulfur mediators. The final step of the 2-thiolation is conducted by MTU1 by using the activated sulfur from the mediators (Umeda et al., 2005). According to studies, deletion mutants of *MTU1* in yeast led to impaired 2-thio modification of the mitochondrial tRNAs for Lys, Glu and Gln (Umeda et al., 2005).

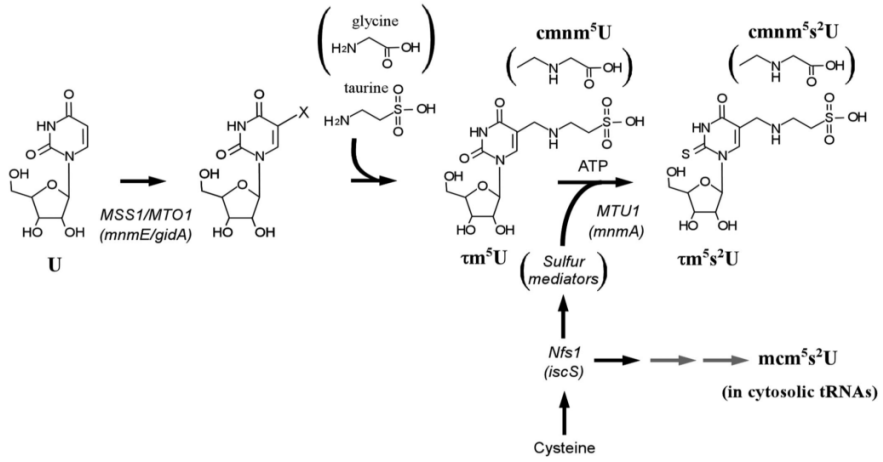



**Figure 4.4: Schematic diagram of the biosynthetic pathway that introduces the τm$^5$ and τm$^5$s$^2$ modifications of mitochondrial tRNAs. The structures in the brackets represent the *E. coli* homologs (Umeda et al., 2005).**

Although mutations in *MTO1* and *TRMU* are associated with hypertrophic cardiomyopathy and acute infantile liver failure respectively, mitochondrial protein synthesis is not affected consistently in patients' cell lines (Zeharia et al., 2009, Ghezzi et al., 2012, Sasarman et al.,

2011). This suggests that mitochondrial dysfunction might not only be caused by the impaired mt-tRNA modification but by an unknown mechanism, also contributing to the phenotype (Armengod et al., 2014).

In addition to that, it has been shown that the mutation m.3243A>G which causes MELAS syndrome, activates retrograde signalling involving ROS, kinase JNK, retinoid X receptor α and transcriptional coactivator PGC1α (Chae et al., 2013), which results in reduced mRNA levels of nuclear-encoded OXPHOS enzymes via transcriptional regulation. Therefore, the mitochondrial dysfunction is further impaired. It has also been shown that ROS production triggers the expression of microRNA 9/9*. As a result, the protein levels of *TRMU*, *GTPBP3* and *MTO1* decrease due to mRNA destabilization given that *TRMU*, *GTPBP3* and *MTO1* mRNAs are direct targets for the microRNA (Meseguer et al., 2015). Considering all the data together, it is hypothesised that the ROS-dependent induction of microRNA9/9* in MELAS cells may significantly contribute to the observed mitochondrial dysfunction.

Although the primary function of mitochondria is to generate ATP, a small fraction of electrons from electron transport chain are transferred directly to $O_2$, resulting in the generation of the superoxide anion, which can give rise to other reactive oxygen species (ROS) as well as reactive nitrogen species (RNS). Therefore, mitochondria are the major source of ROS in the cell. The ROS production is not harmful under physiological conditions as they play a signalling role in the cell. However, under pathological conditions where there is an impairment of the mitochondrial function, the ROS production can be increased. The main mechanism of antioxidant defence against ROS and electrophiles is glutathione (GSH). The de novo synthesis of GSH consists of two sequential enzymatic ATP-dependent reactions. In the first step, cysteine and glutamate are linked to form γ-glutamylcysteine; a reaction catalysed by the γ-glutamylcysteine synthase (γ-GCS). This step is rate-limiting in the synthesis of GSH and highly dependent upon cysteine availability. In the second step, the γ-glutamyl-cysteine is linked to glycine by glutathione synthetase (GS) and the final product is GSH. The synthesis of GSH occurs strictly in the cytosol and is distributed to other organelles (Marí et al., 2009). The mitochondrial GSH (mGSH) arises from the cytosol by the activity of specific carriers named 2-oxyglutarate carrier (OGC) and dicarboxylate carrier (DIC). The mGSH is a critical component of the antioxidant defensive system preventing and repairing oxidative damage generated during normal aerobic metabolism (Marí et al., 2013).

In conclusion, although the mechanism underlying MELAS and MERRF syndromes is the absence of the post-transcriptional modification, it has been shown that other mechanisms,

like increased ROS production, also impair mitochondrial translation. However, cysteine seems to be a key between these underlying processes as it plays a pivotal role in the taurinomethylation and 2-thiolation U34 since cysteine is the precursor of taurine and is also essential for the GSH synthesis.

### 4.1.2 *L-cysteine and N-acetylcysteine*

L-cysteine (Cys) is a semi-essential amino acid encoded by the codons UGU and UGC, and the cysteine's thiol group is of vital importance for a variety of critical enzymatic reactions within the cell. L-cysteine, along with glutamate (Gln) and glycine (Gly), comprise the main building block of glutathione (GSH), the most important antioxidant in the cells. Moreover, L-cysteine plays a pivotal role in a number of cellular processes, such as protein synthesis, iron-sulfur (Fe-S) biogenesis, taurine biosynthesis and regulatory and structural changes in proteins. The transport of cysteine is regulating cellular biosynthesis as well as modulating the availability of sulfur for mitochondrial metabolism. Experimental evidence suggests that cysteine is transported mainly by the alanine-serine-cysteine (ACS) system. The ACS system is a ubiquitous system of $Na^+$-dependent neutral amino acid transport in a variety of cells (Bavarsad Shahripour et al., 2014).

In the neonatal period, the availability of cysteine is limited due to reduced endogenous synthesis of cysteine from methionine by the transsulfuration pathway. The activity of the rate-limiting enzyme in the pathway, cystathionase, is very low at birth and increased slowly during the first few months of life. Therefore, cysteine is an essential amino acid in preterm infants (Zeharia et al., 2009).

N-acetyl cysteine (NAC) is a derivative of cysteine wherein an acetyl group is attached to a nitrogen atom. It is a membrane-permeable cysteine precursor that does not require active transport so it can enter the cell passively. When free NAC enters a cell it is rapidly hydrolysed to release cysteine. Initially, NAC was used as an antidote of acetaminophen (Paracetamol) overdose (Green et al., 2013). However, in recent times it has become a widely utilised nutritional supplement due to its reported antioxidant nature.

Moreover, NAC has been trialled as a treatment for a variety of medical conditions such as chronic obstructive pulmonary disease, contrast induced nephropathy, atrial fibrillation and HIV and influenza A infection, and is considered a relatively well-tolerated and safe medication (Deepmala et al., 2015).

In recent years, NAC has also been suggested as a putative therapeutic target for psychiatric and neurological disorders. According to preclinical studies, NAC may modulate a number of pathophysiological processes such as oxidative stress (Smaga et al., 2012), neurogenesis, apoptosis, mitochondrial dysfunction and inflammation within the brain, and all of which have been shown to be involved to differing degrees in a number of neurological disorders (Deepmala et al., 2015) such as Alzheimer's disease (AD) (Sandhir et al., 2012).

Regarding the regulation of the mitochondrial dysfunction, supplementation with NAC has been shown to regenerate the mitochondrial membrane potential in animal models of inflammatory bowel disease. As a result, the membrane permeability and apoptosis were decreased (Amrouche-Mekkioui and Djerdjouri, 2012). Similar effects on the membrane potential were detected in lung epithelial cells, animal model of myocardial infraction (Basha and Priscilla, 2013) and Huntington's disease (Sandhir et al., 2012).

Also, supplementation with L-cysteine has been shown to improve the thiolation and reverse the mitochondrial defect in patients' cells suffering from Reversible Infantile Respiratory Chain Deficiency (RIRCD) and from reversible infantile hepatopathy due to TRMU deficiency (Boczonadi et al., 2013).

## 4.2 Aims

As discussed previously, studies by our group have shown that supplementation with L-cysteine could be used as potential treatment for patients that suffer from Reversible Infantile Respiratory Chain Deficiency (RIRCD) and from reversible infantile hepatopathy due to TRMU deficiency. The genetic cause of the RIRCD is a homoplasmic $tRNA^{Glu}$ mutation (Boczonadi et al., 2013).

It has been suggested that since the availability of cysteine (which is crucial for normal TRMU activity) in the neonatal period is limited by the low activity of the cystathionase enzyme, dietary cysteine intake may be very important at this age. RIRCD myoblasts showed low in-gel activities of the respiratory chain enzymes, however adding L-cysteine to the culture medium fully reversed this defect. Furthermore, L-cysteine prevented the decrease of mitochondrial translation in *TRMU* deficient cells, *TRMU* down-regulated RIRCD cells and controls, further supporting the hypothesis that low cysteine concentrations may play a role in triggering reversible in vitro mitochondrial translation defects (Boczonadi et al., 2013).

As discussed earlier, the mutations m.3243A>G and m.8344A>G are associated with MELAS and MERRF syndromes respectively. Both mutations lead to absence of $\tau m^5U$ and $\tau m^5s^2U$ modification respectively. Taken together, it was hypothesized that supplementation of cells of patients with MELAS and MERFF syndrome with L-cysteine/N-acetylcysteine could reverse the mitochondrial defect through cysteine acting as a sulfur donor to improve the taurinomethylation and 2-thiolation of U34 and possibly increase the production of GSH leading to an improvement of the impaired mitochondrial translation and function simultaneously.

In the study, four cell lines carrying autosomal recessive mutations in *MTO1* and *TRMU* genes and two further cell lines with nuclear mitochondrial disease of different pathomechanism (*ELAC2* and *COX10*) were also included. *ELAC2* encodes an endonuclease, responsible for the removal of the 3-prime extensions from tRNA precursors, which is an essential step in tRNA biogenesis and mutations of this gene were associated with early onset cardioencephalomyopathy (Haack et al., 2013). *COX10* encodes a cytochrome c oxidase (COX) assembly protein involved in the mitochondrial heme biosynthetic pathway, by catalyzing the farnesylation of a vinyl group, resulting in the conversion of protoheme (heme B) to heme O. The COX10 protein is required for the expression of functional COX (Antonicka et al., 2003a) and mutations in this gene result in multisystem mitochondrial disorders (Antonicka et al., 2003a, Pitceathly et al., 2013).

The aims of the project were firstly to determine safe tolerable levels of L-cysteine and NAC for *in vitro* studies with fibroblasts; secondly to determine whether L-cysteine and NAC have an effect on translation and eventually mitochondrial function and thirdly to determine whether this effect is specific upon the causative genotype of the different mitochondrial deficiencies studied.

## 4.3 Materials and methods

The patients used for this study carried pathogenic mutations in mitochondrial tRNA genes (m.3243A>G MELAS, m.8344A>G MERRF), in nuclear genes affecting the mitochondrial protein synthesis (*MTO1*, *TRMU* and *ELAC2*) or the respiratory chain complex assembly (*COX10*). All cell lines were primary fibroblasts apart from the cell lines carrying the mutations in *MTO1* and *TRMU* genes. The patients carrying the mutations in *TRMU*, *MTO1* and *ELAC2* have been previously reported (Schara et al., 2011, Taylor et al., 2014, Boczonadi

et al., 2013). The precise position of the mutations and the phenotype of the patients used for this study can be found in Table 4.1.

| CELL LINES | GENETIC DEFECT | CLINICAL PRESENTATION |
|---|---|---|
| **MELAS 1** | m.3243A>G (Heteroplasmy: 55%) | diabetes, deafness, pigmentary retinopathy |
| **MELAS 2** | m.3243A>G (Heteroplasmy: 90%) | diabetes, deafness, epilepsy, ataxia, peripheral neuropathy |
| **MERRF 1** | m.8344A>G (Heteroplasmy: 83%) | myoclonic epilepsy |
| **MERRF 2** | m.8344A>G (Heteroplasmy: 95%) | myoclonic epilepsy, ataxia, neuropathy, myopathy |
| **MERRF 3** | m.8344A>G (Heteroplasmy: Unknown) | - |
| ***TRMU*** | c.711_712insG: p.Gln238Alafs*14 / c.1081_1082insAGGCTGTGC | reversible infantile liver failure |
| ***MTO1*** | c.631_631delG: p.Gly211Aspfs*3 / c.1282G>A: p.Ala428Thr | infantile lethal cardio-encephalomyopathy |
| ***ELAC2*** | c.1478C>T: p.Pro493Leu / c.1621G>A: p.Ala541Thr | infantile lethal cardio-encephalomyopathy |
| ***COX10*** | c.2T>C, p.Met1Thr homozygous | infantile lethal Leigh syndrome |

**Table 4.1: Summary of the clinical presentations and pathogenic mutations of the patients, whose fibroblasts were used in this study.**

By the end of supplementation, the cells were collected and measurement of oxygen consumption, BN-PAGE gels, heteroplasmy levels and mtDNA copy number was conducted. All the experiments were done in triplicates.

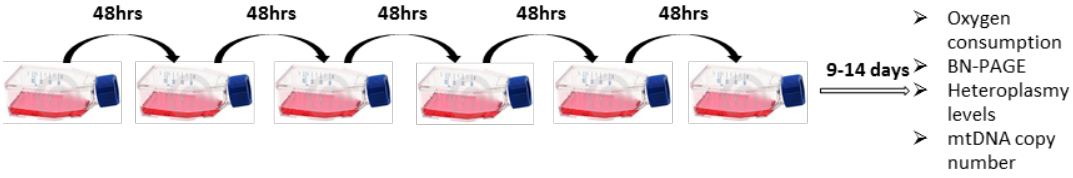



**Figure 4.5: Schematic diagram of the steps followed during the supplementation with L-cysteine and NAC.**

## 4.4 Results

### 4.4.1 *Effect of L-cysteine supplementation in MELAS and MERRF cell lines*

Initially, two different patient cell lines (MELAS1 and MERRF3) together with two control cell lines were supplemented with 1mM of l-cysteine for 9 days. By the end of the supplementation in both treated and untreated cell lines, the levels of oxygen consumption and protein expression levels of the respiratory enzymes were measured.

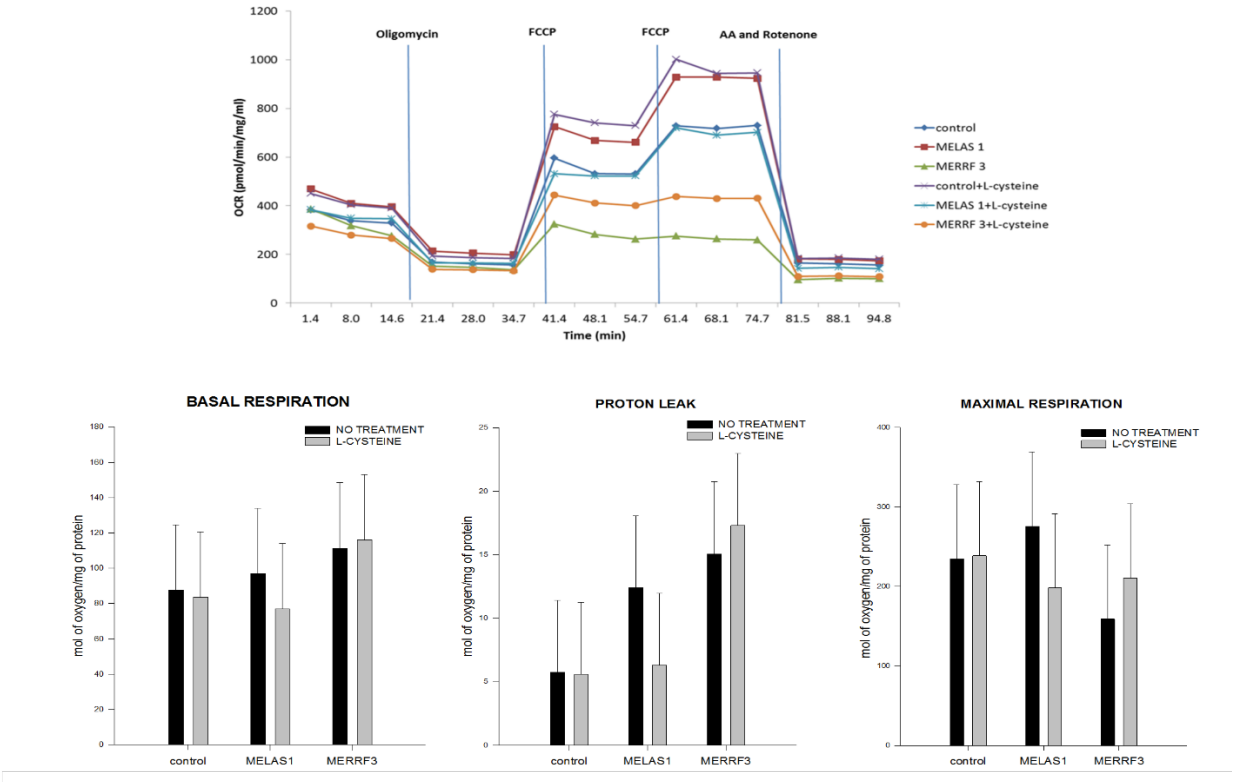



**Figure 4.6: Oxygen consumption in MELAS1 and MERRF3 primary fibroblasts. Black and grey bars represent the mean values of control and patients' cell lines before and after the supplementation with 1mM of L-cysteine respectively. The corrected oxygen**

**consumption by the non-mitochondrial respiration (NMR) and mg of protein is represented as basal respiration, proton leak and maximal respiration. Only MERRF3 presented a slight improvement in terms of oxygen consumption while MELAS1 cell line demonstrated impaired mitochondrial dysfunction after the supplementation.**

As shown in Figure 4.6, the levels of basal respiration of MELAS1 decrease after the supplementation whereas in MERRF3 there is a slight, but none significant increase ($p=0.1848$). Moreover, the levels of maximal respiration in the MERRF3 cell line are elevated after supplementation with L-cysteine ($p=0.4$). In theory, a decrease in maximum respiratory capacity is a strong indicator of potential mitochondrial dysfunction (Brand and Nicholls, 2011).

It is worth noting that the levels of basal respiration in both cell lines (MELAS1: $p=0.87$), MERRF3: $p=0.6$) are slightly higher compared to the control cell line showing that the heteroplasmy levels are possibly low. Indeed, measurement of mtDNA heteroplasmy levels revealed 54% heteroplasmy of the m.3243A>G mutation within the cell line. Different tissues have different bioenergetics thresholds and as patient's bioenergetics capacity declines it eventually fall below the minimum threshold for that tissue and symptoms follow (Wallace and Chalkia, 2013). Typically, the threshold value for MERRF is in the range of 70-98% mutant to wild-type mtDNA (Tuppen et al., 2010) whereas for MELAS in the range of 20-95% characterised by variability in different tissues. Although the heteroplasmy levels of MERRF3 were not measured, it is illustrated from the Figure 4.6 measurement that due to a compensatory mechanism the levels of oxygen consumption rate were, not significantly but, higher compared to the control.

Respiratory chain enzyme complexes were detected by BN-PAGE and are illustrated in Figure 4.7. The expression of complex I increased in the MELAS1 cell line, but it was not significant. On the contrary, the expression levels of the other RC complexes do not present any increase. In the control cell line the relative expression of complex I was slightly increased. Lastly, in MERRF3 there is a small increase in the relative expression of all the complexes but none of the alterations are considered significant.

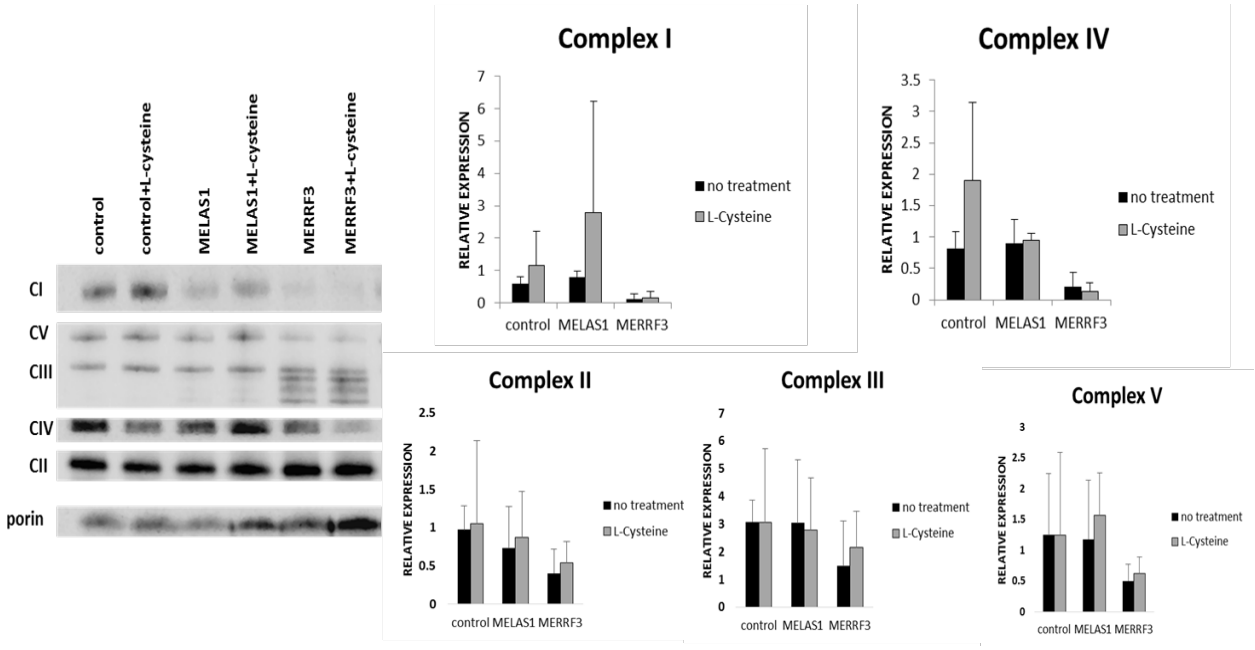



**Figure 4.7: BN-PAGE gel representing the relative expression levels of the OXPHOS complexes before and after the supplementation with 1mM of L-cysteine in MELAS1 and MERRF3 cell line.**

In conclusion, supplementation of MELAS1 and MERRF3 with 1mM of L-cysteine did not significantly improve the function of the mitochondrial respiratory chain in patients' fibroblast cell lines. Therefore, the dose of L-cysteine was increased. Several concentrations of L-cysteine (2mM-10mM) were studied in a control cell line for 9 days. It was observed, that concentrations higher than 5mM inhibited the cell growth and resulted in precipitation of the L-cysteine in the growth medium. Hence, the supplementation of MELAS and MERRF cell lines was repeated using 4mM of L-cysteine, as this was the maximum dose which did not appear to cause any adverse effect on the control cell line. Furthermore, three new cell lines (MELAS2, MERRFF1 and MERRF2) carrying the mutations m.3243A>G and m.8344A>G respectively were included.

Two new cell lines (MERRF1, MERRF2) carrying the mutation m.8344A>G were therefore supplemented with 4mM of L-cysteine for 9 days. Both of the cell lines showed increased levels of basal respiration after the supplementation. In addition to that, the maximal respiration levels were also increased (Figure 4.8A). Interestingly, it appeared that the MERRF2 cells showed a slightly greater increase ($p=0.06$) in basal respiration compared to MERRF1 ($p=0.1$), although these differences were not statistically significant (paired t-test). The degree of mtDNA heteroplasmy for the m.8344A>G mutation, measured before and after the supplementation to exclude that the changes in oxygen consumption caused by a shift in the level of heteroplasmy, revealed no significant change (83% (±5%) and 95% (±5%) for

MERRF1 and MERRF2 respectively after supplementation). It was noted that the greatest increase of oxygen consumption was detected in the cell line with the higher mutation rate (MERRF2).

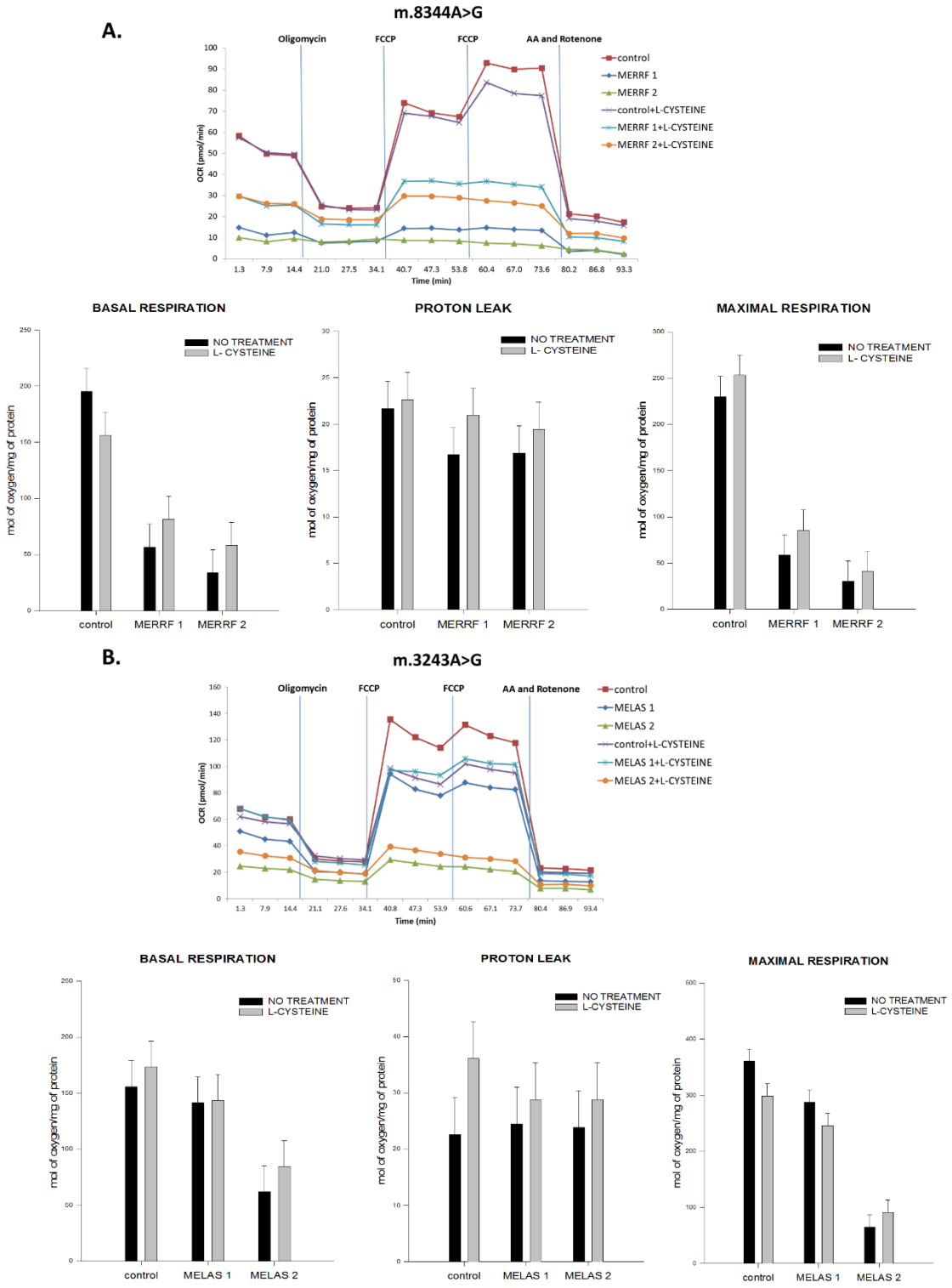



**Figure 4.8: Oxygen consumption in MERRF (A) and MELAS (B) primary fibroblasts. Black and grey bars represent the mean values of control and patients' cell before and**

**after the supplementation with 4mM of L-cysteine respectively. The corrected oxygen consumption by the non-mitochondrial respiration (NMR) and mg of protein is represented as basal respiration, proton leak and maximal respiration. Both MERRF1 and MERRF2 cell lines represented a slight improvement in terms of oxygen consumption. On the contrary, from the MELAS cell lines only MELAS2 illustrated increased levels of oxygen consumption.**

Subsequently, two cell lines (MELAS1, MELAS2) carrying the mutation m.3243A>G were supplemented for 9 days with 4mM of L-cysteine. One of the two primary cell lines (MELAS2) demonstrated increased levels of basal and maximal respiration after the L-cysteine supplementation, while no change was observed in the basal respiration of MELAS1, which also exhibited a decrease in maximal respiration (Figure 4.8B). As mentioned previously, heteroplasmy levels of MELAS1 were 54% pre-supplementation, while the levels of mutated mtDNA in MELAS2 cell line was 88% pre-supplementation. Interestingly, similar to MERRF cell lines, the effect of L-cysteine was more pronounced in the cell line with a higher heteroplasmy level and eventually more severe mitochondrial defect (Basal respiration: MELAS1: p=0.47, MELAS2: p=0.19, paired t-test)

Next, it was studied whether 4mM L-cysteine supplementation affects the steady state of mitochondrial respiratory chain complexes using BN-PAGE (Figure 4.9). Originally, all complexes except for complex II were low in both MERRF and MELAS cell lines confirming a severe defect of mitochondrial protein synthesis. The relative expression of all mitochondrial protein complexes showed a tendency to improve after supplementation (Figure 4.9). However, the very weak or non-detectable bands for complexes I and IV before supplementation in the MERRF cell lines did not allow to reach a conclusion on whether L-cysteine supplementation was beneficial for these complexes. On the other hand, both MERRF cell lines presented increased relative expression of complexes III and V after supplementation with L-cysteine (Figure 4.9A).

In the cell lines carrying the m.3243A>G mutation, the MELAS2 cell line showed increased relative expression in all four complexes containing mtDNA-encoded subunits after L-cysteine supplementation, especially complexes III and V, which both showed a prominent increase. In the cell line harbouring lower m.3243A>G heteroplasmy levels (MELAS1 cell line), the relative expression of complexes I, III and V increased, though complex IV was not significantly compromised prior to supplementation. Interestingly, the relative expression of CIV decreased markedly after the supplementation. It is suggested that this decrease is due to formation of super-complexes that were not visible on the BN blots. It could be claimed that CIV is depleted but a possible depletion of that complex would impair radically the mitochondrial function but the measurements of the oxygen consumption do not indicate so.

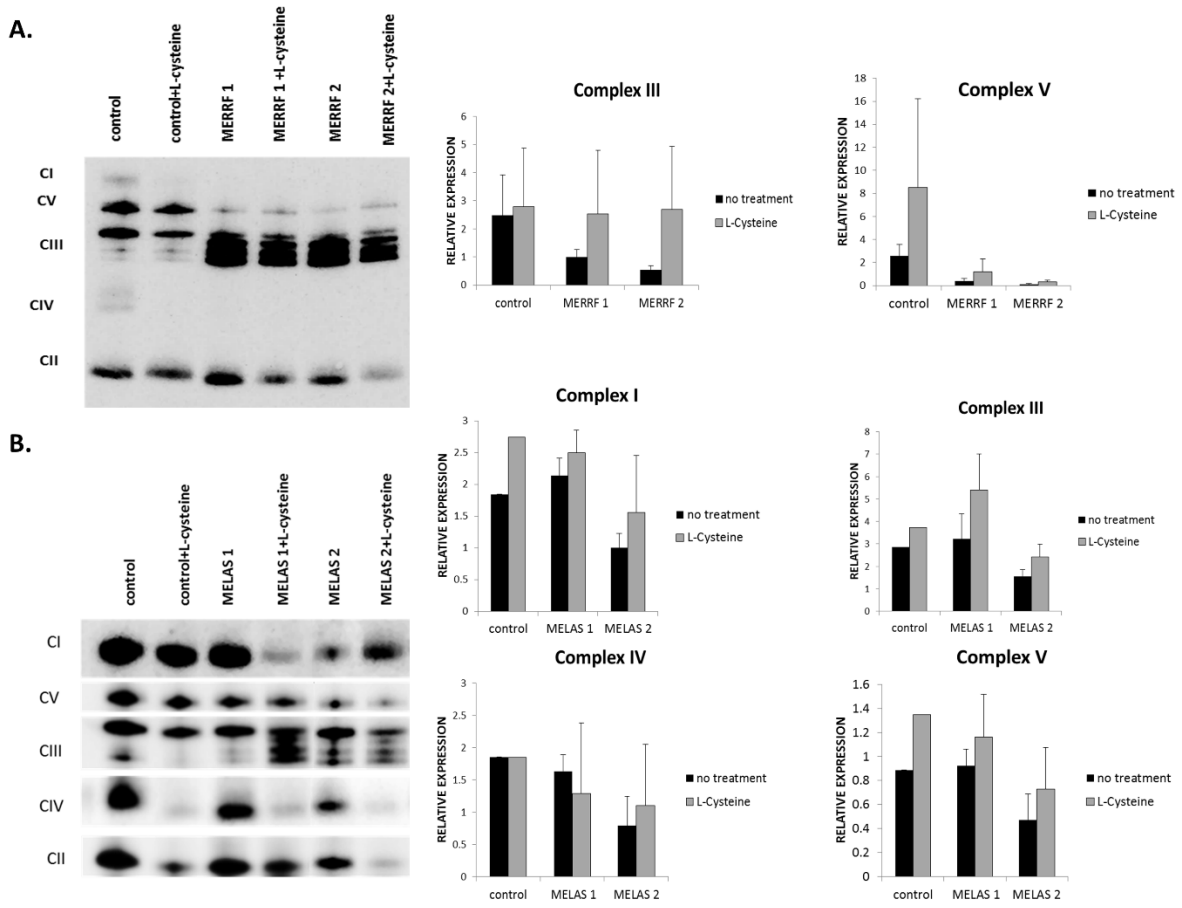



**Figure 4.9: BN-PAGE gels representing the relative expression levels of the OPXHOS complexes before and after the supplementation with 4mM of L-cysteine in MERRF (A) and MELAS (B) primary fibroblasts. Both MERRF cell lines illustrated increased expression levels of CIII and CV. Furthermore, expression levels of all complexes in both MELAS cell lines (apart from CIV) were elevated. CI and CIV could not be detected in MERRF cell lines due to high mutations rate affecting mostly these complexes.**

In summary, although none of the changes in single complex expression levels were statistically significant, the relative expression of all complexes/mitochondrial proteins showed an increasing trend after the supplementation with L-cysteine suggesting that L-cysteine improves the translation of mtDNA encoded proteins. Taken together, the data support that L-cysteine improves the mitochondrial function in cells carrying the m.8344A>G and m.3243A>G mutations, and furthermore, that the L-cysteine supplementation shows a greater predilection to improvement mitochondrial function in cells with higher levels of mtDNA heteroplasmy. Some non-specific bands representing subcomplexes of complex V became slightly weaker after the supplementation with L-cysteine, but this was not quantifiable due to variable involvement of multiple bands.

### 4.4.2 *Effect of L-cysteine supplementation in cell lines with nuclear mutations*

To study whether the positive effect of L-cysteine is specific only for the mitochondrial disorders MELAS and MERFF, three fibroblast cell lines from patients carrying various nuclear defects of mitochondrial protein synthesis (*MTO1*, *ELAC2* and *TRMU*) and one cell line carrying a homozygous mutation in *COX10*, a nuclear-encoded COX assembly gene (Table 4.1) were also supplemented.

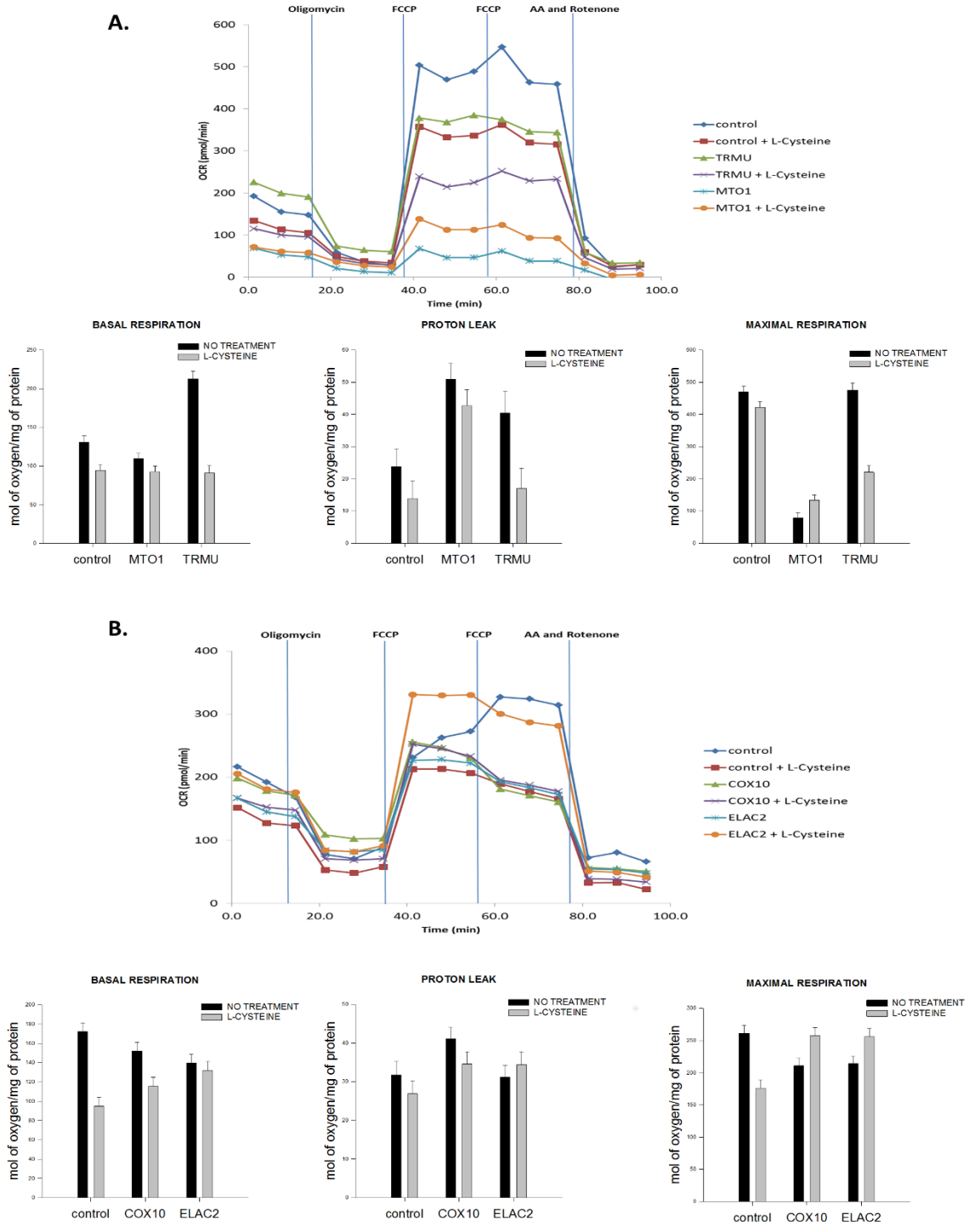



**Figure 4.10: Oxygen consumption before and after the supplementation with 4mM of L-cysteine in primary and immortalised fibroblasts carrying mutation in *MTO1* (A), *TRMU* (A), *COX10* (B) and *ELAC2* (B). Black and grey bars represent the mean value of control and each different cell line before and after the supplementation. The corrected oxygen consumption by the non-mitochondrial respiration (NMR) and mg of protein is represented as basal respiration, proton lean and maximal respiration. L-cysteine supplementation resulted in slight or moderate decreased of oxygen consumption rate in all cell lines with the higher decrease in *TRMU* cell line. (Dr Juliane Muller)**

As shown in Figure 4.10A-4.10B, the basal respiration rate prior to supplementation was not statistically significantly different in any of the four patient cell lines compared to the controls in the resting state, and the cell line carrying the *TRMU* mutation presented higher oxygen consumption rate compared to the control. This observation implies that the fibroblasts carrying pathogenic nuclear DNA mutations do not cause severe mitochondrial dysfunction, presumably mediated by compensatory mechanisms. Supplementation with L-cysteine caused a slight to moderate decrease of basal respiration in all cell lines. Remarkably, the levels of basal and maximal respiration in *TRMU* cells fell back to similar levels compared to the control cell line after the supplementation, which may suggest that less active compensatory upregulation was needed after the supplementation.

Interestingly, the level of maximal respiration in the *MTO1* mutant cell line is significantly increased ($p<0.05$, paired t-test) after the L-cysteine supplementation. The increase in maximal respiration was statistically significant in cells carrying the recessive mutations in *COX10* ($p<0.01$, paired t-test) and *ELAC2* ($p<0.05$, paired t-test).

Prior to supplementation, a significant reduction of the expression of complex I was observed in *MTO1* deficient fibroblasts (Figure 4.11A) together with a reduction in complex IV expression levels in cells carrying the homozygous *COX10* mutation (Figure 4.11B). Relative expression of the remaining complexes was not significantly different compared to the control. Supplementation with L-cysteine resulted in a slight but none significant increase in the relative expression of complexes I, III and V in cells with *MTO1* mutations and an even milder increase of the same three complexes in the *ELAC2* line (Figure 4.11). A uniform decrease of complex IV, as observed in MELAS patients, was present in all patient cells and controls after the supplementation. Overall, the decrease in the relative expression of the respiratory chain enzymes is consistent with the observed decrease in oxygen consumption in all the cell lines.

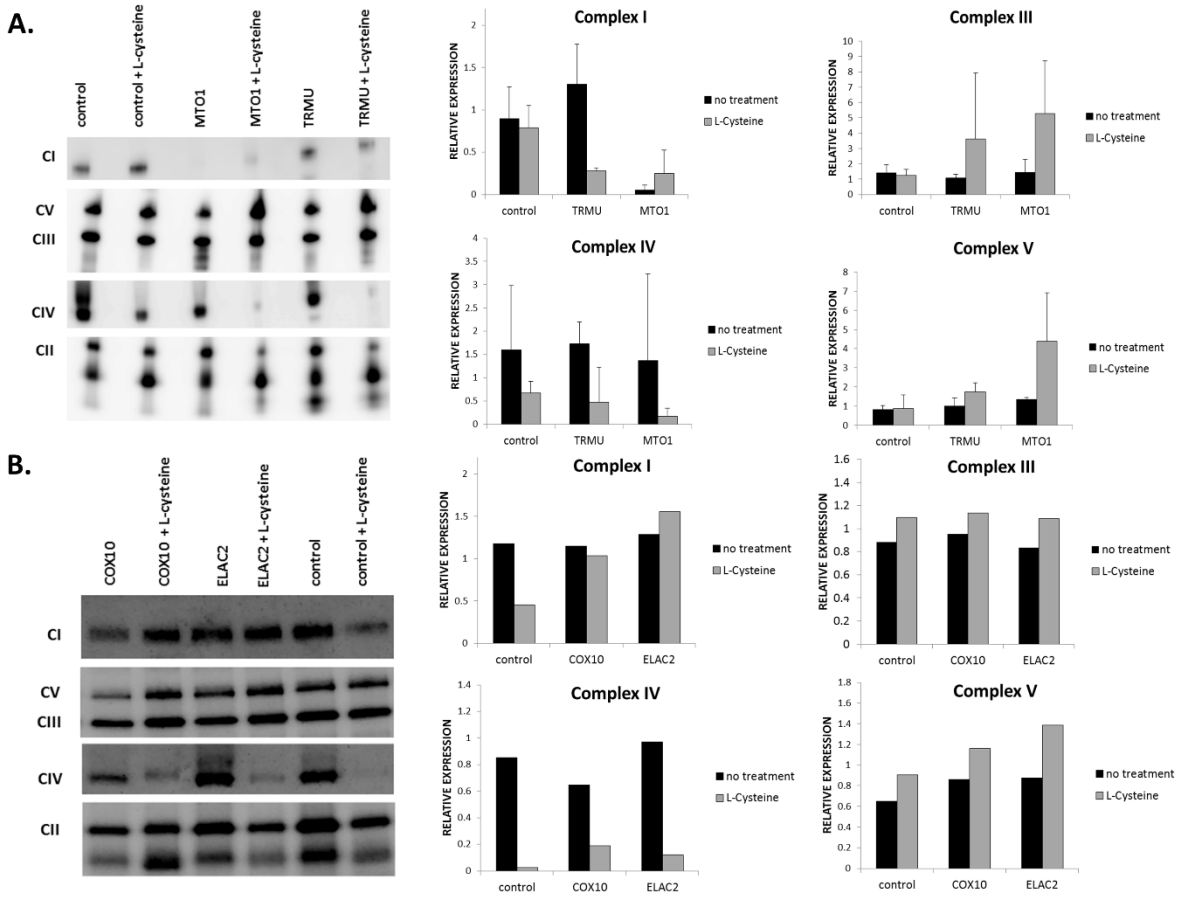



**Figure 4.11: BN-PAGE gels representing the relative expression levels of each different complex of the respiratory chain enzyme before and after the supplementation with 4mM of L-cysteine in primary (COX10, ELAC2) and immortalised fibroblasts (MTO1, TRMU). Expression levels of CIV are decreased in all the cell lines after the supplementation whereas relative expression of CIII and CIV are elevated.**

### 4.4.3 *Effect of NAC supplementation*

Supplementation with 4mM of NAC was performed in the same MELAS and MERRF cell lines used for L-cysteine supplementation.

Surprisingly, as shown in Figure 4.12A-4.12B NAC supplementation with NAC did not have the same positive effect on the overall mitochondrial function as observed previously with L-cysteine. Similarly, the degree of heteroplasmy was not altered by supplementation of NAC in both cell lines. Although, the chemical structure of NAC differs only by an acetyl group compared to the chemical structure of L-cysteine, supplementation with NAC in MERRF and MELAS cell lines not illustrated any positive effect.

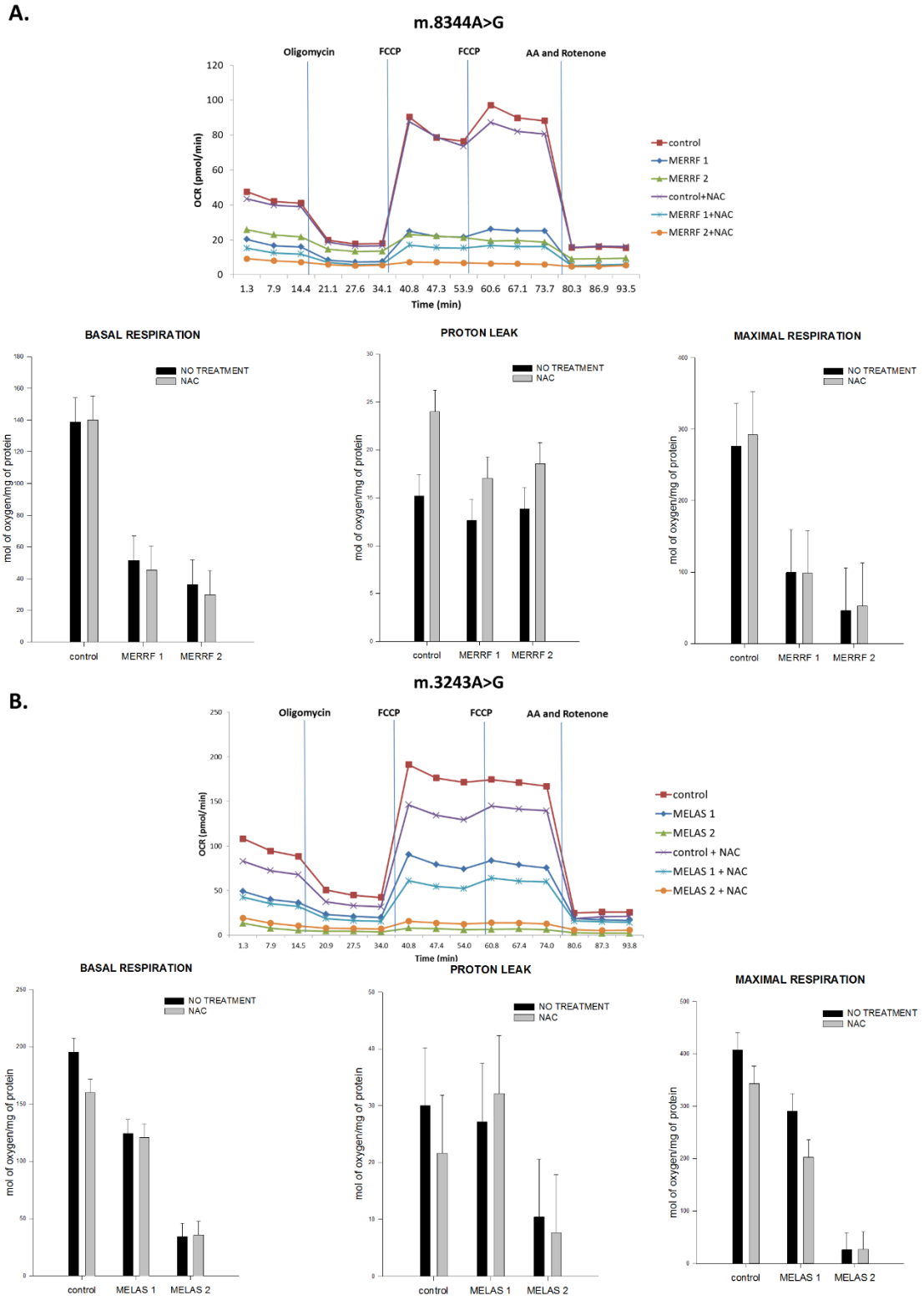



**Figure 4.12: Oxygen consumption in MERRF (A) and MELAS (B) primary fibroblasts. Black and grey bars represent the mean values of control, MERRF and MELAS cell lines before and after the supplementation with 4mM of NAC. The corrected oxygen consumption by the non-mitochondrial respiration (NMR) and mg of protein is represented as basal respiration, proton leak and maximal respiration. Both MERRF and MELAS cell lines presented slightly decreased levels of oxygen consumption**

On the other hand, supplementation with NAC resulted in improved mitochondrial function in both *MTO1* and *TRMU* deficient cells (Figure 4.13A-4.13B) as implied by the higher levels of basal and maximal respiration compared to the control. The increase of basal respiration rate in both *MTO1* and *TRMU* deficient cells was statistically significant ($p<0.01$, paired t-test). Regarding the maximal respiration rate, the increase was only statistically significant in the *MTO1* cell line ($p<0.01$, paired t-test) but not in the *TRMU* cell line. The levels of basal and maximal respiration were decreased in both *ELAC2* and *COX10* deficient cell lines after the supplementation with NAC but not significantly.

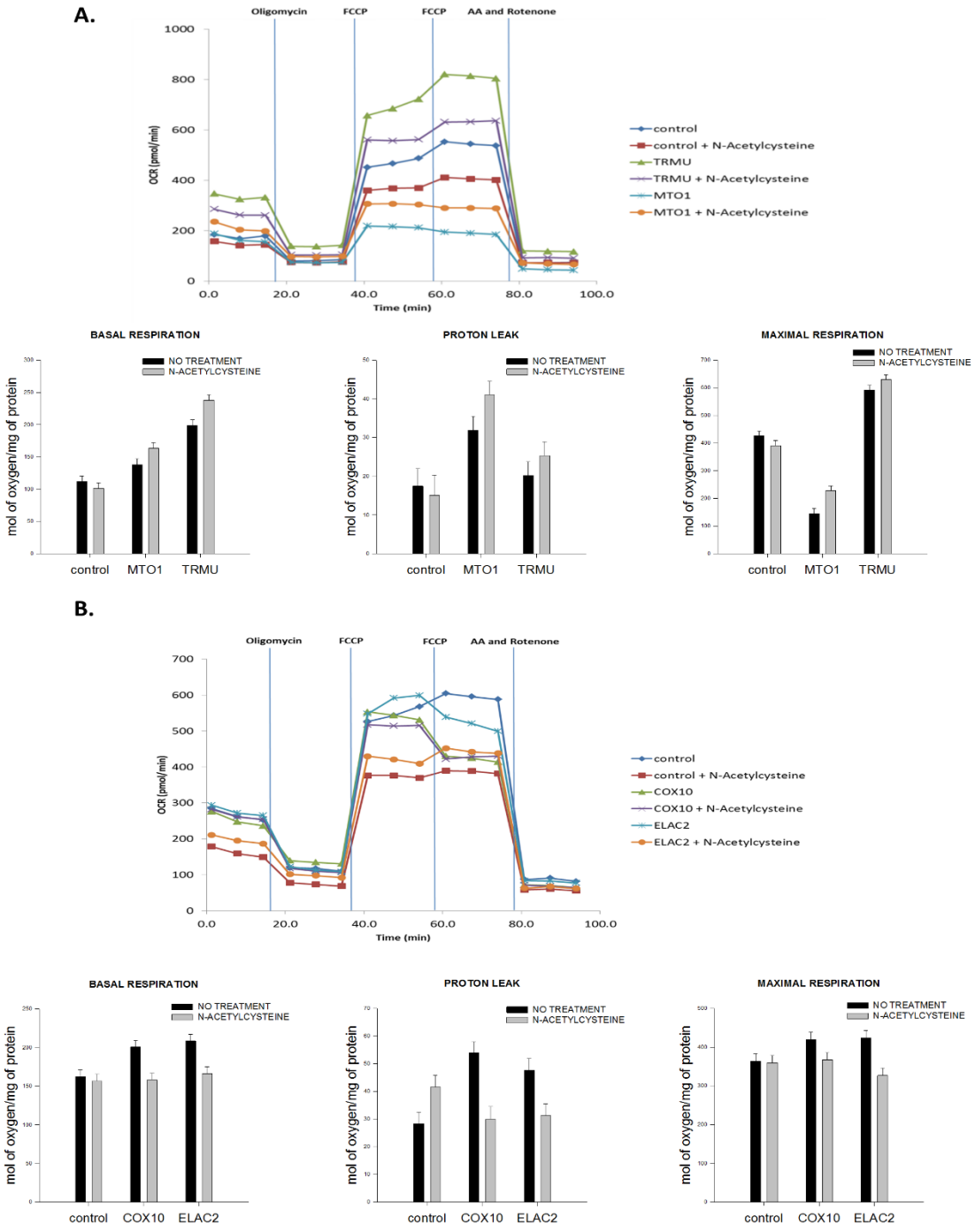



**Figure 4.13: Oxygen consumption before and after supplementation with 4mM of NAC in primary and immortalised fibroblasts carrying mutations in *MTO1* (A), *TRMU* (A), *COX10* (B) and *ELAC2* (B). Black and grey bars represent the mean values of control and each different cell line before and after the supplementation. The corrected oxygen consumption by the non-mitochondrial respiration (NMR) and mg of protein is represented as basal respiration, proton leak and maximal respiration. Immortalised fibroblasts carrying mutations in MTO1 and TRMU illustrated elevated levels of oxygen consumption rate after the NAC supplementation whereas both cell lines carrying mutations in COX10 and ELAC2 were characterised by slightly decreased levels of oxygen consumption.**

### 4.4.4 *Mitochondrial DNA copy number showed no major alteration after supplementation with L-cysteine or NAC*

The quantification of mtDNA copy number before and after the supplementation with L-cysteine revealed a slight increase in copy number in MERRF1 (36%, $p>0.05$), MERRF2 (27%, $p>0.05$), COX10 (51%, $p<0.05$) and MTO1 (113%, $p>0.05$) cell lines, a decrease in TRMU cells (150%, $p>0.05$), and no change in ELAC2 cells (Figure 4.14).

On the other hand, mtDNA copy number tended to decrease after NAC supplementation in almost all the cell lines except for *COX10* and *ELAC2* cell lines (Figure 4.14).

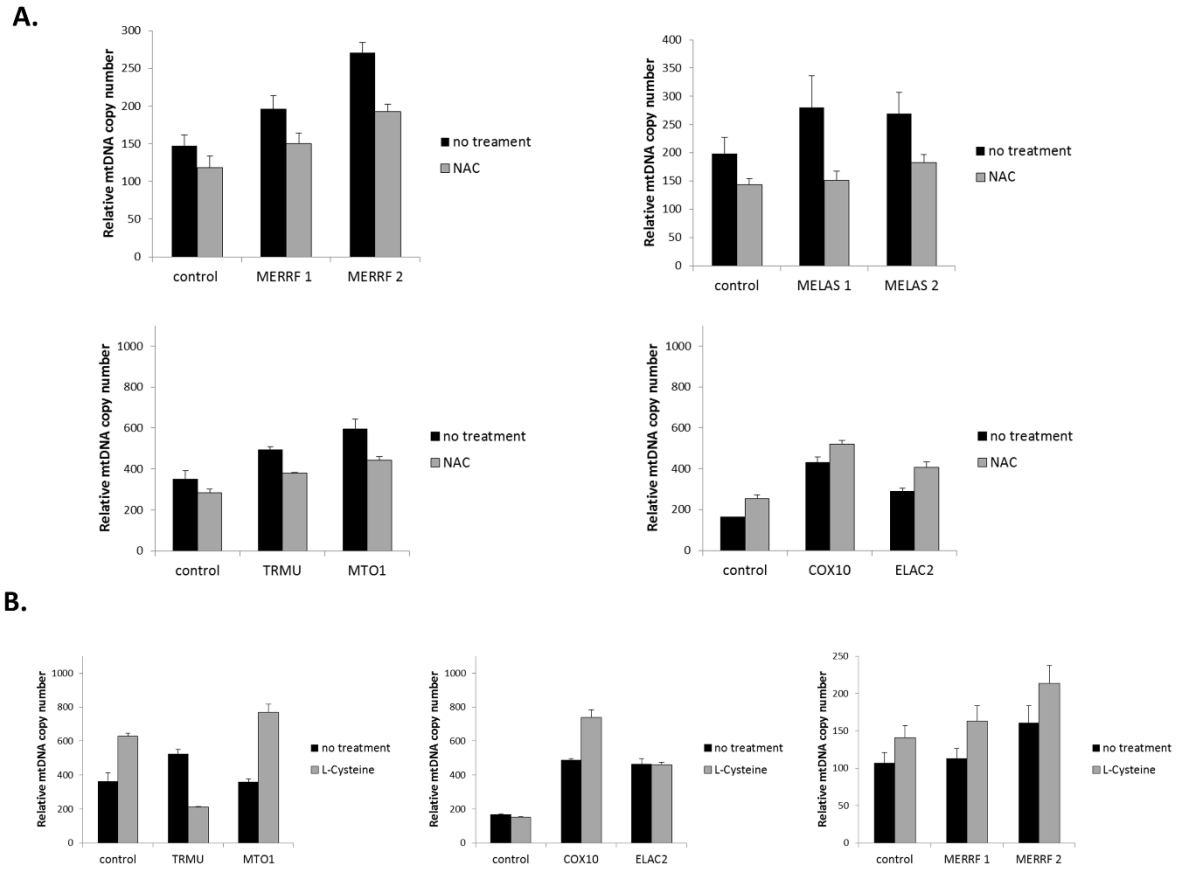



**Figure 4.14: Quantification of relative mtDNA copy number before and after supplementation with 4mM of NAC (A) and L-cysteine (B). MtDNA copy number in cell lines carrying mitochondrial mutations and mutations in *MTO1* and *TRMU* was decreased after supplementation with NAC whereas cell lines carrying mutations in *COX10* and *ELAC2* were characterised by a relative mtDNA copy number after the NAC supplementation. L-cysteine supplementation led to increased mtDNA copy number in MERRF cell lines and cell lines carrying mutations in *MTO1* and *COX10*. The relative mtDNA copy number was significantly decreased in *TRMU* cells and remained stable in *ELAC2* cell after the L-cysteine supplementation.**

## 4.5 Discussion

Both primary and secondary mitochondrial disorders are a large, heterogeneous group of progressive diseases for which there are no effective established treatments to date. Only a few effective therapies are available for only a few mitochondrial disorders.

Two of the most common mitochondrial diseases caused by mutations in the mtDNA, are MELAS and MERRF. The MELAS syndrome is caused by the pathogenic mutations m.3243A>G and m.3271T>C which result in lack of taurine modification at the wobble position of $tRNA^{Leu}$, leading to reduced translation of UUG codons but not UUA (Kirino et al., 2004) and consequently reduced expression of the respiratory chain complexes I and IV (Umeda et al., 2005). Regarding the pathogenic mutation m.8344A>G, which is associated with the MERRF disorder, the lack of both $\tau m^5$ and $s^2$ modification in the in $tRNA^{Lys}$ means that none of the codons for Lysine are able to be decoded (Yasukawa et al., 2001). The precise mechanism of 2-thiolation of uridine at the wobble position of mt-tRNAs for Gln, Glu and Lys and the insertion of the taurinomethylation at carbon 5 post –transcriptionally in humans is not fully understood yet. However, it has been shown that TRMU is the responsible enzyme for the 2-thiolation of carbon 5 of U34 (Guan et al., 2006) and MTO1 with GTPBP3 for the taurinomethylation of carbon 5. Mutations in these genes have been associated with secondary mitochondrial disorders (Zeharia et al., 2009, Ghezzi et al., 2012, Baruffini et al., 2013)

L-cysteine is an amino acid that is required for several key elements of cellular function including the biosynthesis of taurine and glutathione, and it has previously been shown that supplementation with L-cysteine is able to augment mitochondrial translation in myoblasts with reversible infantile mitochondrial diseases in vitro (Boczonadi et al., 2013). Previous studies have shown that a whey-based cysteine donor resulted in significantly reduced oxidative stress in mitochondrial myopathies (Mancuso et al., 2010) and lower levels of reduced cysteine and thiols were detected in plasma of children with mitochondrial diseases (Salmi et al., 2012) further supporting the key role of cysteine in the mitochondrial disorders.

As explained above, NAC has been tested as a potential treatment in several neurological and psychiatric conditions and may be an excellent compound with an acceptable safety profile (Deepmala et al., 2015). Both *in vivo* and i*n vitro* studies support the utility of NAC as potentially viable treatment method of mitochondrial disorders. In *in vivo* models it has been shown to have a beneficial effect in ethylmalonic encephalopathy (Viscomi et al., 2010) and

in fibroblasts carrying pathogenic mutations in the *TRMU* and *TSFM* mutations *in vitro* (Soiferman et al., 2014).

Based on previous data showing that L-cysteine could reverse the mitochondrial defect in two reversible mitochondrial diseases (Boczonadi et al., 2013) and due to the nature of the mitochondrial translational defect in MELAS and MERRF, it was hypothesized that L-cysteine could act as a sulfur donor and precursor of taurine and consequently improve mitochondrial translation.

The presented results show an improvement in mitochondrial function in primary fibroblasts cells carrying the mutations m.3243A>G and m.8344A>G after 9 days of supplementation with 4mM of L-cysteine. Furthermore, in the cell lines where the oxygen consumption rate was elevated after the supplementation (MERRF1, MERRF2, MELAS2), increased relative expression of all the complexes of the respiratory enzymes was observed. However, due to high heteroplasmy levels in MERRF cell lines, it was not possible to study the effect of L-cysteine on the expression levels of complexes I and IV. Surprisingly, it was not detected a similar positive effect in MELAS and MERRF cell lines after the supplementation with 4mM of NAC. It has been shown that in patient cell lines carrying an mtDNA mutation usually the mtDNA population drift towards the wild type and it has been shown that cell lines with higher passage number are likely to contain less mutant mtDNA (van de Corput et al., 1997). In this study the mutation rate in MELAS and MERRF cell lines was approximately the same before and after the supplementation.

Furthermore, it was also studied the effect of L-cysteine and NAC supplementation in four fibroblasts cell lines carrying mutations in the *MTO1*, *TRMU*, *ELAC2* and *COX10* genes. According to the presented data, supplementation with L-cysteine was slightly beneficial to the cell lines carrying mutations in *MTO1*, *COX10* and *ELAC2* genes causing an increase in maximal respiration rate. The immortalised fibroblasts carrying the mutations in *TRMU* illustrated significantly increased oxygen consumption rate prior to supplementation implying the presence of a compensatory mechanism and after the supplementation the levels of basal respiration, proton leak and maximal respiration dropped back to normal compared to the control cell line. Interestingly, NAC supplementation had a positive effect in both *MTO1* and *TRMU* mutated cell lines whereas *COX10* and *ELAC2* illustrated impaired mitochondrial function after the supplementation.

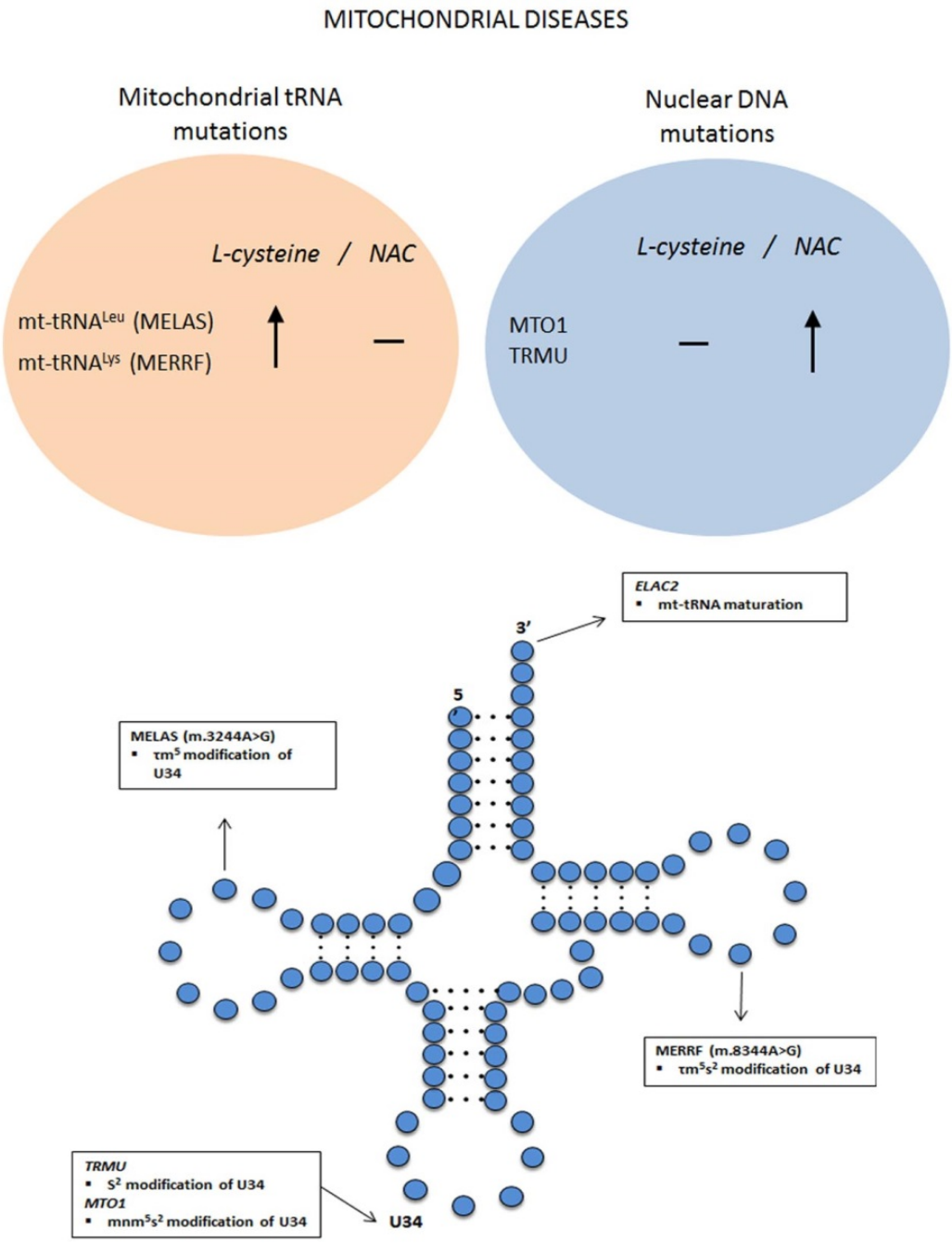



**Figure 4.15: Schematic summary of the results presented**

In summary, it is demonstrated that supplementation with 4mM of either L-cysteine or NAC of both primary and immortalised fibroblasts did not have any toxic effect. However, higher concentrations resulted in inhibition of the cell growth and precipitation of the supplement *in vitro*.

Moreover, supplementation of primary mitochondrial disorders with L-cysteine resulted in improved overall mitochondrial function, preferentially in cell lines with higher heteroplasmy levels but in the secondary mitochondrial disorders it was not observed the same impact on the mitochondrial function. On the contrary, supplementation with NAC was more beneficial to the patients' cell lines carrying mutation in the nuclear genes rather than MELAS and MERRF cell lines (Figure 4.15). According to the presented data, the relative expression of the respiratory chain complexes and demonstrated changes revealing the effect of the supplementation on the mitochondrial translation.

However, the differential effect of L-cysteine and NAC supplementation raises some interesting questions and needs further exploration. It is known that cysteine can follow different metabolic pathways, depending on the needs of the cell each time. Consequently, in different cell types different metabolic pathways are more active. Based on that and on our presented data, it is hypothesised that the differential effect of L-cysteine and NAC supplementation might be due to the different causative genotypes leading to the mitochondrial disorders. The presence of different mutations (primary or secondary) leading to mitochondrial disorders might activate different metabolic pathways of L-cysteine and NAC in each cell. Therefore, the products might be slightly different and hence the total effect on the cell metabolism.

It is accepted that the cell model used for this study comprises a limitation in interpreting the effect of the supplementation. The bulk of mitochondrial diseases are tissue specific and the defect might therefore not be detectable in all cell types. For example, as discussed below, the fibroblasts carrying the *TRMU* mutation illustrated significantly increased mitochondrial function compared to the control and the oxygen consumption rate of all the cell lines carrying nuclear mutations were relatively high given the phenotype of the patients. Furthermore, either healthy or disease fibroblasts rely on glycolysis as their main source of energy. Therefore, the expression levels and activity of the respiratory chain enzymes are relatively low and possibly difficult to detect or measure them (Robinson, 1996, Cameron et al., 2004). Myoblasts would possibly comprise a more appropriate cell model, as muscle is

one of the mostly affected tissues in mitochondrial diseases, but are difficult to obtain from patients in comparison to fibroblasts derived from a skin biopsy.

However, NAC and L-cysteine may be still useful not only in protecting the cell from high levels of ROS production due to mitochondrial dysfunction, but also enhance the mitochondrial translation when is needed. Further work should aim to determine the degree of correlation in mitochondrial functional studies between fibroblasts and myoblasts from the same individuals, together with determining whether there is a tissue specific effect of L-cysteine and NAC in patients with primary and secondary mitochondrial disorders respectively. Finally, similar studies can be recapitulated in mice and only after clinical trials can be considered.

# Chapter 5. Tissue specificity

## 5.1 Overview

### 5.1.1 *Tissue specificity*

In the bulk of mitochondrial diseases, the relationship between the percentage of a pathogenic mtDNA mutation and the development of a specific phenotype is still puzzling (Rossignol et al., 2003). It has been shown that the same mtDNA mutation can result in different clinical manifestations and conversely, the same clinical feature can be occurred by different mtDNA mutations. Furthermore, different biochemical defects of the same respiratory chain enzyme may lead to different clinical phenotypes. The same variability in the expression of a mtDNA mutation or biochemical defect can be observed between different patients and also between different tissues in a given individual (tissue specificity) (Rossignol et al., 2003, Boczonadi and Horvath, 2014).

This variability between the cells and tissues of an individual can be partly explained by the complex characteristics of mitochondrial genetics. As has been discussed previously, every mammalian cell consists of a mobile network of mitochondria containing thousands of copies of mtDNA. Hence, mutated and wild-type copies of mtDNA can coexist (heteroplasmy) within one cell. The heteroplasmy levels may also vary between different individuals or even between cell or tissues within the same patient (Chinnery et al., 1999) and that can explain the observed clinical variability between individuals carrying the same mtDNA mutation.

Theoretically, according to the maternal inheritance pattern of human mtDNA, the mutations accumulate slowly through the generations until a mutant threshold level is reached, followed by the manifestation of a disease (Poulton et al., 2010). The responsible mechanism for the prevention of the transmission of defective mtDNA to offspring (Ylikallio and Suomalainen, 2012) is the genetic bottle-neck of the mtDNA in the developing primordial germ cells (Poulton et al., 2010). Due to the presence of the mtDNA genetic bottleneck, only a small subset of the mtDNA copies, originally located in the oocyte, populate the germ cells that will become the next generation. The bottleneck might be responsible for a strong reduction in mtDNA copy number in primordial germ cells which is followed by amplification and random segregation of mtDNA species during the germ cell division (Cree et al., 2008). Other studies claim that the bottle-neck results from the replication of only a subset of mtDNAs in

primary oocytes that undergo folliculogenesis (Wai et al., 2008). Both ways, heteroplasmy levels can be shifted rapidly during generations and for this reason members from the same family carrying the same pathogenic mtDNA mutation might be characterised by conspicuous clinical variability. Studies conducted in a mouse model of mtDNA disease have shown that during germ cell or oocyte development the severely defective mtDNA molecules (Fan et al., 2008, Stewart et al., 2008). Therefore, this purifying selection might be the reason why moderately pathogenic mtDNA mutations, which do not cause complete inhibition of the OXPHOS system, can lead to late-onset diseases, while there is a selection against highly deleterious mtDNA mutations (Fan et al., 2008).

Finally, studies have shown that mtDNA mutations may also occur sporadically in somatic tissues (Greaves and Turnbull, 2009). In that case, the mitochondria undergo clonal expansion, reaching a threshold (Greaves and Turnbull, 2009) level and eventually leading to a mitochondrial disorder at a later age (Fan et al., 2008).

### 5.1.2 *Tissue specificity in MELAS syndrome*

One of the most frequent maternally inherited mitochondrial disorders is MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes) syndrome. As discussed previously (*1.3.1*), the most common mutation associated with MELAS syndrome is m.3243A>G in the MT-TL1 gene encoding the $tRNA^{Leu(UUR)}$, as it is detected in 80% of the affected individuals (El-Hattab et al., 2015). The mutation results in the absence of the $\tau m^5U$ modification of the uridine at the wobble position of tRNA for Leu and consequently leads to reduced translation of UUG codons and lower expression of the UUG-rich protein ND6 (Kirino et al., 2004).

The clinical manifestation of the MELAS syndrome is characterised by multi-organ involvement and a wide spectrum of clinical features. One of the primary features of MELAS is stroke-like episodes however these do not occur in the majority of the affected individuals. A study where different tissues from individuals with mitochondrial disorders were compared in terms of OXPHOS deficiency, showed that the frontal cortex of individuals carrying the heteroplasmic mutation m.3243A>G (heteroplasmy levels >89%) was characterised by deficient OXPHOS (decreased expression levels of complex I and IV) and significantly decreased ATP synthase expression levels (Fornuskova et al., 2008). Therefore, the investigation of the mitochondrial function of neurons from MELAS patients might enlighten the neurological manifestation of the disease and enable us to deeply understand the effect of the mutation on neuronal cell type function.

### 5.1.3 *Leukoencephalopathy with vanishing white matter disease*

One of the more prevalent genetically determined leukodystrophies in children is leukoencephalopathy with vanishing white matter (VWM) (van der Knaap et al., 2006). Vanishing white matter disease is a fatal disorder, clinically characterized by progressive motor dysfunction, mainly cerebellar ataxia and less prominent cognitive decline. Brain magnetic resonance imaging (MRI) from affected patients have shown a diffuse leukoencephalopathy with progressive white matter lesion and cystic degeneration.

Mutations in the genes *EIF2B1-EIF2B5*, encoding the five (α-ε) subunits of the eukaryotic translation initiation factor 2B (eIF2B), have been associated with the disease (Leegwater et al., 2001, van der Knaap et al., 2002). Although the genes consisting of the eIF2B factor are ubiquitously expressed, mutations in those genes cause only a defect in the cerebral and cerebellar white matter.

Although vanishing white matter may manifest during any decade of life, the early onset of the disease is usually between 2 and 6 years of age and leading to death often in childhood. On the other hand, the late-onset cases are characterised by slowly progressive encephalopathy in adults, and onset can be even as late as the $6^{th}$ decade of life (Labauge et al., 2009).

The eukaryotic translation initiation factor 2B is necessary for the initiation and regulation of the cytosolic translation in various conditions (Scheper et al., 2006). It is referred as guanine nucleotide exchange factor (GEF) as it catalyses the guanine nucleotide exchange on eIF2 which results in conversion of inactive eIF2·GDP into active eIF2·GTP. The bulk of the eIF2B mutations lead to impaired GEF activity *in vitro* (Li et al., 2004, Richardson et al., 2004).

The neuropathology and progress of the disease has been reported earlier in human brain autopsies, and recently by MRI. In young pre-symptomatic patients, the MRI is characterised by mild abnormalities in the subcortical white matter indicating impaired myelination and extended abnormalities in the periventricular white matter with myelin vacuolization (van der Knaap et al., 1998, van der Lei et al., 2010). Progressively the MRIs are characterised by extension of white matter abnormalities and cystic degeneration. At the end stage of the disease, the white matter is cavitated, with areas of loss of all structures while the intact areas of white matter are characterised by vacuolization , a lack of myelin and increased number of OPCs (Oligodendrocyte Progenitor Cells) which do not develop into mature myelin-forming cells (Dooves et al., 2016). Finally, a limited number of oligodendrocytes have an abnormal

and foamy appearance (Hata et al., 2014). Studies have shown that similar clinical phenotype was detected in mice carrying homozygous point mutations in Eif2b4 (p. Arg484Trp) and Eif2b5 (p.Arg191His) associated with VWM (Dooves et al., 2016).

Although the cause of the myelin vacuolization is still not known, it is believed that the astrocytes play a major role in the pathomechanism of the disease (Dooves et al., 2016). It has been shown that human VWM astrocytes have an abnormal and immature morphology (Bugiani et al., 2011). According to another study, the number of immature astrocytes increases long before the clinical onset of the disease or before other histologic abnormalities manifest implying a strong correlation of the astrocytes with the disease progression (Dooves et al., 2016). In addition, it has been shown that human VWM astrocytes are characterised by GFAP expression which possibly affects the cytoskeletal architecture of astrocytes and their interaction with other cells including oligodendrocytes (Middeldorp and Hol, 2011).

### 5.1.4 *Cellular disease models*

The study of these very tissue specific human mitochondrial disorders has been delayed by the inaccessibility of the relevant affected tissues and cell types. One of the affected tissues in a subset of mitochondrial diseases is the central nervous system but obtaining neuronal cells from humans is only possible post mortem. However, the recent developments of the technology enable us to generate different type of cells from the affected individuals.

In 2006, Takashi et al were the first to generate pluripotent stem cells directly from fibroblasts cultures with only four reprogramming factors (Oct3/4, Klf4, Sox2, c-Myc). These induced pluripotent stem cells (iPSCs) carry the same characteristics as the embryonic stem cells (ESCs). Therefore, iPSCs have the ability to grow indefinitely while maintaining pluripotency and the ability to differentiate into the desired cell type (Takahashi and Yamanaka, 2006). A few groups have managed to generate patient-derived iPCSs carrying various heteroplasmic mtDNA mutations for *in vitro* cellular disease modelling.

Fujikura et al generated patient-derived iPSCs carrying the m.3243A>G mutations from two patients with isolated diabetes mellitus, as the clinical phenotype. The generated iPSCs cell lines could be categorised to mutation rich (80%-90% heteroplasmy levels) and mutation free (undetectable levels of m.3243A>G mutation). The authors managed to differentiate the mutation rich patient-derived iPCSc cell line into endodermal lineage in which no changes in the heteroplasmy levels were detected (Fujikura et al., 2012).

Later, Cherry et al generated and characterized iPSCs from a patient carrying a heteroplasmic 2.5kb mtDNA deletion leading to Pearson syndrome. In this study it was reported that a few of the generated iPSC lines were characterised by gradually decreasing heteroplasmy levels over the culture period and eventually low mutation iPSC lines were obtained. However, examination of the mitochondrial function in the mutation-rich iPSC cell line (~65% heteroplasmy levels) showed decreased basal respiration levels compared mutation-poor iPSC lines (Cherry et al., 2013).

Folmes et al generated and characterised patient-derived iPSCs carrying the heteroplasmic m.13513G>A mutation from a patient with MELAS phenotype. The patient fibroblasts presented impaired mitochondrial function and from the three iPSC lines generated, two were characterised by high heteroplasmy levels (50%-60%) while the mutation was undetectable in the third cell line. Interestingly, similar to previous studies (Cherry et al., 2013), one of the mutation-rich iPSC line exhibited a gradual decrease of the heteroplasmy levels over the culture period and therefore a subpopulation of low level mutation iPSC line was obtained (Folmes et al., 2013).

Hamalainen et al generated and characterised three isogenic iPSC lines (heteroplasmy levels >80%) and three isogenic mutation free iPSC lines derived from the same MELAS patient (m.3243A>G). The variable and instable heteroplasmy rate of the m3243A>G mutation enable generation of isogenic lines with different mutation rate. Afterwards, the iPSC lines underwent neuronal differentiation and the neurons derived from the mutation-rich iPSC lines were characterised by down-regulation of mitochondrial respiratory chain complex I and accelerated mitophagy via the PARKIN-PINK1 pathway. In addition, iPSC lines with high heteroplasmy levels also presented two-fold increase of the mtDNA copy number compared to the parental fibroblasts and a twofold increased in the number of visible nucleoids compared to the mutation-low or control iPSC lines (Hämäläinen et al., 2013).

Finally, another study reported the generation and characterisation of patient-derived iPSCs from myoblasts obtained from a patient with a novel heteroplasmic mutation (m. 5541C>T) associated with MELAS syndrome. The derived iPSC lines were differentiated into neurons (CNS and PNS lineages) and a significant loss of terminally differentiated neurons was observed compared to their progenitors. According to the authors, the progenitors of both CNS and PNS lineages do not exhibit increased mitochondrial respiration state and as a result they are not affected by the mutation whereas the terminally differentiated cells present severe mitochondrial dysfunction (Kodaira et al., 2015, Hatakeyama et al., 2015).

The generation of patient-derived iPSC lines enable us to study different cell types affected by mitochondrial diseases, to explore new drug candidates that are applicable to mitochondrial diseases and even the implementation of regenerative therapeutics may be possible in the future. However, there are still a few difficulties to overcome. The iPSCs (similar to ESCs) contain rounded mitochondria with poor cristae structure and are characterised by low respiration capacity and reduced mtDNA copies as a result of their adaptation to glycolysis (Prigione et al., 2010, Suhr et al., 2010). It has been also reported that human iPSCs are characterised by new mtDNA variants at a single base level which do not exist at the parental somatic cells and are acquired during the cellular reprogramming or differentiation (Prigione et al., 2011) and finally the generation, characterization and differentiation of an iPSCs to neural cells requires lot of time (at least 4-6 months). Therefore, the development of the direct conversion of mouse and human somatic cells (fibroblasts) to induced neural progenitor cells (iNPCs) facilitated the generation of specific cell types (Yang et al., 2011). The method of direct conversion utilizes the overexpression of cell type-specific transcription factors to cause lineage changes bypassing pluripotent cellular stage and eventually resulting in the desired cell type. The use of direct conversion enables us to reduce the required time for acquiring neural cells, skip the developmental stage of iPSCs and therefore help to focus on disease studies rather than stem cell studies (Kim, 2015). One of the major advantages of this method is the fact that certain diseases, affecting the CNS mostly, can be modelled and examined in a short time. To date, in most cases the availability of isolated post-mortem tissue is limited. Moreover, it is not known how inflammatory and necrotic environment can affect the tissues (Meyer et al., 2014).

## 5.2 Aims

1. Due to the tissue specificity and the severe neurological manifestation of MELAS syndrome in combination with the inaccessibility of human brain tissue fibroblasts from a heteroplasmic (m.3243A>G) MELAS patient were converted directly to neuronal cell types. The aim was firstly to generate induced neuronal progenitor cells (iNPCs) carrying the m.3243A>G mutation via the direct conversion, secondly characterise and investigate the mitochondrial function of those cells to explore how the m.3243A>G mutation affects neurons.
2. Based on the similarities in clinical presentation of a patient carrying the p.Arg113His mutation in *EIF2B5* to the clinical presentation of MELAS and on the identified isolated complex I defect in his muscle biopsy (see 5.3.2) it was hypothesized that the mitochondrial translation machinery may be affected in VWM. Therefore, fibroblasts,

myoblasts and patient derived-iNPCs were examined for mitochondrial translation defect.

Although many different protocols regarding the direct conversion of fibroblasts to iNPCs have been published, we have followed the protocol published by Meyer et al (Meyer et al., 2014). Briefly, the fibroblasts isolated from the affected individual were transduced with retroviral vectors containing four reprogramming factors (Oct3/4, Sox2, Klf4 and c-Myc). Following, the next 6-10 days the transduced cells underwent morphological changes and eventually generated tripotent iNPCs cells. The protocol and reagents used for the direct conversion are described in Chapter 3.

## 5.3 Results

### 5.3.1 *Direct conversion of fibroblasts of a patient with MELAS (m.3243A>G)*

The fibroblasts carrying the heteroplasmic mutation m.3243A>G were isolated from the patient referred as MELAS 1 in Chapter 4. The clinical presentation of the patient is described in Table 4.1. Measurement of heteroplasmy levels revealed 54% presence of m.3243A>G mutation in the MELAS 1 fibroblast cell line.

The cells were transduced Day 0 with retroviral vectors containing four reprogramming factors (Oct3/4, Sox2, Klf4 and c-Myc) kindly provided by Dr. Kathrin Meyer (The Research Institute, Nationwide Children's Hospital, Columbus). The cells were incubated overnight and the next day fresh DMEM/Glutamax growth medium was added for one day. On day 3 the cells were grown with conversion medium which consists of DMEM/F12 enriched with N2 supplement, B27 supplement, EGF (epidermal growth factor) and FGF2 (fibroblast growth factor 2) growth factors and heparin. Approximately a week after the transduction, morphological changes should be detected as the cells from the flat fibroblastic shape usually become smaller with distinct extensions. Additionally, they should start to form sphere-like structures that can be picked up and cultured further as monolayers (Meyer et al., 2014).

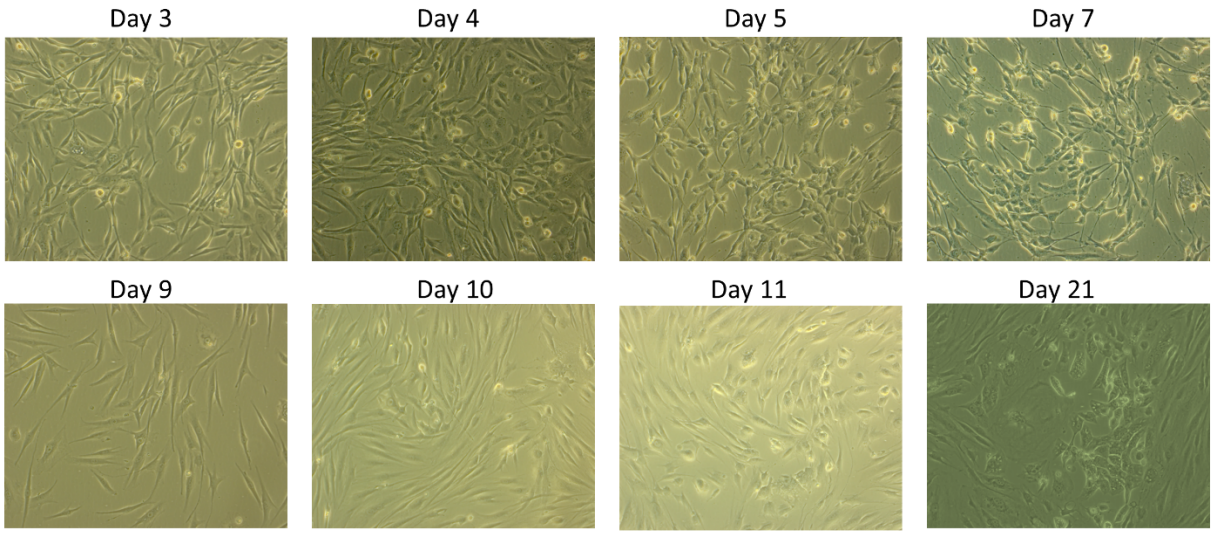



**Figure 5.1: Consecutive microscope images of the transduced fibroblasts carrying the heteroplasmic mutation m.3243A>G from day 3 after the transduction till day 21.**

As shown in Figure 5.1, from day 3 till day 5 the transduced cells started progressively to change morphology and accumulated to form sphere-like structures. However, on day 7 although the cells kept their rounded-shape, they started losing their distinct extensions. Therefore, the cells were lifted and seeded on six-well culture plate coated by fibronectin. Fibronectin is used as a substrate to enhance the adherence and proliferation of many cell types. Moreover, on that day the growth medium was switched from conversion to NPC medium. As discussed in 3.10, the NPC medium consists of DMEM/F12 enriched with N2 and B27 supplement and FGF2 growth factor. On day 9 the cells were observed under the microscope but their morphology was more similar to fibroblasts rather than iNPCs and no neurospheres could be found. The cells were kept in culture for 10 days onwards, however unfortunately their morphology remained stable (fibroblast-like) and after day 20 the cells started to die.

The inefficient direct conversion of heteroplasmic m.3243A>G fibroblasts to iNPCs can be due to different reasons. It has been previously shown that above a certain threshold level (depending on the mtDNA mutation) of heteroplasmy the cellular reprogramming might be blocked although the cells might keep their pluripotent state (Yokota et al., 2015). As mentioned above, the heteroplasmy levels are not so high but the ATP levels and the non-canonical function of the respiratory chain due to the mutation might have been an inhibitory factor for the direct conversion.

Secondly, the appearance of the cells on day 7 might indicate that the cells were over stressed due to transduction and therefore only the non-transduced cells managed to survive and

proliferate. It is known that the cells carrying pathogenic mitochondrial mutations are characterised by oxidative stress due to ROS production. Therefore, the additional stress due to the cellular reprogramming might have resulted in accumulative stress that acted as obstacle for the direct conversion.

Finally, the efficiency of the retrovirus vector transfection might have been poor and this may have underlain the failure of conversion to iNPCs

### 5.3.2 *Mitochondrial dysfunction in fibroblasts and myoblasts carrying the homozygous mutation p.Arg113His in EIF2B5*

The patient is a 62-year-old man, only child of non-consanguineous English parents with no family history of neurological disease. At the age of 50 he developed balance problems and unsteadiness and from the age of 58 he is mostly wheelchair bound. His clinical examination revealed bilateral dysdiadochekinesis more on the left side with intention tremor. His vision, deep tendon reflexes and muscle tone were normal. He presented memory problems and had fluctuating cognitive decline. His MRI at the age of 54 showed prominent white matter T2 hyperintesities with periventricular perivascular space dilatation. The corpus callosum and spinal cord were markedly atrophic. Based on the fluctuation and the MRI, the possibility of MELAS was suggested. Genetic screening for the m.3243A>G, *ABCD1* (adrenoleykodystrophy) and *NOTCH3* (CASADIL, Cerebral Autosomal-Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) were negative.

Next, his muscle biopsy showed an isolated complex I deficiency with increased complex II expression suggesting a mitochondrial proliferation while the levels of the rest respiratory chain enzymes were normal. At the age of 58 years his MRI was compatible with vanishing white matter disease and the genetic analysis confirmed the homozygous p.Arg113His mutation in the *EIF2B5* gene.

Initially we measured the oxygen consumption of the fibroblasts carrying the homozygous mutation p.Arg113His in *EIF2B5*. As it shown in Figure 5.2 the oxygen consumption measurement did not illustrate any significant impairment of mitochondrial function. However, both basal and maximal respiration levels are slightly decreased in the patient's cell line compared to the controls though the alteration are not statistically significant ($p=0.1$, $p=0.26$ respectively, unpaired t-test).

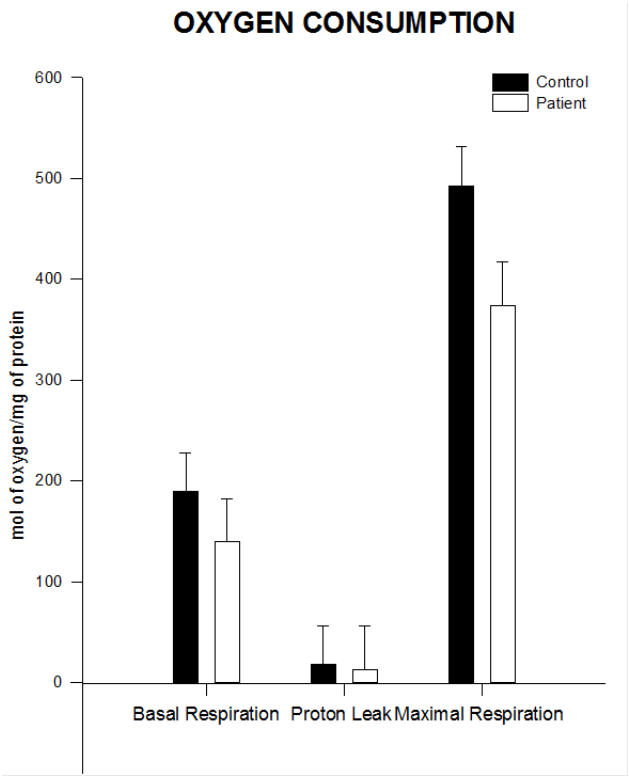



**Figure 5.2: Oxygen consumption in fibroblasts carrying the homozygous mutation p.Arg113His om EIF2B5. Black and white bars represent the mean values of control and patient's primary fibroblast cells respectively. The corrected oxygen consumption by the non-mitochondrial respiration (NMR) is represented as basal respiration, proton leak and maximal respiration.**

Following, the protein expression levels of the respiratory level chain enzymes measured by BN-PAGE are illustrated in Figure 5.3. As shown the BN-PAGE measurement did not show any alteration in the relative expression of the respiratory chain enzymes. The expression levels of all the complexes were normal, apart from complex V where the relative expression was slightly higher in the patient. The protein expression levels are normalised to porin expression levels.

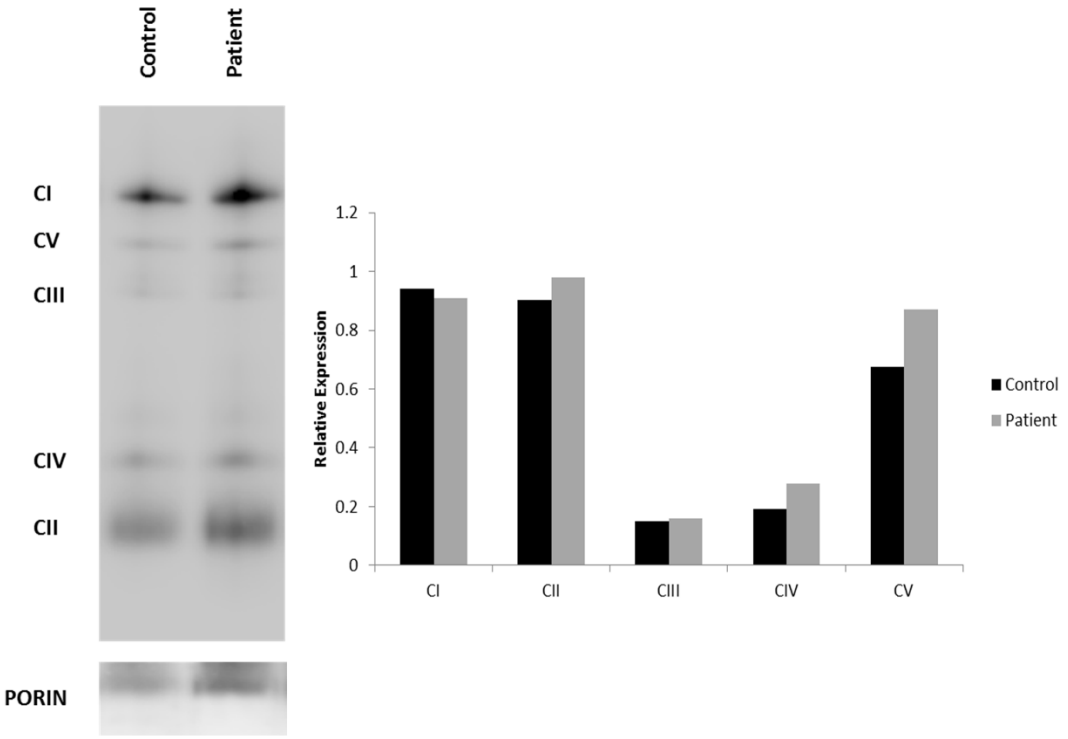



**Figure 5.3: BN-PAGE representing the relative expression of OXPHOS complexes in the control and patient primary fibroblasts. The quantification of the expression levels of the respiratory chain enzymes were normalized to porin expression levels.**

Based on the data obtained from the fibroblasts carrying the homozygous mutation, it is hard to reach any conclusion regarding the effect of the mutation on the mitochondrial function of fibroblasts. In addition, as it was discussed previously, the biochemical measurement revealed complex I deficiency in the patient's muscle biopsy. Therefore, we measured the oxygen consumption and expression levels of the respiratory chain enzymes in myoblasts isolated from the patient.

As shown in Figure 5.4 the oxygen consumption measurement in the myoblasts illustrated also no significant impairment of mitochondrial function. Although the basal respiration levels were slightly increased compared to the control, the maximal respiration levels were decreased implying slight impairment of the mitochondrial function ($p=0.22$, upaired t-test).

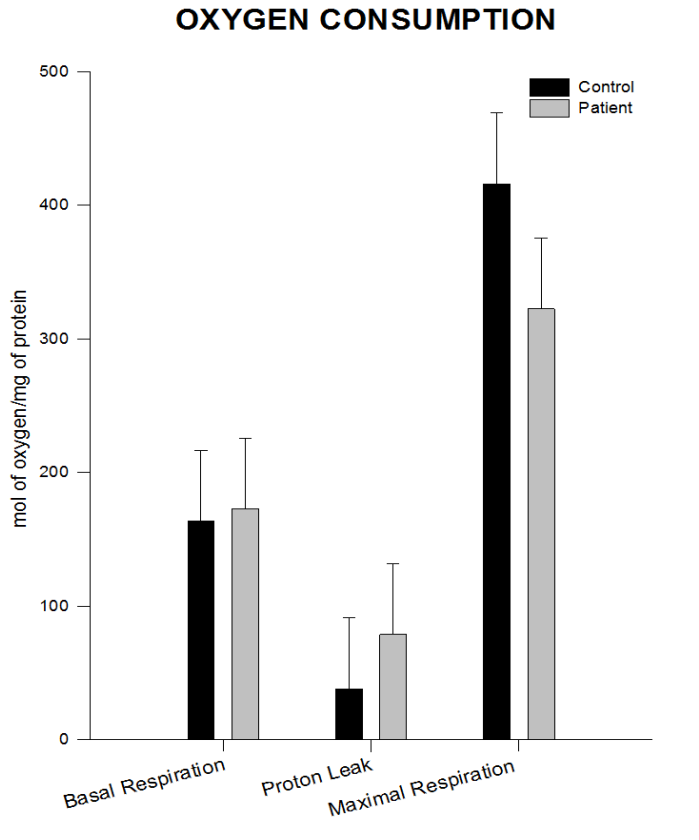



**Figure 5.4: Oxygen consumption in primary myoblasts carrying the homozygous mutation p.Arg113His in *EIF2B5*. Black and grey bars represent the mean values of control and patient's primary myoblast cells respectively. The corrected oxygen consumption by the non-mitochondrial respiration (NMR) is represented as basal respiration, proton leak and maximal respiration.**

Interestingly, the levels of proton leak are increased (p=0.09, unpaired t-test) compared to the control possibly demonstrating a defective ATP synthesis machinery.

Finally, we extracted the whole protein from fibroblasts and myoblasts carrying the homozygous mutation and measured the expression levels of all the respiratory chain enzymes.

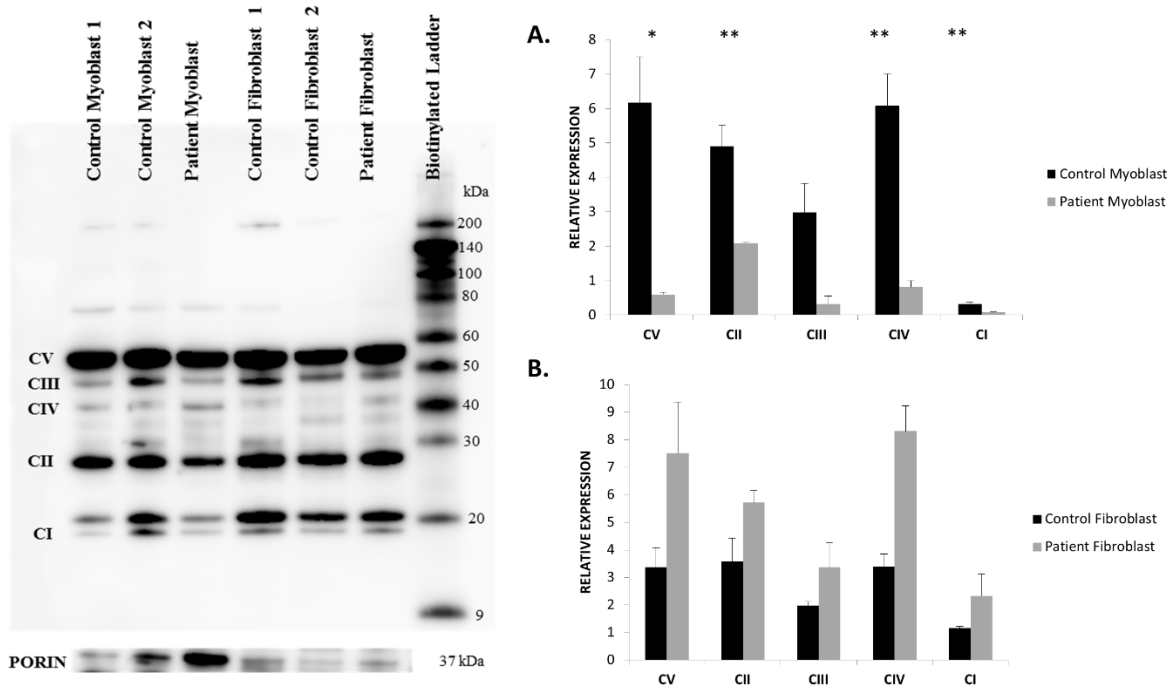



**Figure 5.5: Quantification of the protein levels of the complexes of the respiratory chain enzymes by SDS-PAGE in both primary myoblasts (A) and fibroblasts (B). The relative expression of the complexes is normalised to porin expression levels.**

As shown in Figure 5.5, in myoblasts the relative expression of all respiratory chain enzymes is significantly decreased except from complex III. On the other hand, in fibroblasts the expression levels of the respiratory chain enzymes are increased, especially of complexes I and IV. Furthermore, as shown in the figure, the expression levels of porin in the myoblasts are elevated compared to the controls. This may imply increased mitochondrial biogenesis rate and a compensatory mechanism of the mutated cells towards the mitochondrial defect. The striking abnormalities in the myoblasts compared to fibroblasts may indicate a defect of the mitochondrial translation in muscle and suggest a tissue specific manifestation.

### 5.3.3 *Direct conversion*

The most affected cells in VWM are the oligodendrocytes and astrocytes. It has been shown that the quantity of astrocytes is increased long before the manifestation of the disease (Dooves et al., 2016). In order to examine the hypothesis whether the homozygous p.Arg113His mutation in the *EIF2B5* gene cause a mitochondrial translation defect in more

affected cell types, it was attempted to directly convert the mutated fibroblasts to iNPCs. The protocol followed was the same to the one used for converting MELAS fibroblasts.

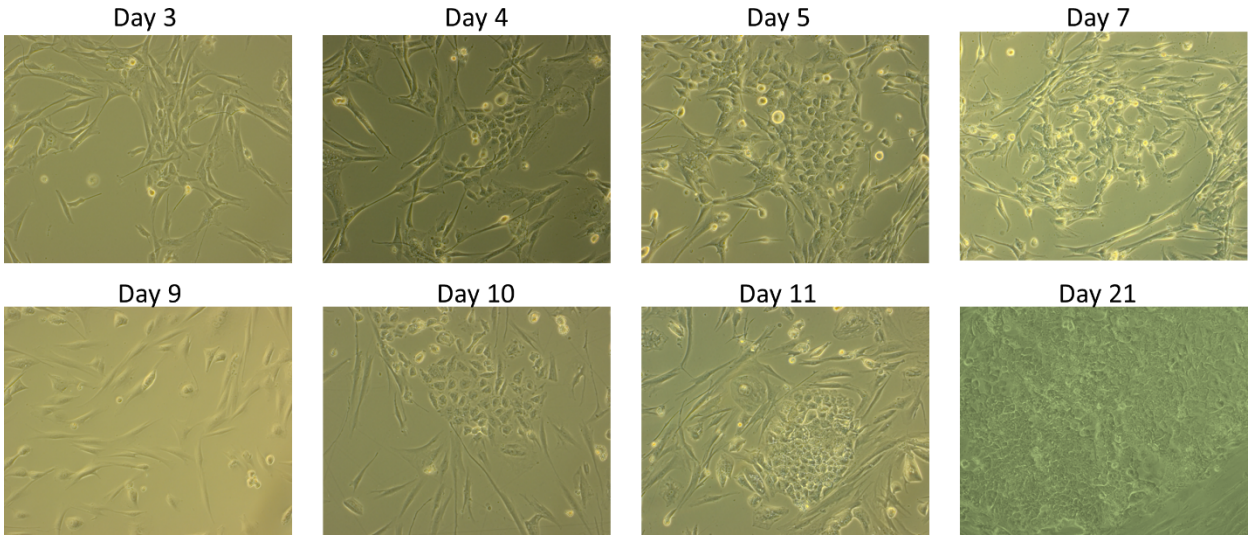



**Figure 5.6: Consecutive microscope images of the transduced fibroblasts carrying the homozygous mutation p.Arg113His from day 3 after the transduction till day 21.**

As shown in Figure 5.6, the cells showed morphological changes from day 3 after the transduction and started accumulating next to each other, to structure the neurospheres. On day 7 the cells were split, seeded on fibronectin coated wells and supplemented with NPC growth medium. On day 9 a mixture of round-shape cells and fibroblasts were observed under the microscope. The cells were kept in culture and although they were accumulating progressively, they did not form neurospheres as expected. After day 21, the cells discarded.

The tendency of accumulation indicates a pluripotent state of the cells however the direct conversion is considered inefficient as no neurospheres were formed. This could be possibly explained by the poor quality of the retroviral vectors used for the direct conversion. On the other hand, the impaired cytosolic translation machinery might have also affected the efficiency of the direct conversion as the cells might have not been able to adapt to the increased needs required for the reprogramming.

## 5.4 Discussion

The multi-organ involvement and tissue specificity of mitochondrial disorders due to mitochondrial translational deficiencies complicates the clinical diagnosis of the disease and the development of a potential treatment. Regularly, the affected post-mortem human tissue is of limited availability for further investigation and the necrotic environment might affect the quality of the tissue (Meyer et al., 2014). One of the main types of clinical manifestations of

mitochondrial disorders are the involvement of neuronal cell types. Therefore, the rapid development of cell reprogramming technology enabled scientists to generate cells of the desired type from somatic cells like fibroblasts. Although the generation and differentiation of iPSCs is already an established way to model neurons from an individual carrying the desired mutation, direct conversion of fibroblasts to iNPCs enables us to model and investigate how pathogenic mutations affect the CNS. Furthermore, the generation of iNPCs via direct conversion is less time-consuming compared to the generation of iPSCs and differentiation into neurons is possible.

In this chapter, it was illustrated my attempt of direct conversion of fibroblasts carrying the heteroplasmic mutation m.3243A>G and the homozygous mutation p.Arg113His in *EIF2B5* to induced neuronal progenitor cells. Regarding the MELAS 1 cell line, although the cells initially presented morphological changes implying that the transduction was effective, on day 9 their morphology was more similar to fibroblasts and they did not manage to form neurospheres. Regarding the VWM cell line, the cells seemed to have come to a pluripotent state but did not manage to form neurospheres. The outcome of the direct conversion both of MELAS 1 and VWM fibroblasts might due to a variety of reasons. It has been shown in previous studies, that heteroplasmic mitochondrial DNA mutation that lead to mitochondrial respiratory dysfunctions could block cellular reprogramming (Yokota et al., 2015). According to Yokota et al, the generation of iPSCs was drastically depressed by high proportions of mutant DNA. Therefore, the heteroplasmy levels of MELAS 1 cell lines might have acted in an inhibitory way towards the direct conversion. On the other hand, the differentiation of myoblasts to CNS and PNS lineages showed severe mitochondrial dysfunction in the terminally differentiated cell (Kodaira et al., 2015, Hatakeyama et al., 2015). Based on these studies in combination with the presented data. it is hypothesised that the impaired decreased ATP levels of cells characterised by mitochondrial respiratory dysfunction, might be another crucial inhibitory parameter of the direct conversion in both cell lines I tried to convert. Due to the mitochondrial dysfunction the produced ATP levels in MELAS 1 (and possibly VWM cells) might have not been high enough to support the direct conversion of fibroblasts to iNPCs. Another reason of the insufficient conversion might have been the additional stress caused by the transduction on these neuronal cell types. Regarding the VWM cell line, the impaired cytosolic translation machinery might have also acted in a prohibitive way towards the efficient generation of iNPCs. Finally, it cannot be excluded the possibility that the poor quality of the retrovirus used might have influenced the insufficient direct conversion in both cell lines.

Based on our results it could be claimed that the protocol followed might not be the appropriate for these disease models. However, as shown in figure 5.7 a random control primary fibroblast cell line has been successfully converted in-house to iNPCs confirming the validity and accuracy of the protocol. It is worth mentioning that the retrovirus used for control cell line conversion belonged to a different batch of retroviruses used for the MELAS and VWM conversion.

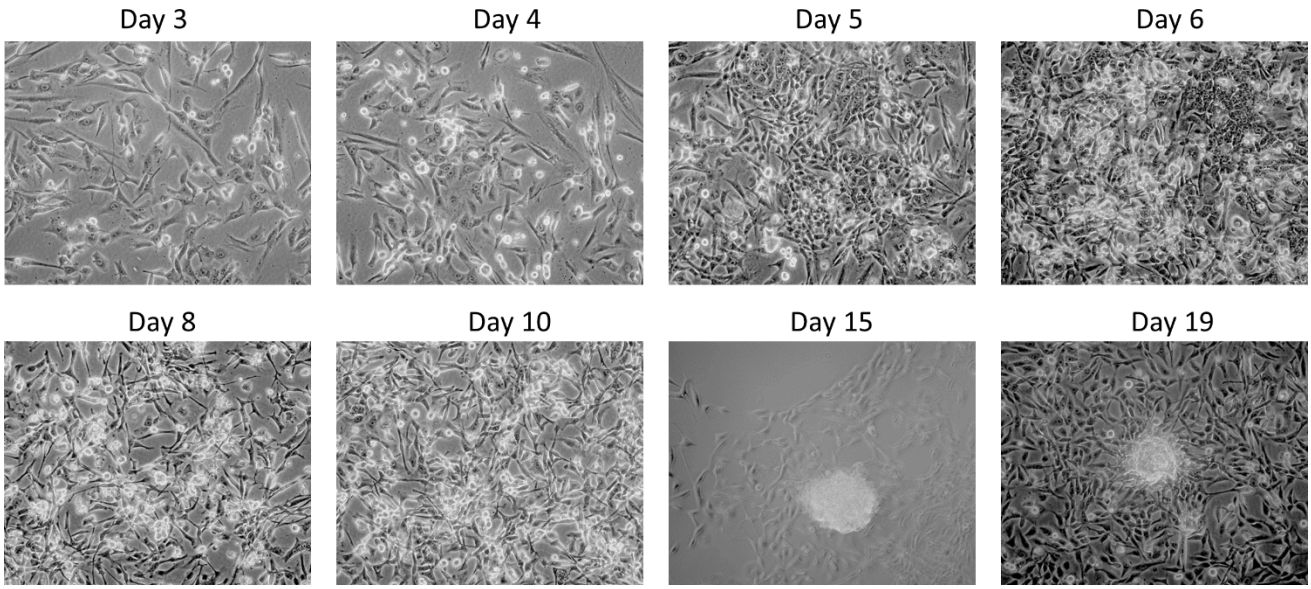



**Figure 5.7: Consecutive microscope images of a transduced control primary fibroblasts cell line showing the direct conversion to iNPCs and the formation of neuroshperes (Dr. Veronika Boczonadi).**

Finally, in this chapter I investigated the hypothesis of impaired mitochondrial translational machinery in patients affected by VWM. According to the oxygen consumption measurement and respiratory chain enzyme expression levels, it is concluded that the fibroblasts carrying the homozygous mutation p.Arg113His do not present any significant mitochondrial dysfunction. On the contrary, the myoblasts showed significantly reduced expression levels of the majority of the respiratory chain enzymes possibly implying a mitochondrial translational defect. Although further investigation needs to be done to verify whether the impairment of the mitochondrial translational machinery and the isolated complex I deficiency in the patient's muscle biopsy are due to a significant defect of mitochondrial protein synthesis in the VWM phenotype. As mitochondrial dysfunction has not been studied previously in patients with VWM, the observation is of importance. However, it needs further investigation as it is still not clear if the mitochondrial dysfunction is partly responsible for the

manifestation of the disease or it is a secondary phenomenon due to malfunction of the cytosolic translation machinery.

# Chapter 6. Novel mutation in MiD49 is associated with mitochondrial myopathy

## 6.1 Overview

In humans, mitochondria undergo constant fission and fusion to maintain their function (Westermann, 2010). These coordinated activities regulate the mitochondrial morphology and intracellular distribution and impose their cell-type specific appearance. The mitochondrial fission requires the recruitment of a large GTPase from the cytosol onto the outer mitochondrial membrane, called dynamin related protein 1 (Drp1). It has been suggested that Drp1oligomerizes around the circumference of the mitochondrion and it's GTP hydrolysis activity directs the constriction and scission (Ingerman et al., 2005, Mears et al., 2011). However, it is still not fully understood how Drp1 is recruited to the mitochondrion and which factors promote the recruitment.

To date, four different adaptors (Fis1, Mff, MiD49 and MiD51) have been suggested to act as recruiters of Drp1 to the outer mitochondrial membrane. Although early studies suggested that Fis1 is necessary for mitochondrial fission (Yoon et al., 2003), later studies showed that the number of the mitochondrial Drp1 puncta in Fis1-null cells significantly decreased (Losón et al., 2013) compared to wild type cells. Moreover, the levels of Drp1 in purified mitochondrial fractions from Fis1-null cells were not significantly decreased implying that F is1 has little or no role in recruiting Drp1 and consequently in mitochondrial fission (Losón et al., 2013). On the contrary, the recruitment of Dpr1 to the outer mitochondrial membrane is significantly reduced in Mff-null cells and the expression levels of Drp1 in the mitochondrial fraction of Mff-null cells are profoundly reduced compared both to wild type and Fis1-null cells (Losón et al., 2013) indicating that Mff is essential to the mitochondrial fission. Further studies investigating the proteins located in close proximity to Dpr1 have verified that Fis1 is localised in mitochondria but not in close proximity with Drp1 (Osellame et al., 2016). Taken these findings together it is believed that both Fis1 and Mff recruit Drp1 to the mitochondria but Mff plays the predominant role (Losón et al., 2013). Studies have shown that Fis1 and Mff also regulate the mitochondrial fission in peroxisomes (Koch and Brocard, 2012). To our best knowledge no mutations in Fis1 and Mff have been associated with disease in humans to date.

Recent studies have suggested that MiD49 and MiD51 which are encoded by the genes *SMCR7(MiD49*) and *SMCR7L*(*MiD51*) respectively, might act as receptors of Drp1. In 2000,

during a random cellular localisation of uncharacterised human proteins it was found that overexpression of *MiD51* led to unique changes in mitochondrial distribution and to an elongated mitochondrial network (Palmer et al., 2011).

It has been shown that the two genes are paralogues to each other and share 45% of their sequence identity (Palmer et al., 2011). The gene encoding MiD49 consists of three exons whereas *MiD51* consists of six exons. Moreover, they are differentially expressed in different tissues and during different developmental stages (Liu et al., 2013). Both of them are anchored in the outer mitochondrial membrane (Palmer et al., 2011) but their crystal structures show both similarities and differences. Although both of them contain a nucleotidyl transferase domain, only MiD51 is able to bind nucleotide diphosphates (ADP and GDP) whereas MiD49 is believed to bind an unknown ligand. MiD51 is dimeric in contrast to MiD49 which is monomeric but both of them share motifs interacting with Drp1. Their structural differences might suggest a differential regulation of *MiD51* versus *MiD49* mediated fission (Losón et al., 2014, Losón et al., 2015, Richter et al., 2014). It has been suggested that the MiD proteins have the ability to recruit Drp1 independently to Mff and Fis1(Palmer et al., 2013).

Initially, the function of both MiD proteins in mitochondrial fission was elusive. Palmer et al showed that knockdown of both genes led to decreased Drp1association with the mitochondrial surface and increased fusion events due to Drp1 sequestration in the cytosol (Palmer et al., 2011). Moreover, overexpression of both genes led to increased levels of Drp1 association to the mitochondrial surface as expected, but interestingly the cells overexpressing MiD proteins were characterised by increased fusion events and an elongated mitochondrial network (Palmer et al., 2011). Due to this fact, Liu et al claimed that *MiD49* and *MiD51* possibly promote mitochondrial fusion rather than fission. According to their study, cells overexpressing *MiD51* presented a mitochondrial fused phenotype, which was not reserved when *Mfn2* was blocked (Liu et al., 2013). However, in 2013 Palmer et al showed that overexpression of both MiD proteins followed by blocking both *Mfn1* and *Mfn2*did not lead to fusion events, illustrating the direct correlation of MiD proteins with mitochondrial fission events (Palmer et al., 2013). According to Losón et al, increased fusion events followed by *Mid49/51* overexpression, are due to the recruitment of an inactive phosphorylated form of Drp1 at Ser-637 (Palmer et al., 2013, Losón et al., 2013).

As it was discussed previously, MiD49 and MiD51 are specific mitochondrial adaptors of Drp1 in contrast to Mff and Fis1 which are also present in peroxisomes. However, increased

expression of the MiD proteins induced the peroxisome elongation due to loss of Drp1 from the mitochondria (Palmer et al., 2013).

The loss of either MiD49 or MiD51 adaptors have been reported to have contradictory effects on the mitochondrial fission. According to Palmer et al loss of either gene does not affect profoundly the amount of Drp1 at the mitochondrial surface or the mitochondrial morphology whereas loss of both genes leads to enhanced mitochondrial longevity (Palmer et al., 2011, Palmer et al., 2013). Osellame et al showed that individual deletion of *MiD49* or *MiD51* did not affect the levels of MiD51 and MiD49 respectively but simultaneous loss of *MiD49/51* on MEFs led to increased mitochondrial fusion and decreased levels of Dpr1 association with the mitochondrial outer membrane. They also showed that loss of both of the adaptors did not affect the ER-mitochondrial contact sites or the F-actin morphology (Osellame et al., 2016). On the other hand both knockdown of either gene and simultaneous knockdown resulted in similar enhancement of mitochondrial length and connectivity indicating that both *MiD49* and *MiD51* are equally valuable to the cell (Losón et al., 2013).

Interestingly, MEFs lacking *MiD49/51* presented apoptotic resistance as they retained more cytochrome c within the mitochondria and it seems that both proteins are important to the apoptotic recruitment of Drp1 to the mitochondrial surface. Also, loss of *MiD49/51* impairs OPA1-depedent cristae remodelling during apoptosis. These data taken together indicate that both genes possibly have a vital role in the intrinsic apoptotic pathway of the cell(Osellame et al., 2016).

Despite many years of research, it is still unknown how the adaptors engage with Drp1, and how they regulate the mitochondrial fission or role in the apoptosis.

## 6.2 Materials

A 15-year old boy from a consanguineous Jewish family with a healthy sister, developed progressive muscle weakness and exercise intolerance and high CK (1200U/L) at the age of 6 years His muscle biopsy revealed mitochondrial myopathy with numerous ragged red fibres and COX negative fibres (Figure 6.1). Quantification of the mtDNA copy number of the muscle biopsy showed increased mtDNA copy numbers compared to the control and no mtDNA deletions were detected in the patient's sample. The biochemical measurement detected deficiencies of multiple respiratory chain enzymes and the clinical investigation revealed pure muscular phenotype with no peroxosimal dysfunction and other organ involvement. Whole exome sequencing identified a homozygous nonsense mutation

(c.247C>T, p.Q81*) in exon 3 of *MiD49*. Although the exome sequencing revealed more than one candidate mutations in different genes, none of these mutations were segregated in the family.

Skin and skeletal muscle biopsy were obtained from the patient to investigate the effect of the mutation on the mitochondrial function.

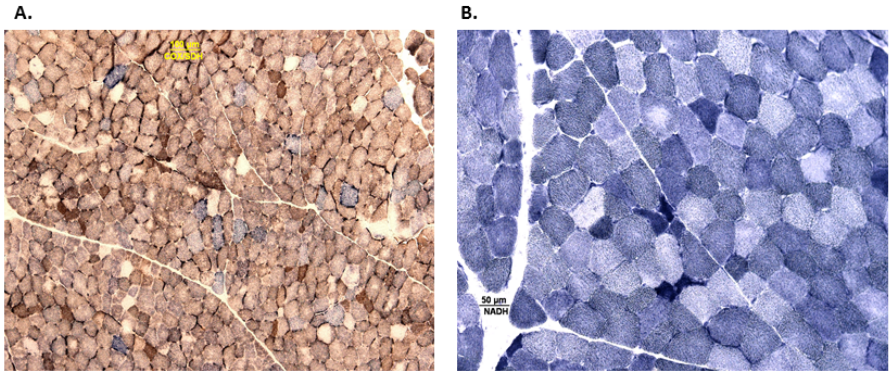



**Figure 6.1: A. Simultaneous staining of muscle fibres for cytochrome oxidase (COX) and succinic dehydrogenase (SHD), B. Nicotinamide adenine dinucleotide (NADH) staining of muscle fibres. Dark blue muscle fibres represent increased mitochondrial proliferation and pale muscle fibres illustrate deficient muscle fibres.**

## 6.3 Results

Based on previous studies showing that *MiD49* knockdown resulted in increased fusion events and consequently in an elongated mitochondrial network (Palmer et al., 2011, Palmer et al., 2013, Liu et al., 2013), Dr Verónica Eisner Sagüés (Mitochondrial Communication and Function Laboratory, School of Biological Sciences, Department of Cellular and Molecular Biology, Catholic University of Chile) kindly evaluated the fusion events in control and patient's fibroblasts by photoactivation under confocal microscopy. As shown in Figure 6.2 the fibroblasts carrying the homozygous nonsense mutation presented increased fusion events resulting in imbalanced mitochondrial dynamics.

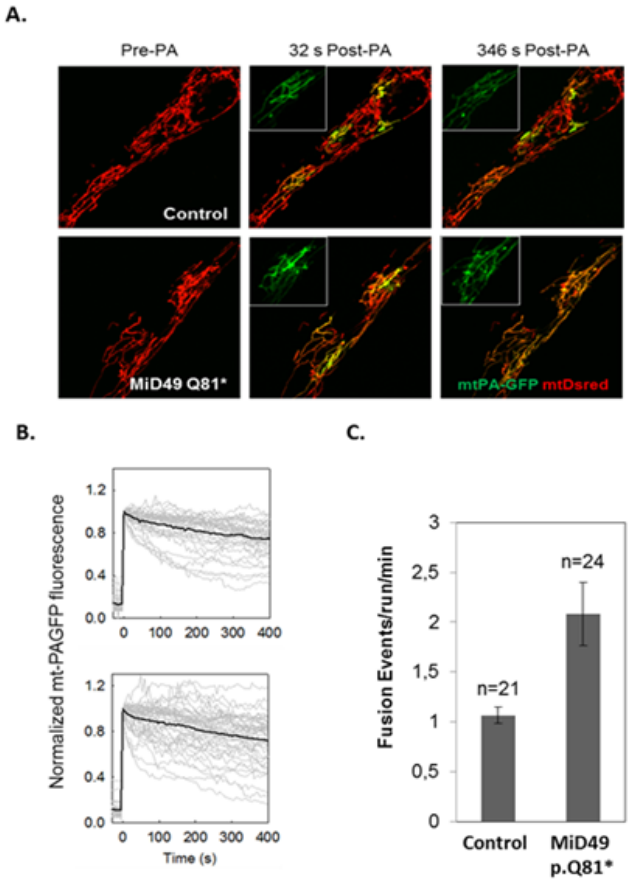



**Figure 6.2: Mitochondrial continuity and fusion events evaluation in human fibroblasts from control and MiD49 p.Q81* fibroblasts. A. Cells were transfected with mtDsRed and mtPA-GFP codifying plasmids and images by confocal microscopy. Representative cells before and after photoactivation of 5x5 regions interest (ROIs: white squares). The images display the continuity among mitochondrial evidenced by the diffusion of photoconverted PA-GFP towards neighbouring mitochondrial out of ROIs. B. mtPA-GFP fluorescence decay evaluated inside the photoactivation area.Gray curves, individual regions; black curves represent the mean. C. Frequency of fusion events. Data from at least 4 independent experiments. n= # cells (Source Dr Verónica Eisner Sagüés)**

Next, in order to examine how the disturbed mitochondrial dynamics in the fibroblasts affects the mitochondrial function, oxygen consumption of fibroblasts carrying the homozygous nonsense mutation was measured. As shown in Figure 6.3, the levels of basal respiration are slightly increased (22% increase) compared to the control (wild type) fibroblasts while the levels of maximal respiration are slightly decreased (3% decrease) compared to the control fibroblasts.

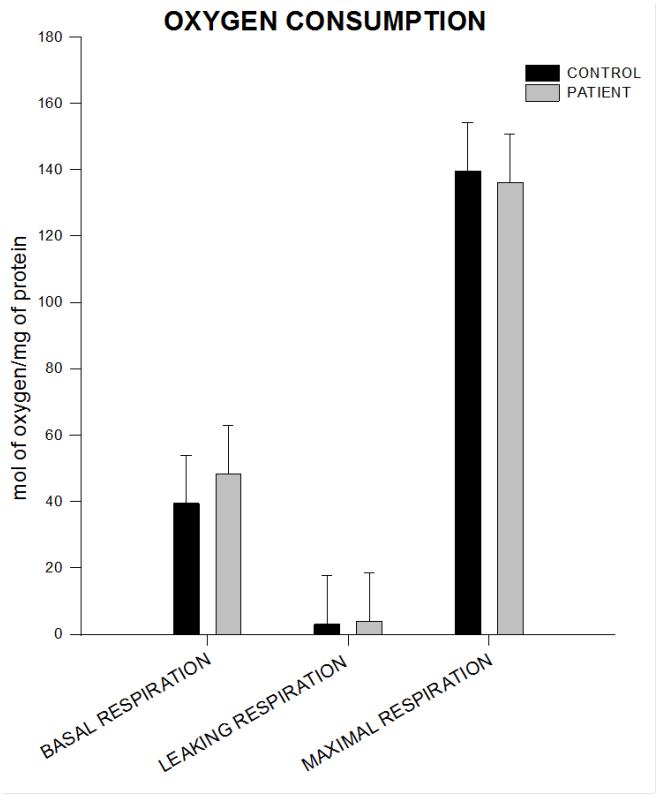



**Figure 6.3: Oxygen consumption in fibroblasts carrying the homozygous nonsense mutation c.247C>T inMiD49. Black and grey bars represent the mean values of control and patient's primary fibroblast cells respectively. The corrected oxygen consumption by the non-mitochondrial respiration (NMR) is represented as basal respiration, leaking respiration and maximal respiration.**

Since these alterations are not statistically significant it is concluded that the fibroblasts carrying the homozygous nonsense mutation do not exhibit any mitochondrial dysfunction. In support of the result of oxygen consumption, the expression levels of the respiratory chain enzymes of the patient's fibroblasts measured by BN-PAGE, were not significantly increased compared to the control (Figure 6.4).

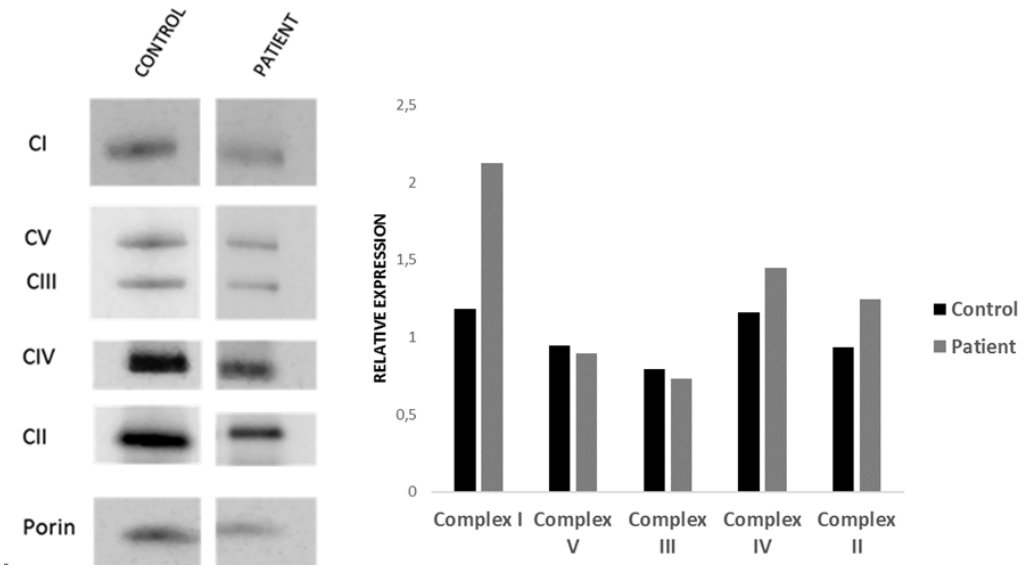



**Figure 6.4: BN-PAGE representing the relative expression of OXPHOS complexes in the control and patient primary fibroblasts.**

Next, the expression levels of proteins involved in the mitochondrial fission and fusion such as MiD49, Mfn2, OPA1, Drp1 and MiD51 and of the protein complexes of the respiratory chain in the whole protein lysate isolated from the patient's fibroblasts were measured.

As shown in Figure 6.5, the relative expression of MiD49 is significantly decreased in the patient's fibroblasts compared to the control (p=0.01, unpaired t-test).

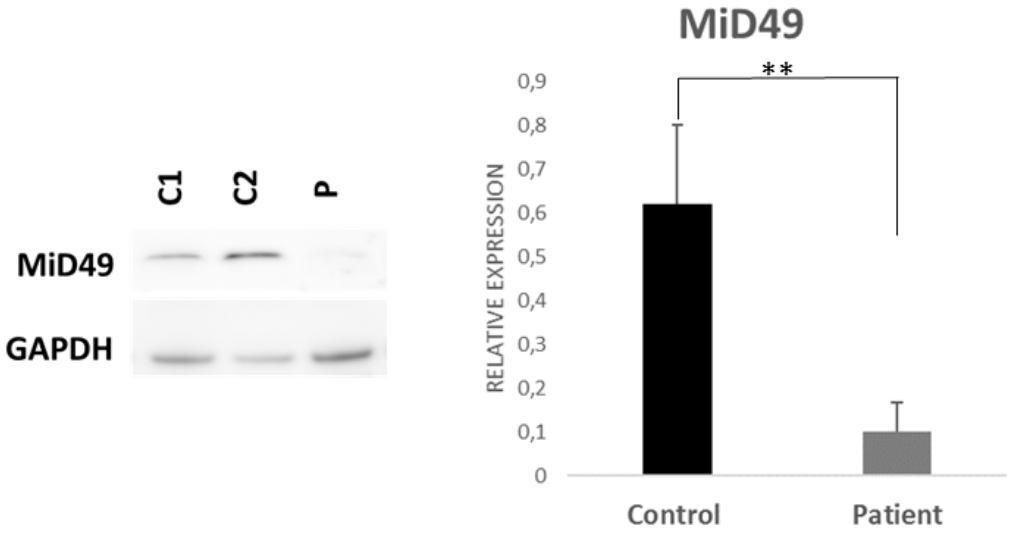



**Figure 6.5: Immunoblotting detected significantly decreased protein expression levels of MiD49 in the patient's fibroblasts.**

Due to the increased fusion events in the fibroblasts carrying the homozygous nonsense mutation, it was expected that the relative expression of Mfn2 and OPA1 would be also

increased (Figure 6.6). Furthermore, the decreased expression of MiD49 resulted in slightly decreased expression of Drp1 and MiD51. However, none of these alterations in the protein expression levels were statistically significant. The data are consistent with previous publications where knockdown of MiD49 in either HEK of MEF cells resulted in increased expression of Mfn2 and decreased levels of Drp1 (Palmer et al., 2011, Palmer et al., 2013, Osellame et al., 2016).

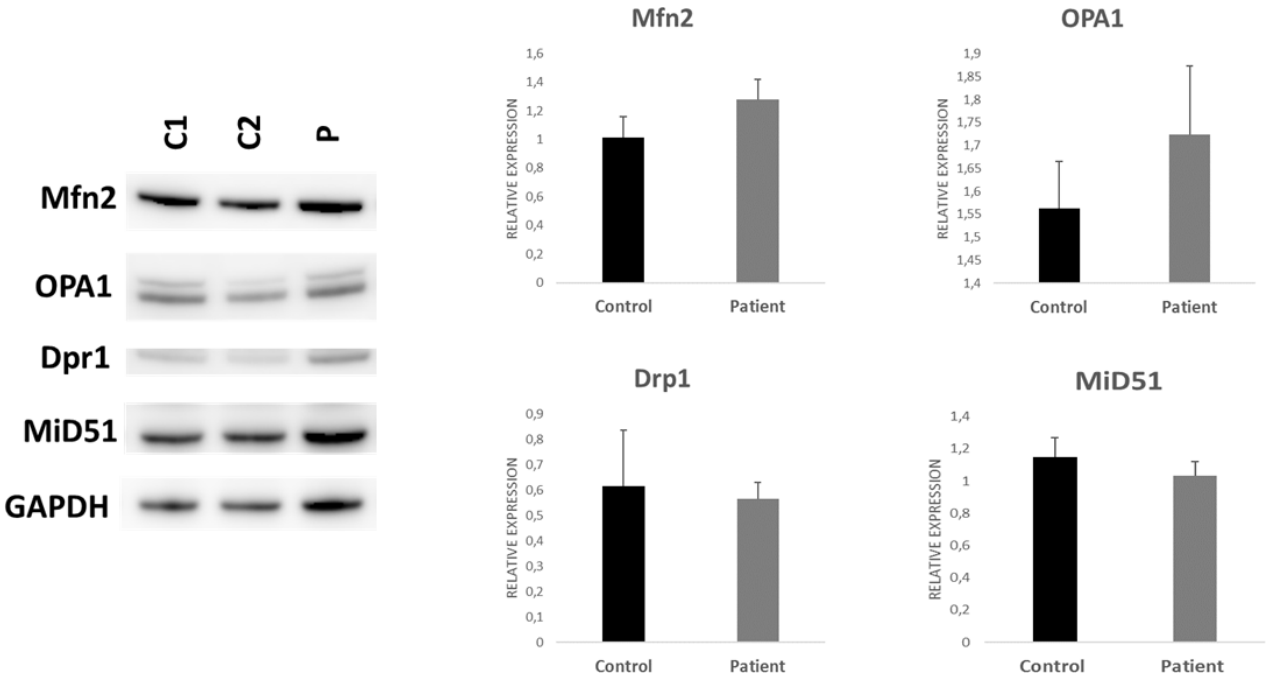



**Figure 6.6: Quantification of mitochondrial fusion (Mfn2 and OPA1) and fission (Drp1 and MiD51) protein expression levels by immunoblotting illustrated increased but not significant levels of Mfn2 and OPA1, Also, relative expression of Dpr1 and MiD51 was decreased.**

The elevated protein expression levels detected in all the OXPHOS complexes (Figure 6.7) and specifically the significant increase in the relative expression of CII and CIV ($p=0.02$, $p=0.016$ respectively, unpaired t-test) agreed with the increased relative expression of the respiratory chain enzymes measured by BN-PAGE in mitochondria isolated from fibroblasts.

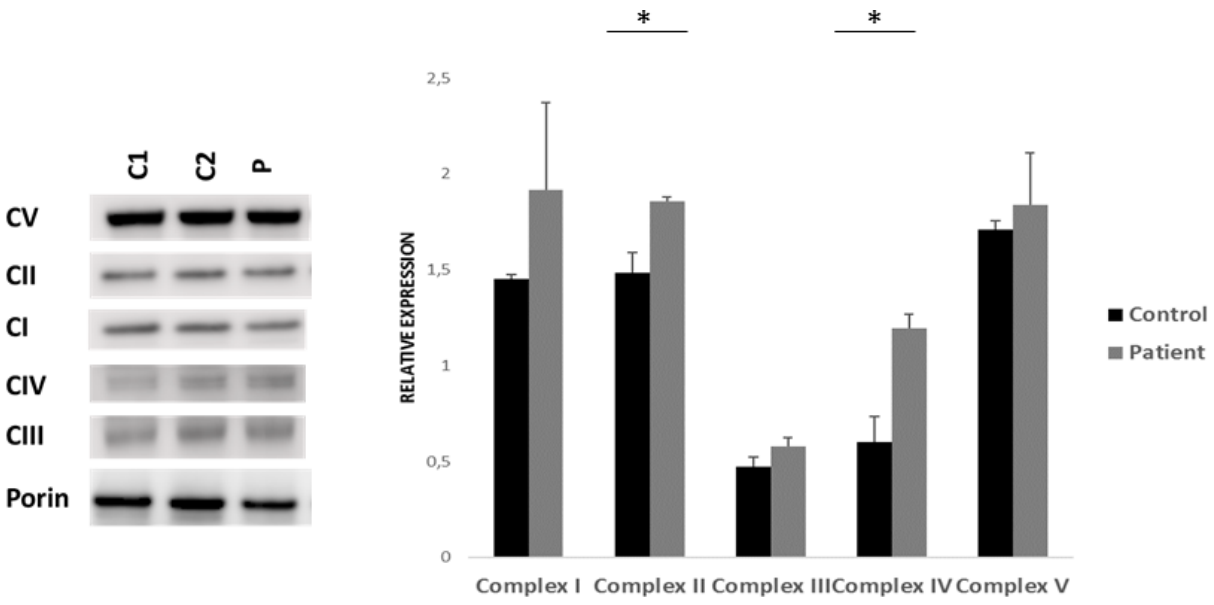



**Figure 6.7: Quantification of expression levels of the OXPHOS components in total protein lysates extracted from cultured primary fibroblasts.**

Following, the quantification of the relative mtDNA copy number in the fibroblasts illustrated significantly increased mtDNA copy number (p=0.01, unpaired t-test) in the patient's fibroblasts compared to the control, similar what was observed in the patient`s skeletal muscle (Figure 6.8).

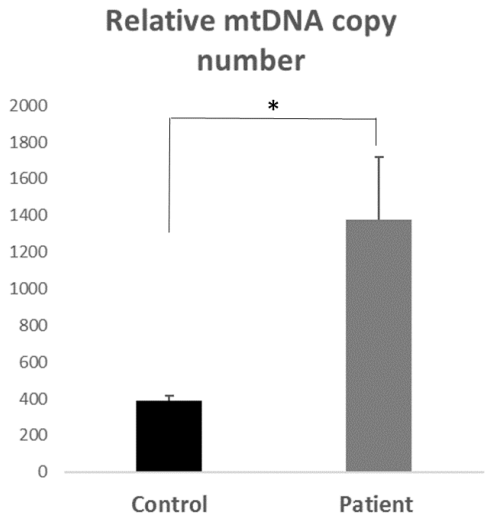



**Figure 6.8: Quantification of the mtDNA copy number showing the significant increase of the mtDNA copies in the patient's primary fibroblasts.**

Based on these data, the increased mtDNA copy number in fibroblasts accompanied with increased relative expression of the protein complexes of the respiratory chain and no indication of significant mitochondrial dysfunction might indicate a possible compensatory mechanism in fibroblasts.

Since the clinical examination of the patient revealed pure mitochondrial myopathy, the total protein from the patient's muscle biopsy was extracted and the relative expression of the complexes of the respiratory chain and of MiD49, Mfn2, OPA1, MiD51 and Drp1 were measured.

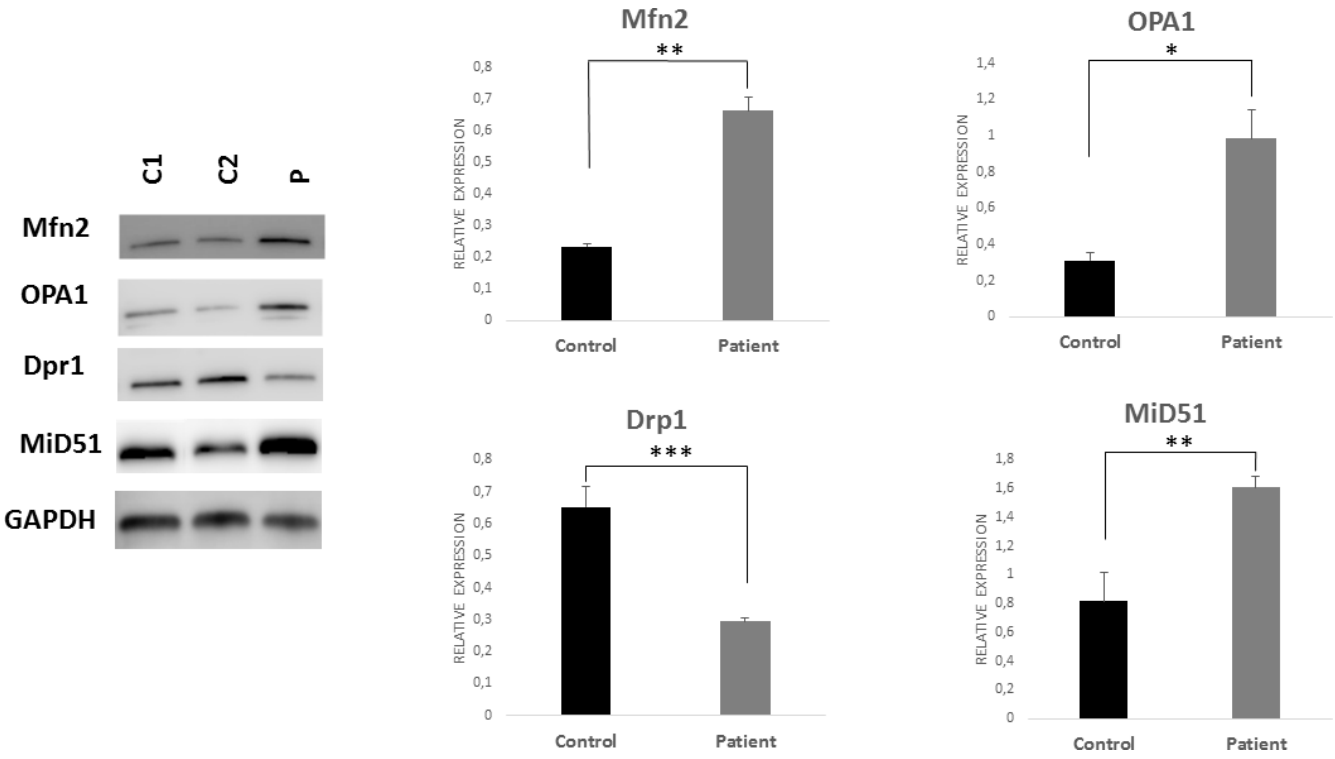



**Figure 6.9: Quantification of Mfn2, OPA1, Drp1 and MiD51 protein expression levels from total protein lysates extracted from the muscle biopsy.**

As shown in Figure 6.9, the relative expression of Mfn2 ($p=0.002$, unpaired t-test) and OPA1 ($p=0.02$, unpaired t-test,) in the patient's sample is significantly elevated compared to the control sample illustrating overexpression of proteins responsible for mitochondrial fusion at the outer mitochondrial membrane. On the contrary, Drp1 expression levels are significantly decreased ($p=0.0005$, unpaired t-test) in the patient's skeletal muscle showing the limited activity of mitochondrial fission. Although previous studies have shown that knockdown of *MiD49* in MEF cells does not affect the levels of MiD51 (Osellame et al., 2016), here it has been shown significantly increased expression of MiD51 in the patient's skeletal muscle.

Interestingly, the MiD49 protein was still detected on SDS-PAGE in the patient's muscle sample (Figure 6.10) but it was significantly decreased compared to the control skeletal muscle samples. It is commonly accepted that a gene may encode different variants of the same protein called protein isoforms. On the website of the National Centre for Biotechnology Information (NCBI), it has been reported that MiD49 protein can be identified

in three different isoforms and all the reported isoforms contain exon 3, where the mutation is located. Therefore, theoretically the stop mutation detected in the patient should result in non-functional isoforms of MiD49 and the protein should not be detectable on SDS-PAGE. Based on that, different antibodies were used to detect the protein levels of MiD49. However, the result from the different antibodies showed that MID49 is still detectable but significantly decreased. It is hypothesized that It the different antibodies used for detection of MiD49 might not have been specific for the protein and the band detected might be MiD51 protein due to their sequence similarity.

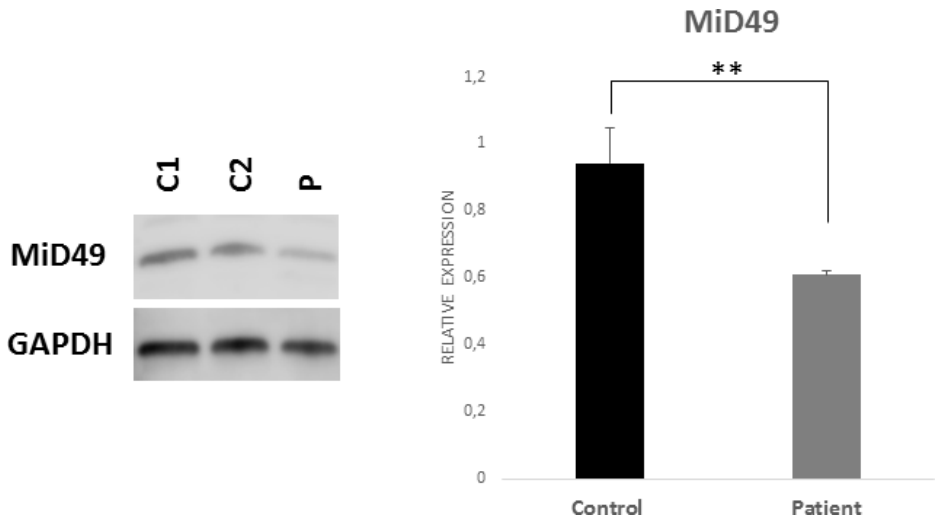



**Figure 6.10: Immunoblotting detected significantly decreased relative expression of MiD49 in the muscle**

Although the expression of MiD49 is not fully depleted in the patient's muscle biopsy, the relative expression of the OXPHOS complexes is significantly decreased compared to the control (Figure 6.11) indicating that the imbalanced mitochondrial dynamics may affect the respiratory chain activity. As shown in Figure 6.11 the relative expression of CI, CII, CIII and CIV is significantly decreased while the relative expression of CV is decreased but not significantly.

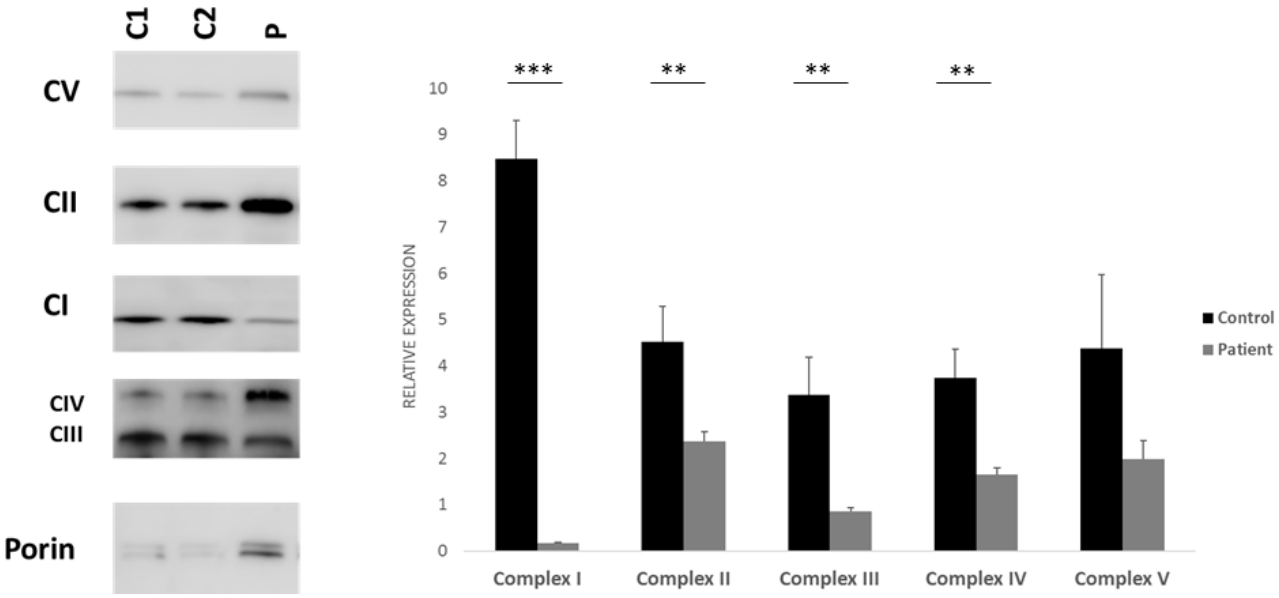



**Figure 6.11: SDS-PAGE gel showing the expression of the OXPHOS components in total protein lysates extracted from muscle**

## 6.4 Discussion

One of the unique characteristics of mitochondria is their ability to build large interconnected and intracellular networks (Westermann, 2010) via fusion and fission. These two balanced activities rule the mitochondrial morphology and distribution and eventually the cell type-specific appearance. Drp1plays a major role in mitochondrial fission which oligomerizes around the mitochondrion and directs the fission. However, it is still unclear how Drp1 is recruited to the mitochondrion. To date, four different adaptors have been suggested to act as mediators of Dpr1 recruitment; initially Fis and Mff and recently MiD49 and MiD51. To date there has not been any human disease linked with mutations in any of these genes.

In this chapter it has been shown that fibroblasts carrying the novel homozygous mutation p.Q81* in *MiD49* carried elongated mitochondrial with increased mtDNA copy number and presented significantly increased fusion events compared to the control fibroblasts indicating that MiD49 plays a vital role in mitochondrial dynamics. The oxygen consumption levels in the patient's fibroblasts accompanied with increased expression levels of the OXPHOS complexes might suggest a compensatory mechanism in the fibroblasts. The significantly increased levels of protein involved in mitochondrial fusion (Mfn2 and OPA1) and significantly decreased levels of Drp1 expression levels in the muscle biopsy, suggest that reduced levels of MiD49 result in imbalanced mitochondrial dynamics.

In conclusion, p.Q81* (c.247C>T) in MiD49 is the first reported mutation in any of the four adaptors of Drp1 linked to human disease. Moreover, mutations in that gene have not been reported in any other cohort of patients to my knowledge. These findings illustrate the vital role of MiD49 in mitochondrial fission and further studies are needed to enlighten the specific role of MiD49 in mitochondrial fission and apoptosis.

# Chapter 7. Mutations in C12orf65 are associated with disturbed mitochondrial translation and Behr syndrome

## 7.1 Overview

### 7.1.1 *Potential role of C12orf65*

The protein group class I release factor family (RFs), consisting of RF1Lmt/mtRF1a, RF1mt, C12orf65 and ICT1 proteins, is necessary for efficient protein translation. The main functions of these proteins are the recognition of the stop codons at the A-site of the ribosome and the hydrolysis of the ester-bond between the last tRNA and the newly synthesized polypeptide chain. Subsequently, the newly synthesized protein is released. To date, the specific function of each different protein belonging to that protein family is still unknown (Richter et al., 2010).

The presence of specific domains and/or sequence motifs is an essential criterion for proteins to be assigned to a release factor family. These domains are: the Codon Recognition (CR) domain, consisting of the helix alpha-5 and the 'anticodon tripeptide motif', and the Peptidyl tRNA hydrolase (PTH) domain. The PTH domain is characterized by a GGQ (Gly-Gly-Gln) motif at the active site of the protein, responsible for the ester-bond hydrolysis (Richter et al., 2010).

C12orf65 is a nuclear encoded protein that belongs to the class I peptide release factors family. It is located at 12q24.31 and consists of 3 exons. The protein is characterized by the loss of the two stop codon recognition functional elements (CR domain and 'anticodon tripeptide motif') while retains the PTH domain. Immunoprecipitation studies have shown that C12orf65 is a soluble protein that does not exhibit ribosomal-specific PTH activity and is localised in the mitochondrial matrix (Antonicka et al., 2010, Richter et al., 2010). It has been suggested to be involved in the process of peptidyl-tRNAs that have been prematurely released during the polypeptide elongation (Antonicka et al., 2010).

To date, it is the only gene from the class I release factors family which has been associated with a human disease. Down-regulation of C12orf65 resulted in significant changes in the mitochondrial membrane potential and mitochondrial mass, indicating that it is essential for cell vitality and mitochondrial function (Kogure et al., 2012).

Studies have shown that overexpression of *ICT1* in *C12orf65* mutated cells partially rescued the phenotype in the cells, indicating that *ICT1* and *C12orf65* might have overlapping

functions (Antonicka et al., 2010). Furthermore, C12orf65 protein is predicted to contain a homologous C-terminal alpha-helix to a recently described ICT1 C-terminal helix (Duarte et al., 2012). The existence of C12orf65 homolog in only five out of 28 bacterial groups suggests that the eukaryotic protein derived from a duplication of a canonical RF. Moreover, the wider phylogenetic distribution of ICT1 in combination with the common structural features between ICT1 and C12orf65, is a strong argument that the latter derived from a duplication of ICT1 (Duarte et al., 2012).

In 2010, Antonicka et al described two patients from two unrelated families with mutations in *C12orf65*. They were characterized by combined OXPHOS deficiencies associated with a decrease in the synthesis of all mitochondrially encoded polypeptides. Furthermore, both patients presented optic atrophy from 5-7 years and in the following years developed complex neurological symptoms. Although their clinical presentation was reminiscent to Leigh syndrome the progression of the disease was much slower compared to the typical Leigh syndrome. Sequence analysis in patient 1 revealed a homozygous 1bp deletion (248delT) in *C12orf65* resulting in premature stop codon at position 84 of the protein. Similarly, in patient 2 was found a homozygous 1bp deletion (210delA) in *C12orf65* resulting in premature stop codon at position 84 (Antonicka et al., 2010).

The second report on mutations in the *C12orf65* gene described two male siblings from a consanguineous Japanese family. Measurement of enzymatic activities of the respiratory chain complexes in one of the patients showed decrease activity of complexes I and IV. The clinical investigation revealed optic atrophy and neuropathy in both patients. Exome sequencing identified a homozygous nonsense mutation (c.394C>T, p.Arg132*) in *C12orf65* resulting in premature stop codon at position 132 (Shimazaki et al., 2012).

In 2013, Buchert et al reported a homozygous nonsense mutation p.Gln139* in two siblings from a consanguineous family resulting in a truncated protein (Buchert et al., 2013). Moreover, a homozygous p.Val116* truncating mutation has been reported in 3 members of a large consanguineous Indian family. The mitochondrial potential, respiration rate and the enzymatic activity of the mitochondrial chain complexes in the affected members were reduced. The clinical phenotype was characterized by neuropathy and optic atrophy (Tucci et al., 2014). The same mutation was identified also in a pair of female monozygotic twins (Imagawa et al., 2016). Next, two compound heterozygous (p.Pro34llefs*25 and p.Gly72*) frameshift mutations were identified in two siblings (Heidary et al., 2014). Two further

homozygous mutations p.Lys138Argfs*16 and c.282+2T>A have been reported in consanguineous families with childhood-onset optic atrophy (Spiegel et al., 2014).

| Clinical presentation of patients in this study | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Onset/alive or died†** | **Optic atrophy** | **Neuropathy** | **Pyramidal signs** | **Opthalmo-paresis** | **Ataxia** | **Cognitive dysfunction** | **Biochemical Markers** | **Mutation** |
| **Patient 1** | 5y/13y | +(5y) | +++ | ++ | ++ | + | + | CI,CII/III,CIV defect in muscle | Hom. p.Pro34Ilef |
| **Patient 2** | 6y/7y | +(6y) | + | + | + | + | - | n.d. | |
| **Patient 3** | 5y/16y | +(5y) | +++ | ++ | - | + | + | n.d. | Hom. C.282G> |
| **Clinical presentation of previously reported patients** | | | | | | | | | |
| **Antonicka et al., P1** | 1y/8y† | ++(5y) | ? | ? | ++ | ++ | + | CI, CIV, CV defect in fibroblasts | Hom. p.Val83Gly |
| **Antonicka et al., P2** | 15m/20y | + | ++ | - | ++ | ? | ? | n.d. | Hom. p.Gly72Ala |
| **Antonicka et al., P3** | 3y/22y† | +(3y) | ++ | ? | ++ | - | ? | n.d. | |
| **Shimazaki et al., P1** | 7y/32y | +(7y) | + | + | - | - | - | CI, CIV defect in fibroblasts | Hom. p.Arg13 |
| **Shimazaki et al., P2** | 7y/42y | +(7y) | ++ | ++ | - | - | - | n.d. | |
| **Buchert et al., P1** | ?/27y | - | + deformed hands/ feet | ? | + | ? | + | n.d. | Hom. p.Gln13 |
| **Buchert et al., P2** | ?/24y | - | ? | ? | + | ? | + | n.d. | |
| **Tucci et al., P1** | 8y/34y | + | + | + | + | ? | + | n.d. | Hom. p.Val11 |
| **Tucci et al., P2** | c./53y | + | + | + | ? | ? | + | n.d. | |
| **Tucci et al., P3** | c./51y | + | + | + | ? | ? | + | CV defect in lymphoblasts | |
| **Heidary et al., P1** | c./8y | + | ? | + | + | + | + | CIV defect in muscle | Com. Het. p.Pro34Ilefs*2 |
| **Heidary et al., P2** | c./5y† | + | ? | + | + | + | + | CIV defect in muscle | p.Gly72Alafs* |
| **Spiegel et al., P1** | c./? | + | + | + | - | - | - | n.d. | Hom. |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | p.Lys138Argfs³ |
| **Spiegel et al., P2** | c./? | + | + | + | - | - | + | CI and CIV defect in muscle | Hom. c.282+2T |

**Table 7.1: Clinical presentation of Patients 1-3 in this study and the previously reported patients carrying pathogenic mutations in *C12orf65***

### 7.1.2 *Behr Syndrome*

Behr syndrome was first described by Carl Behr in 1909 (Behr, 1909). The clinical manifestation of the disease is characterized by infantile optic atrophy and various neurological disorders such as ophthalmoparesis, nystagmus, ataxia, peripheral neuropathy and developmental delay.

Most of the reported cases are sporadic or show autosomal recessive inheritance. However, a few reported cases presented autosomal dominant inheritance pattern (Felicio et al., 2008). Mutations in *OPA3*, *OPA1* and *C19orf12* have been correlated to Behr syndrome (Sheffer et al., 1992, Anikster et al., 2001, Marelli et al., 2011, Bonneau et al., 2014, Kleffner et al., 2015).

## 7.2 Materials and Methods

Patient 1, a 13 year old boy and patient 2 his 7 years old sister, were born to non-consanguineous Irish parents. Patient 1 had normal early development and the first symptom was optic atrophy at 5years. In the following years he developed weakness and atrophy of the right arm and leg, mild ataxia and learning difficulties. Detailed metabolic work-up was normal. The clinical examination revealed optic atrophy, ophthalmoparesis and bilateral nystagmus. The muscle biopsy revealed mitochondrial myopathy and deficiencies in complexes I, II/III and IV of the respiratory chain. No mtDNA deletions were detected in muscle DNA. Direct sequencing of the *OPA1*, *TK2*, *RRM2B* and *PEO1* genes revealed no candidate pathogenic mutations.

Patient 2 has optic atrophy since the age of 6 years, followed by mild foot weakness and balance problems. The neurological examination revealed broad nasal bridge, bilateral optic atrophy and ophthalmoparesis.

Patient 3, a 16 years-old boy, was born to non-consanguineous healthy parents of Hungarian Roma ethnic origin. His other three siblings were healthy. At 5 years he developed visual impairment, and increasing clumsiness. The clinical examination showed bilateral optic atrophy and slight nystagmus. A diagnostic muscle biopsy was not performed in this case.

Whole exome sequencing was performed in genomic DNA of patients 1 and 2 and direct sequencing of C12orf65 in patient 3 based on the clinical phenotype. DNA was

fragmented and enriched by Illumina TruSeq 62Mb exome capture and sequenced (Illumina HiSeq 2000, 100 bp paired-end reads). The in-house bioinformatics pipeline included alignment to the human reference genome (UCSC hg19), reformatting, and variant detection (Varscan v.2.2, Dindel v1.01), as described previously (Horvath et al., 2012). On-target variant filtering excluded those with minor allele frequency greater >0.01 in several databases: dbSNP135, 1000 genomes (February 2012 data release), the National Heart, Lung and Blood Institute (NHLBI, NIH, Bethesda, MD) Exome Sequencing Project (ESP) 6500 exomes, and 343 unrelated in-house controls. Rare homozygous and compound heterozygous variants were defined, and protein altering and/or putative 'disease causing', along with their functional annotation, were identified using ANNOVAR (Wang et al., 2010). Candidate genes were prioritized if previously associated with a disease phenotype (Lieber et al., 2013). Putative pathogenic variants were confirmed by Sanger sequencing.

Fibroblasts cell cultures from patients 1, 2 and controls were obtained from the Biobank of the Medical Research Council, Centre for Neuromuscular Disease, Newcastle. Informed consent was obtained from all subjects. Measurement of the oxygen consumption was conducted.

Whole blood was obtained from patient 3 and his unaffected family members. Informed consent was obtained from all subjects. DNA and RNA extraction from whole blood, PCR and Sanger sequencing were conducted.

The following primers were used for genomic DNA and cDNA analysis of *C12orf65*:

| Genomic DNA | Forward Primer (5'-3') | Reverse Primer (5'-3') | Annealing Temperature ($^{o}$C) | Product Size (bp) |
|---|---|---|---|---|
| **Exon 2** | GCATAATCTTGAGGGCAGATG | GGCCCAAGCCAGAAAAATA | 60 | 486 |
| **Exon 3** | GCGAACAGGTTGAATTTAATGA | CACTATAATAATGCTGGTGATGGA | 60 | 397 |
| **cDNA** | | | | |
| **Exon2/3** | GCAACCAACAAAACCAGCAA | CAGGACTGTTTTCACCATTGTAG | 63 | 160/478 |

**Table 7.2: Sequence of primers used from amplification of the genomic DNA and cDNA**

## 7.3 Results

Whole exome sequencing revealed a homozygous nonsense mutation (p. Pro34Ilefs*25) in patients 1 and 2. The mutation was segregated with the disease in the family and was not detected in 190 ethnically-matched control alleles. According to bioinformatics tools, it is predicted to cause a complete loss of the C12orf65 protein.

| Patient ID | Variant Type | On Target (a) | Rare/Novel Protein Altering Variants (b) | Rare Compound Heterozygous Protein Altering | Rare Compound Heterozygous Protein Altering & Mitochondrial | Rare Compound Heterozygous Mitochondrial Genes | Rare Homozygous Protein Altering | Rare Homozygous Protein Altering & Mitochondrial | Rare Homozygous Mitochondrial Genes |
|---|---|---|---|---|---|---|---|---|---|
| **Patient 1** | SNV | 79,650 | 255 | 13 | 1 | *ENOSF1* | 8 | 1 | *C12orf65* |
| | Indel | 8,901 | | | | | | | |
| **Patient 2** | SNV | 81,683 | 244 | | | | | | |
| | Indel | 9,169 | | | | | | | |

**Table 7.3: Rare/novel variants (homozygous MAF<0.01, compound heterozygous MAF product <0.0001 and single heterozygous MAF<0.001) with exclusion of common variants found to be shared in an in-house panel of 394 individuals, 1000 Genomes and NHLBI-ESP 6500 databases**

Measurement of oxygen consumption in fibroblasts from patients 1 and 2 showed decreased levels of both oxygen consumption rate and maximal capacity respiration levels (Figure 7.1). As it is illustrated in Figure 7.1 patient 1 presents lower levels of both oxygen consumption and maximal respiration levels ($p=0.008$ and $p<0.001$ respectively) compared to patient 2. Nonetheless, the levels of maximal respiration are also significantly decreased in patient 2 ($p<0.001$). Hence, the capacity of the electron transport chain in both affected is significantly limited compared to the control verifying the mitochondrial dysfunction caused by the nonsense mutation identified.

Although an increase of mitochondrial mass is often observed in mitochondrial myopathies, the quantification of the mtDNA copy number detected lower levels in patient 1 compared to controls. However, no mtDNA deletion was detected in the skeletal muscle of patient. This may reflect downregulation of the mitochondrial mass. Interestingly, it has been suggested that down-regulation of C12orf65 results in ROS production, apoptotic cell death and reduced levels of mitochondrial mass (Kogure et al., 2012). The mtDNA copy number in patient 2 was not measured.

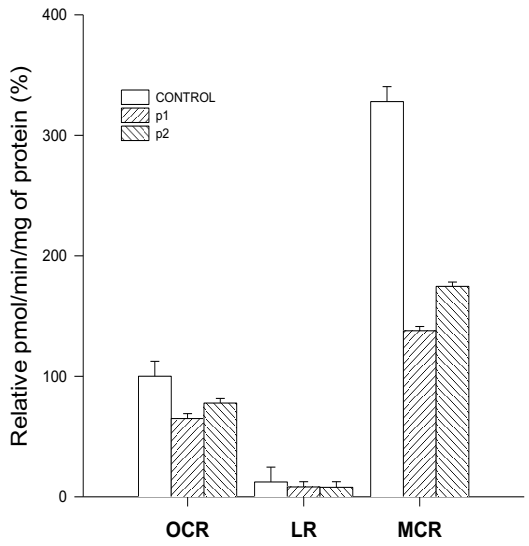



**Figure 7.1: Oxygen consumption in fibroblasts, white and stripped bars represent the mean values from control (C1 and C2) and patients (P1 and P2), respectively. Corrected oxygen consumption by the non-mitochondrial respiration and mg of protein is represented as oxygen consumption (OCR), leaking respiration (LR) and maximal capacity respiration (MCR) respectively**

In patient 3 direct Sanger sequencing revealed a homozygous splice site mutation (c.282G>A). This mutation affects the last codon of exon 2 and results in loss of a splice site with retention on intron 2. Unaffected members were heterozygous for the c.282C>A variant which was not detected in 200 Hungarian Roma alleles.

As is illustrated in Figure 7.2 showing the amplification of cDNA in patient 3 and his mother, the mutation is causing retention in intron 2 giving a larger band at 478bp compared to his mother that gives a normal splicing product at 160 bp.

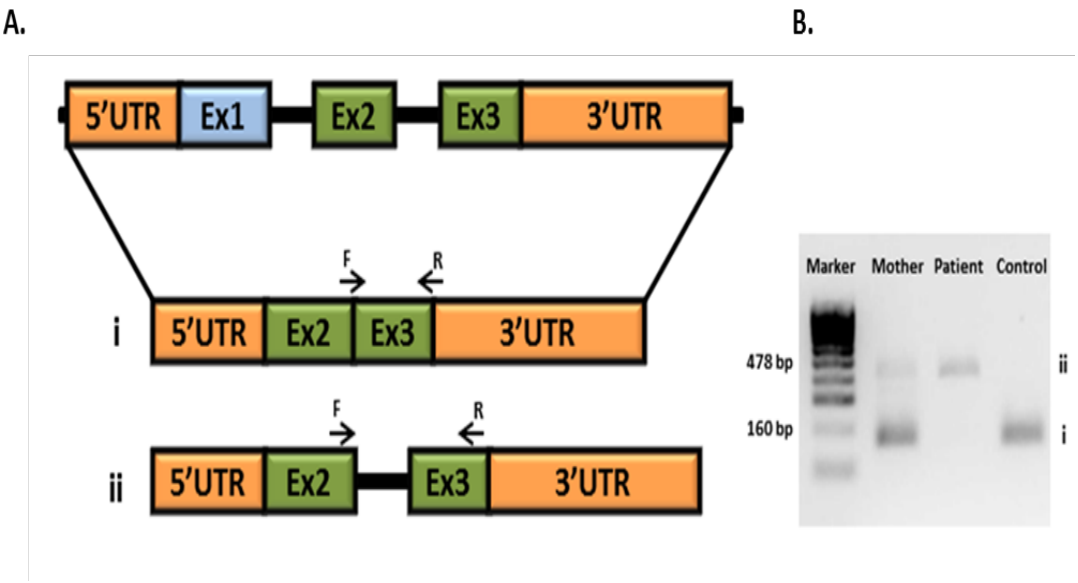



**Figure 7.2: A. Schematic structure of C12orf65 cDNA showing the splice defect (ii) caused by the c.282 G>A mutation in patient 3. B. Agarose gel showing the amplification of cDNA in patient 4 and his mother, i) normal splicing producing a 160bp product, ii) mutation causing intron 2 to be retained giving a larger band of 478 bp.**

The retention of intron 2 results in a non-functional structural form of C12orf65 protein that is degraded from the cell and consequently the mitochondrial function is impaired.

## 7.4 Discussion

Mutations in *C12orf65* have been correlated with distinct phenotypes such as classical Leigh syndrome, hereditary spastic paraplegia (SPG55) or complicated Charcot-Marie Tooth disease (CMT6) resulting in a phenotypic heterogeneity. However, optic atrophy and neuropathy are two shared clinical symptoms among the different phenotypes described. Furthermore, it has been suggested that there is a correlation of the mutation site in *C12orf65* gene with the disease severity (Buchert et al., 2013, Tucci et al., 2014) and consequently with the phenotype.

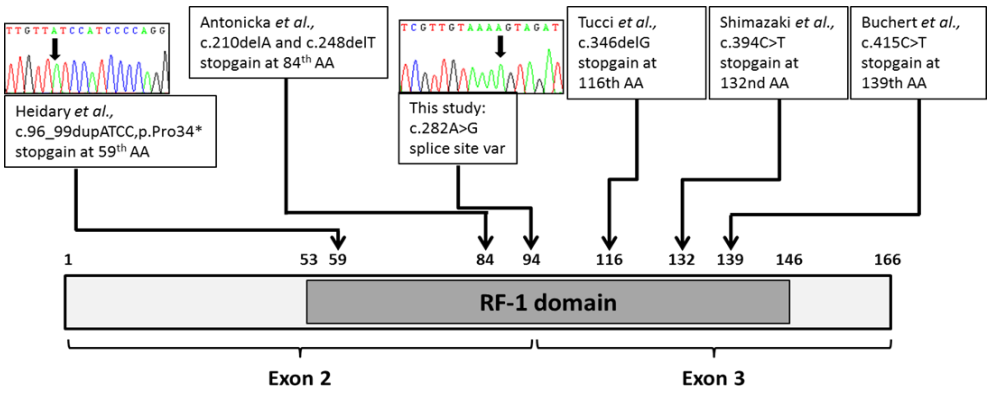



**Figure 7.3: Schematic structure of *C12orf65*, showing the localisation of the RF-1 domain, position of identified mutations and the exon structure. Exome sequencing identified pathogenic C12orf65 mutations in 4 patients**

The two different homozygous nonsense mutations, presented in the described patients, match the original historical description of Behr's syndrome (Behr, 1909). The first symptom in all patients was childhood-onset optic atrophy, followed by spastic paraparesis, distal weakness and motor neuropathy. Furthermore, all of them presented combined respiratory deficiency (Pyle et al., 2014).

Although the precise role of *C12orf65* in the mitochondrial translation process is not fully known, it is pronounced that absence of the protein causes severe mitochondrial dysfunction. Despite the fact that Behr syndrome is characterised by genetic heterogeneity, the mitochondrial dysfunction is the common pathway leading to characteristic symptoms of the syndrome (Yu-Wai-Man and Chinnery, 2014).

In summary, Behr's syndrome due to C12orf65 mutations is a clinically recognizable disease presentation and should be considered in multiple mitochondrial respiratory chain deficiencies (Pyle et al., 2014).

# Chapter 8. General Discussion

Mitochondrial disorders comprise a large, heterogeneous group of disorders that affect both children and adults. Mitochondrial diseases are amongst the most common inherited neuromuscular disorders with a minimum prevalence of around 1:5000 live births (Schaefer et al., 2008). They usually affect more than one organ, leading to complex multisystemic dysfunctions. However, there are cases where the manifestation of the disease is tissue specific (Chinnery and Hudson, 2013). Although, tissues in which the metabolic demand is higher, such as skeletal muscle, the central nervous system or heart are typically affected (Chinnery and Hudson, 2013), the phenotype is variable and heterogeneous due to dual genetic control (mtDNA and nDNA), level of heteroplasmy, tissue energy demand, maternal inheritance and mitotic segregation. In the past, it was believed that mitochondrial disorders were strictly caused by a biochemical defect of the respiratory chain (Filosto and Mancuso, 2007). However, nowadays it is widely accepted that mitochondrial disorders can be caused by either mitochondrial or nuclear genes that regulate mitochondrial homeostasis and functioning such as mitochondrial translation and mitochondrial fusion and fission (Filosto and Mancuso, 2007).

One of the greatest challenges of mitochondrial disorders is the development of successful treatments. Although clinicians and scientists have been working for decades on developing treatments for mitochondrial disorders, there is no established treatment to date, due to the complex genetic and phenotypic heterogeneity of the diseases. In certain cases, the use of pharmacological treatments, which are analyzed thoroughly in Chapter 1, have an effect on certain mitochondrial disorders. Amongst the pharmacological treatments tested on different mitochondrial disorders, L-cysteine has been shown to improve the thiolation and therefore the OXPHOS activity in mitochondrial translation disorders caused by mitochondrial tRNA mutations that affect the thiolation of specific mitochondrial tRNAs (Boczonadi et al., 2013). Therefore, during my PhD, I studied the effect of L-cysteine and N-acetyl-cysteine supplementation in a variety of mitochondrial disorders. Based on my data, it seems that L-cysteine was beneficial to cell lines carrying mtDNA mutations (m.3243A>G and 8344A>G) and NAC on the other hand, was beneficial to cell lines carrying nDNA (*TRMU*, *MTO1*, *ELAC2* and *COX10*) mutations. The differential effect observed between the two supplements it was hypothesized it was due to the nature of the mutation leading to the mitochondrial disease. The supplementation with antioxidants and especially L-cysteine and NAC have been

proposed before (Deepmala et al., 2015) to be effective on a variety of mitochondrial and neurodegenerative diseases. The results presented in my study underline the beneficial effect of those supplements on a subset of mitochondrial disorders. In the future, further exploration needs to be done to clarify the exact molecular mechanism of L-cysteine and NAC; their impact on U34 thiolation, on mitochondrial translation and on the levels of GSH. Moreover, considering the beneficial effect on cell models, the results should be tested on animals. Although supplementation with L-cysteine and NAC is not a panacea for all the mitochondrial disorders, it is a small step towards the treatment of a certain subset of mitochondrial disorders.

A second major challenge clinicians and scientists need to fight is the accurate and immediate diagnosis of a mitochondrial disorder. The development of new technologies such as Next Generation Sequencing has shed light on the genetic and clinical heterogeneity of mitochondrial diseases by identifying novel genes. It is already known that the same mutation might lead to different clinical features and vice versa different mutations might lead to the same clinical phenotype. Therefore, it is challenging for the clinicians to diagnose betime and accurately a mitochondrial disorder.

Mutations in *C12orf65* have been associated with different distinct phenotype such as classical Leigh syndrome or complicated Charcot-Marie Tooth disease. However, it has been noted that optic atrophy and neuropathy are two clinical characteristics shared by all the patients carrying mutations in that gene (Pyle et al., 2014). Based on published studies describing the clinical phenotype of patients carrying mutations in *C12orf65* gene in combination with the patients described and analyzed in my PhD (Chapter 7) I reached the conclusion that mutations in that gene lead to Behr syndrome as all the different characteristics described fit the original historical description of that syndrome.

In addition to that, during my PhD, with the use of NGS, it was found that a novel mutation in the nuclear gene *MiD49* is associated with mitochondrial myopathy. *MiD49* gene encodes a protein which functions as an adaptor of Drp1, which is responsible for the mitochondrial fission. According to my data, the novel homozygous nonsense mutation p.Q81* is associated with significantly increased fusion events in fibroblasts illustrating the vital role of the protein in mitochondrial dynamics. Moreover, the significantly decreased levels of the protein in the muscle led to significantly decreased expression of the OPXHOS complexes. The clinical investigation of the patient carrying the homozygous nonsense mutation revealed pure

mitochondrial myopathy with no other organ involvement and his muscle biopsy revealed also mitochondrial myopathy with numerous ragged red fibers.

In summary, the above examples underline the necessity of NGS for the correct diagnosis of a mitochondrial disorder and identification of novel genes leading to mitochondrial dysfunction. By unravelling new genes associated with mitochondrial diseases and classifying mitochondrial disorders it will help the better understanding of the pathways involved and the identification of novel and specific therapies.

Finally, tissue specificity is one of the most characteristic clinical features of mitochondrial disorders. The same variability in the expression of a mtDNA mutation or biochemical defect can be observed between different patients and also between different tissues in a given individual, a state called tissue specificity. Despite many years of research on mitochondria, the tissue specificity has not been fully explained yet. However, it can be partially explained by the different heteroplasmy levels found in different tissues in each individual. For example, MELAS syndrome is a multisytemic disorder characterized by tissue specificity and the CNS is highly affected in the majority of m.3243A>G carriers. During my PhD, I tried to implement the new method of direct conversion of fibroblasts carrying either mitochondrial or nuclear mutations to iNPCs. Therefore, I used fibroblasts from patients carrying the m.3243A>G mutation and fibroblasts from a patient diagnosed with VWM. Although VMW is not directly related to mitochondrial dysfunction, according to my results myoblasts carrying the mutation p.Arg113His in *EIF2B5* showed significantly decreased relative expression levels of OXPHOS enzymes indicating possible mitochondrial dysfunction. One of the major clinical features in patients diagnosed with VWM is the foamy appearance of oligodendrocytes and the abnormal morphology of astrocytes.

In both MELAS and VWM fibroblast cells, the direct conversion to iNPCs proved insufficient and the possible reasons are comprehensively evaluated in Chapter 6. However, the innovative method used for generation of iNPCs needs less time compared to the traditional method of cell differentiation through the iPSCs state and it was attempted for the first time on fibroblasts with mitochondrial mutations. To sum up, methods like these enables the scientists to study and delve into the mechanism of tissue specificity.

Despite many years of research, the rapid diagnosis and efficient treatment of mitochondrial disorders remains challenging. During my PhD, I have broadened our knowledge on specific aspects of mitochondrial disease, which I hope, will contribute to future advances in the field.

# Appendix A: Publications in peer-reviewed scientific journals

1. **Bartsakoulia M**, Müller SJ, Gomez-Duran A, Patrick Yu Wai Man, Boczonadi V, Horvath R: Cysteine Supplementation May be Beneficial in a Subgroup of Mitochondrial Translation Deficiencies, *Journal of Neuromuscular Disorders* 9/2016, DOI: 10.3233/JND-160178

Journal of Neuromuscular Diseases 3 (2016) 363–379
DOI 10.3233/JND-160178
IOS Press



Research Report

## Cysteine Supplementation May be Beneficial in a Subgroup of Mitochondrial Translation Deficiencies

Marina Bartsakoulia[a,1], Juliane S. Müller[a,1], Aurora Gomez-Duran[a,b], Patrick Yu Wai Man[a,c,d], Veronika Boczonadi[a] and Rita Horvath[a,*]
[a]*Wellcome Trust Mitochondrial Research Centre and the John Walton Muscular Dystrophy Research Centre, Institute of Genetic Medicine, Newcastle University, Central Parkway, Newcastle upon Tyne, UK*
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[d]*NIHR Biomedical Research Centre at Moorfields Eye Hospital and UCL Institute of Ophthalmology, London, UK*



**Abstract.**
**Background:** Mitochondrial encephalomyopathies are severe, relentlessly progressive conditions and there are very few effective therapies available to date. We have previously suggested that in two rare forms of reversible mitochondrial disease (reversible infantile respiratory chain deficiency and reversible infantile hepatopathy) supplementation with L-cysteine can improve mitochondrial protein synthesis, since cysteine is required for the 2-thiomodification of mitochondrial tRNAs.
**Objectives:** We studied whether supplementation with L-cysteine or N-acetyl-cysteine (NAC) results in any improvement of the mitochondrial function *in vitro* in fibroblasts of patients with different genetic forms of abnormal mitochondrial translation.
**Methods:** We studied *in vitro* in fibroblasts of patients carrying the common m.3243A>G and m.8344A>G mutations or autosomal recessive mutations in genes affecting mitochondrial translation, whether L-cysteine or N-acetyl-cysteine supplementation have an effect on mitochondrial respiratory chain function.
**Results:** Here we show that supplementation with L-cysteine, but not with N-acetyl-cysteine partially rescues the mitochondrial translation defect *in vitro* in fibroblasts of patients carrying the m.3243A>G and m.8344A>G mutations. In contrast, N-acetyl-cysteine had a beneficial effect on mitochondrial translation in *TRMU* and *MTO1* deficient fibroblasts.
**Conclusions:** Our results suggest that L-cysteine or N-acetyl-cysteine supplementation may be a potential treatment for selected subgroups of patients with mitochondrial translation deficiencies. Further studies are needed to explore the full potential of cysteine supplementation as a treatment for patients with mitochondrial disease.

Keywords: Cysteine supplementation, mitochondrial translation, mt-tRNA modification, mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy ragged red fibres (MERRF)

### INTRODUCTION

Most mitochondrial diseases are disabling, progressive or fatal, affecting the brain, liver, skeletal muscle, heart and other organs [1, 2]. Currently there are no effective cures and treatment is at best symptomatic [3]. Although defective oxidative phosphorylation is the common final pathway, it

[1]MB and JSM contributed equally to this study.
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2. Cottenie E, Kochanski A, Jordanova A, Bansagi B, Zimon M, Horga A, Jaunmuktane Z, Saveri P, Rasic VM, Baets J, **Bartsakoulia M**, Ploski R, Teterycz P, Nikolic M,

Quinlivan R, Laura M, Sweeney MG, Taroni F, Lunn MP, Moroni I, Gonzalez M, Hanna MG, Bettencourt C, Chabrol E, Franke A, von Au K, Schilhabel M, Kabzińska D, Hausmanowa-Petrusewicz I, Brandner S, Lim SC, Song H, Choi BO, Horvath R, Chung KW, Zuchner S, Pareyson D, Harms M, Reilly MM, Houlden H. Truncating and missense mutations in IGHMBP2 cause Charcot-Marie Tooth disease type 2. *American Journal of Human Genetics*, 95(5):590-601

3. Pyle A, Ramesh V, **Bartsakoulia M**, Boczonadi V, Gomez-Duran A, Herczegfalvi A, Blakely EL, Smertenko T, Duff J, Eglon G, Moore D, Man PY, Douroudis K, Santibanez-Koref M, Griffin H, Lochmüller H, Karcagi V, Taylor RW, Chinnery PF, Horvath R. Behr's Syndrome is Typically Associated with Disturbed Mitochondrial Translation and Mutations in the *C12orf65* Gene. *Journal of Neuromuscular Diseases*, 1(1):55-63.

# Appendix B: Abstracts for scientific meetings

Abstract for poster presentation at UK Neuromuscular Translational Research Conference (London, March 2014)

Title:

Behr's syndrome is a mitochondrial disease due to autosomal recessive mutation in the C12orf65 gene

Authors:

Angela Pyle,[1†] Ramesh Venkateswaran,[2†] Marina Bartsakoulia, [1] Veronika Boczonadi,[1] Agnes Herczegfalvi,[3] Emma L. Blakely,[4] Tania Smertenko, [1] Jennifer Duff, [1] David Moore,[1] Patrick Yu Wai Man,[1] Veronika Karcagi,[5] Hanns Lochmüller,[1] Mauro Santibanez-Koref,[1] Helen Griffin,[1] Robert W. Taylor,[4] Patrick F. Chinnery,[1]and Rita Horvath[1]

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Abstract:

Behr's syndrome is a classical phenotypic description of childhood-onset optic atrophy combined with various neurological symptoms, including ophthalmoparesis, nystagmus, spastic paraparesis, ataxia, peripheral neuropathy and learning difficulties. Here we describe 4 patients with the classical Behr's syndrome phenotype who carry homozygous nonsense mutations in the C12orf65 gene encoding a mitochondrial protein. C12orf65 mutations have been previously reported with various clinical presentations, such as Leigh syndrome, SPG55, CMT6, syndromic intellectual disability, but a thorough review of these previous reports

indicates that the phenotype of all patients with C12orf65 mutations is compatible with Behr's syndrome. We think that C12orf65 mutations are more frequent than previously suggested and C12orf65 screening should be considered not only in mitochondrial respiratory chain deficiencies, but also in the inherited peripheral neuropathies, spastic paraplegias and ataxias, especially with pre-existing optic atrophy.

## Behr's syndrome is a mitochondrial disease due to autosomal recessive mutation in the *C12orf65* gene

**Angela Pyle,[1]† Ramesh Venkateswaran,[2]† Marina Bartsakoulia, [1] Veronika Boczonadi,[1] Agnes Herczegfalvi,[3] Emma L. Blakely,[4] Tania Smertenko, [1] Jennifer Duff, [1] David Moore,[1] Patrick Yu Wai Man,[1] Veronika Karcagi,[5] Hanns Lochmüller,[1] Mauro Santibanez-Koref,[1] Helen Griffin,[1] Robert W. Taylor,[4] Patrick F. Chinnery,[1]and Rita Horvath[1]**

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### Introduction

Behr's syndrome is a childhood-onset optic atrophy combined with various neurological symptoms. To date, *OPA3* mutations have been identified in Iraqi Jews presenting Behr's and Costeff syndrome and more recently autosomal dominant *OPA1* mutations were reported in Behr's syndrome without metabolic abnormalities.

C12orf65 belongs to a family of four mitochondrial class I peptide release factors including mtRF1, mtRF and ICT1. Only C12orf65 has been associated to date with human disease. C12orf65 protein may play a role in recycling abortive peptidyl-tRNA species, released from the ribosome during the elongation phase of translation. Down-regulation of the gene results in significant changes in the mitochondrial membrane potential and mitochondrial mass, indicating that it is essential for cell vitality and mitochondrial function.

### Patients and Methods

| | Onset/alive or died† | Optic atrophy | Neuro-pathy | Pyra-midal signs | Ophthal-moparesis | Ataxia | Learning difficulties | Mitochondrial studies | Mutation |
|---|---|---|---|---|---|---|---|---|---|
| Patient 1 | 5y/13y | + (5y) | +++ | ++ | ++ | + | + | Complex I, III, IV defect in muscle mtDNA depletion(10% of normal) | Homorygous p.Pro34Ilefs*25 |
| Patient 2 | 6y/7y | + (6y) | + | + | + | + | - | n.d. | |
| Patient 3 | 6y/22y | + (6y) | +++ | ++ | - | - | + | Complex IV defect in muscle mtDNA depletion(10% of normal) | Homorygous p.Pro34Ilefs*25 |
| Patient 4 | 5y/16y | + (5y) | +++ | ++ | - | + | + | n.d. | Homozygous c.282G>A (splice site alteration) |

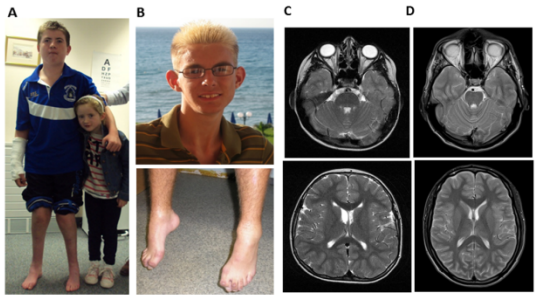



***Fig 1***: Clinical presentation of patients 1, 2 (A) and 3 (B) presented in this study. Brain MRI of patient 1 (C) and 3 (D) detected bilateral, symmetric hyperintensities in the posterior pons and medullary region.

### Methods

- ✓ Whole exome sequencing with Illumina HiSeq 2000, 100 bp paired-end reads.
- ✓ Data filtering according to in-house bioinformatics pipeline.
- ✓ Direct Sanger sequencing of genomic DNA and cDNA.

**Funding** erc MRC Medical Research Council wellcome trust centre for Mitochondrial Research

### Results and discussion

| Patient ID | Variant Type | On Target (a) | Rare/Novel Protein Altering Variants (b) | Rare Compound Heterozygous Protein Altering | Rare Compound Heterozygous Protein Altering & Mitochondrial | Rare Compound Heterozygous Mitochondrial Genes | Rare Homozygous Protein Altering | Rare Homozygous Protein Altering & Mitochondrial | Rare Homozygous Mitochondrial Genes |
|---|---|---|---|---|---|---|---|---|---|
| Patient 1 | SNV | 79,650 | 255 | 13 | 1 | *ENOSF1* | 8 | 1 | *C12orf65* |
| | Indel | 8,901 | | | | | | | |
| Patient 2 | SNV | 81,683 | 244 | | | | | | |
| | Indel | 9,169 | | | | | | | |
| Patient 3 | SNV | 71,970 | 216 | 16 | 0 | - | 8 | 2 | *C12orf65, APEX2* |
| | Indel | 7,089 | | | | | | | |

***Fig 2:*** Mutations detected in patients 1-3 but only the homozygous truncating mutation in *C12orf65* segregated with the disease in each family

The homozygous mutation detected in patient 4 (c.282A>G) affects the last codon of exon 2 and results in the loss of a splice site with retention on intron 2. (Fig 3)

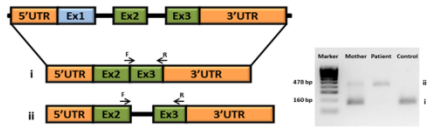
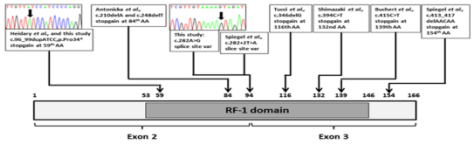



***Fig 3***: Diagram and electrophoresis gel showing the retention of an intron




| | Onset/alive or died† | Optic atrophy | Neuro-pathy | Spasti city | Ophthal mopar | Ataxia | Learning diff. | Mitochondrial studies | Mutation |
|---|---|---|---|---|---|---|---|---|---|
| Antonicka et al. P1 | 1y/8y† | ++ (5y) | ? | ? | ++ | ++ | + | Complex I, IV, V defect in fibroblasts | Homozygous p.Val83Glyfs*1 |
| Antonicka et al., P2 | 15m/20y | + | ++ | - | ++ | ? | ? | n.d. | Homozygous p.Gly72Alafs*12 |
| Antonicka et al., P3 | 3y/22y† | + (3y) | ++ | ? | ++ | - | ? | n.d. | |
| Shimazaki et al., P1 | 7y/32y | + (7y) | + | + | - | - | - | Complex I, IV defect in fibroblasts | Homozygous p.Arg132* |
| Shimazaki et al., P2 | 7y/42y | + (7y) | ++ | ++ | - | - | - | n.d. | |
| Buchert et al., P1 | ?/27y | - | + | ? | + | ? | + | n.d. | Homozygous p.Gln139* |
| Buchert et al., P2 | ?/24y | - | ? | ? | + | ? | + | n.d. | |
| Tucci et al., P1 | 8y/34y | + | + | + | + | ? | + | n.d. | Homozygous p.Val116* mutation |
| Tucci et al., P2 | childhood/ 53y | + | + | + | ? | ? | + | n.d. | |
| Tucci et al., P3 | childhood/ 51y | + | + | + | ? | ? | + | Complex V defect in lymphoblasts | |
| Heidary et al., P1 | childhood/ 8y | + | ? | + | + | + | + | Complex IV defect in muscle | Compound heterozygous p.Pro34Ilefs*25 and p.Gly72Alafs*12 |
| Heidary et al. P2 | childhood/ 5y† | + | ? | + | + | + | + | Complex IV defect in muscle | |
| Spiegel et al., F1 | childhood/ ? | + | + | + | - | - | - | n.d. | Homozygous p.Lys138Argfs*16 |
| Spiegel et al., F2 | childhood/ ? | + | + | + | - | - | + | Complex I and IV deficiency in muscle | Homozygous c.282+2T>A (splice site alteration) |

***Fig 4***: Clinical presentation of previously reported patients

Although C12orf65 deficiency was originally reported as a "classical" mitochondrial disease, detailed reanalysis of the phenotype described in previous reports and in our patients revealed that all may be compatible with Behr's syndrome. Our study strongly suggests that *C12orf65* is an important causative gene that underlies this distinct clinical phenotype.

Abstract for poster presentation at EUROMIT International Meeting on Mitochondrial Pathology (Tampere, Finland, June 2014)

Title:

Studying the effect of L-cysteine in MELAS and MERRF

Authors:

Marina Bartsakoulia[1], Veronika Boczonadi[1], Aurora Gomez-Duran[1], Patrick Yu Wai Man[1], Patrick F.Chinnery[1], Rita Horvath[1]

Affiliations:

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Abstract:

Mitochondrial disorders comprise a large group of heterogeneous disorders characterized by impaired cellular energy production. Mutations located within the mt-tRNA genes are a common cause of mitochondrial disorders.

We have previously reported that the symptoms in reversible infantile respiratory chain deficiency (RIRCD), and reversible infantile hepatopathy, could be explained by low thio-modification of the mt-tRNAGlu by TRMU. Since the availability of cysteine (which is crucial for normal TRMU activity) in the neonatal period is limited by the low activity of the cystathionase enzyme, dietary cysteine intake may be very important at this age. RIRCD myoblasts showed low activities, however adding L-cysteine to the culture medium fully reversed this defect. Furthermore, L-cysteine prevented the decrease of mitochondrial translation in TRMU deficient cells, TRMU down-regulated RIRCD cells and controls, further supporting the hypothesis that low cysteine concentrations may play a role in triggering a reversible in vitro mitochondrial translation defect.

Two other mutations m.3243A>G and m.8344A>G also lead to impaired posttranscriptional modifications, such as thiolation and taurino-methylation, of mitochondrial tRNAs for Leu and Lys and lead to MELAS and MERRF respectively. The pathogenic mutation m.3243A>G in tRNALeu, results in lack of taurine modification at the wobble position of the tRNA, leading to reduced UUG (Leu) translation and complex I deficiency. The pathogenic mutation

of m.8344A>G, results in reduced thiolation and taurine wobble modification- of tRNALys which leads to mitochondrial dysfunction. We hypothesized that L-cysteine might have beneficial effect in these patient cells carrying the mutations.

In this study, we supplemented MERRF and MELAS cells from patients with L-cysteine and consequently we investigated mitochondrial complex assembly by BN-PAGE, oxygen consuption and “in gel” activities. Our data indicated increased levels of mitochondrial complexes after L-cysteine supplementation both in the control and in the patients’ cells.

# Studying the effect of L-cysteine in MELAS and MERRF

***Marina Bartsakoulia*[1], Veronika Boczonadi[1], Aurora Gomez-Duran[1], Patrick Yu Wai Man[1], Patrick F.Chinnery[1], Rita Horvath[1]**

[1]Wellcome Trust Centre for Mitochondrial Research, Institute of Genetic Medicine, Newcastle University, Newcastle upon Tyne, UK

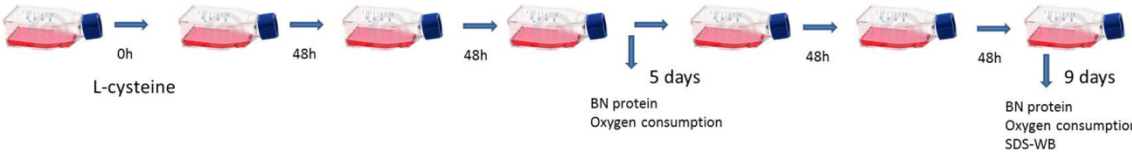
## INTRODUCTION

In mammalian mitochondria mt-tRNAs, uridine at the anticodon wobble position of the $tRNA^{Leu}$(UUR) and $tRNA^{Lys}$ undergoes a post-transcriptional modification which is responsible for precise codon recognition. In particular, the uridine at position 34 (U34) , which is the wobble position of tRNAs Lys, Glu and Gln, is modified at carbons 2 and 5. Carbon 2 is modified exclusively through thiolation ($s^2$), whereas carbon 5 can be taurino-methyl modified($\tau m^5$). The $\tau m^5 s^2$ offers rigidity to the U34 wobble base of tRNAs Lys, Glu and Gln [(1)].

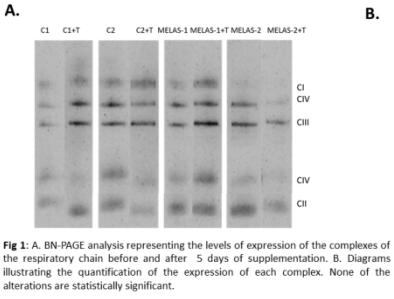
We have previously reported that the symptoms in reversible infantile respiratory chain deficiency (RIRCD) and reversible infantile hepatopathy, could be explained by low thio-modification of the mt-$tRNA^{Glu}$ by TRMU. RIRCD myoblasts showed low activities, however adding L-cysteine to the culture medium fully reversed this defect. Furthermore, L-cysteine prevented the decrease of mitochondrial translation in TRMU deficient cells, TRMU down-regulated RIRCD cells and controls, further supporting the hypothesis that low cysteine concentrations may play a role in triggering a reversible in vitro mitochondrial translation defect [(2)].

## PATIENTS AND METHODS

Fibroblasts from two patients suffering from MELAS (m.3243 A>G) disease with heteroplasmy level 70% (MELAS-1) and 82% (MELAS-2) respectively and from one patient suffering from MERRF (m.8344 A>G) disease were analyzed. The cells were supplemented for different periods of time with 1mM L-cysteine.




### 5 days of supplementation



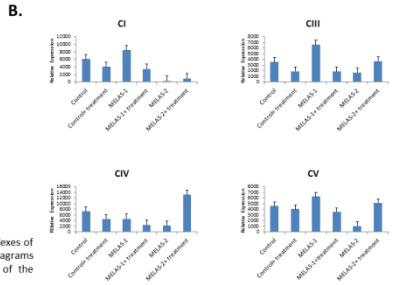
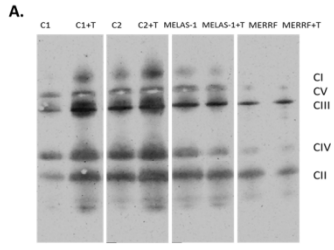




**Fig 1**: A. BN-PAGE analysis representing the levels of expression of the complexes of the respiratory chain before and after 5 days of supplementation. B. Diagrams illustrating the quantification of the expression of each complex. None of the alterations are statistically significant.

### 9 days of supplementation



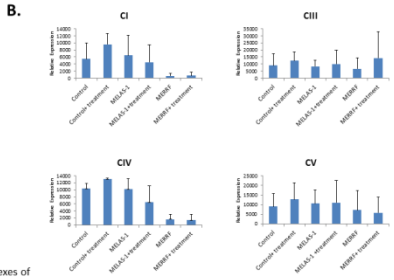
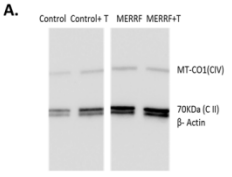




**Fig 2**: A. BN-PAGE analysis representing the levels of expression of the complexes of the respiratory chain before and after 9 days of supplementation. B. Diagrams illustrating the quantification of the of each complex. None of the alterations are statistically significant.



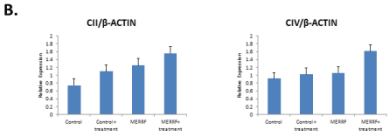




**Fig3** : A. Western blot analysis showing the levels of expression of Complexes II and IV. B. Diagrams illustrating the quantification of the complexes compared to β-actin.

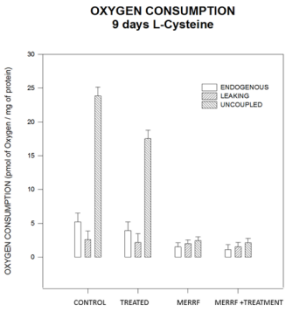



**Fig 4**: Oxygen consumption in fibroblasts before and after 9 days of supplementation. The white bar represents the endogenous respiration and the stripped bars the leaking respiration and the uncoupled respiration respectively.

## DISCUSSION

- The mostly affected complexes of the OXPHOS system in MERRF and MELAS are Complex I and Complex IV.
- In MELAS after 5 days of supplementation we observed increased levels of all Complexes in one of the two cell lines, while 9 days of supplementation resulted in a slight increase of Complex III and V in the second MELAS cell line.
- In MERRF 9 days of supplementation resulted in increased levels of all Complexes on BN-PAGE/ SDS-PAGE.
- A single measurement of oxygen consumption did not detect increased activity of OXPHOS in MERRF.
- Studies on further cell lines are ongoing.

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Abstract for poster presentation at the 8th Annual Neuromuscular Translational Research Conference, (Newcastle Upon Tyne, UK, 2015)

Title:

Studying the effect of L-cysteine in mitochondrial diseases

Authors:

Marina Bartsakoulia[1], Juliane S. Müller[1], Veronika Boczonadi[1], Aurora Gomez-Duran[1], Patrick Yu Wai Man[1], Patrick F.Chinnery[1], Rita Horvath[1]

Affiliations:

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**Background:** Mitochondrial disorders comprise a large group of heterogeneous disorders characterized by impaired cellular energy production. Mutations located within the mt-tRNA genes are a common cause of mitochondrial disorders.

We have previously reported that L-cysteine prevented the decrease of mitochondrial translation in cells of patients with reversible infantile respiratory chain deficiency and TRMU deficiency, supporting the hypothesis that low cysteine concentrations may play a role in triggering a reversible in vitro mitochondrial translation defect.

**Aim and Methods:** Based on these observations we expanded the supplementation with L-cysteine to other mitochondrial conditions affecting posttranscriptional modifications of mt-tRNAs on the molecular level. Absence of post-transcriptional modifications at the wobble positions of mitochondrial tRNAs for Leu and Lys has been correlated to Mitochondrial Encephalomyopathy and Lactic Acidosis with Stroke-like episodes (MELAS) and Myoclonic Epilepsy with Red Ragged Fibres (MERRF) syndromes, respectively. We tested whether supplementation of growth media with L-cysteine can reverse the defect in cells from patients with MELAS and MERRF, as well as in patients with other types of mitochondrial translation defects (COX10, MTO1 and ELAC2 mutations). Mitochondrial complex assembly was assessed by BN-PAGE, in-gel activity assay, and oxygen consumption (Seahorse analysis).

**Results and conclusions:** Our data indicate increased levels of mitochondrial complexes after L-cysteine supplementation in some, but not all of the studied patient cell lines. We are testing

whether our approach of supplementation with L-cysteine could be used in the future to treat some mitochondrial conditions.

# STUDYING THE EFFECT OF L-CYSTEINE IN MITOCHONDRIAL DISEASES

**Marina Bartsakoulia[1], Juliane S. Müller[1], Veronika Boczonadi[1], Aurora Gomez-Duran[1], Patrick Yu Wai Man[1], Patrick F.Chinnery[1], Rita Horvath[1]**

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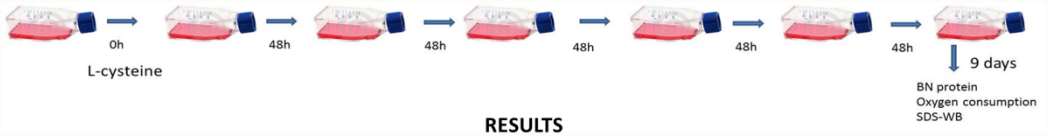
## BACKGROUND

Mitochondrial disorders comprise a large group of heterogeneous disorders characterized by impaired cellular energy production. Approximately 1 in 5000 people is affected and currently there is no effective treatment available. Mutations located in within the mt-tRNA genes are a common cause of mitochondrial disorders. We have previously reported that low cysteine concentrations may play a role in triggering a reversible mitochondrial translation defect in patients with reversible infantile respiratory chain deficiency (RIRCD), which is caused from a mutation in the mitochondrial $tRNA^{Glu}$. RIRCD myoblasts showed low activities, however adding L-cysteine to the culture medium fully reversed this defect. Our hypothesis is that increasing cysteine availability might reverse a mitochondrial translation defect either in mitochondrial diseases affecting posttranslational mt-tRNA modifications or in other mitochondrial translation disorders.

## PATIENTS AND METHODS

Based on these observations we supplemented with 4mM of L-cysteine or N-acetylcysteine the following primary fibroblast cell lines: Two (2) patient cell lines with Mitochondrial Encephalomyopathy and Lactic Acidosis with Stroke-like episodes (MELAS), two (2) patient cell lines with Myoclonic Epilepsy with Red Ragged Fibres (MERRF), three (3) patient cell lines with nuclear defects (MTO1, ELAC2, COX10) and three (3) control cell lines. We tested whether the supplementation of growth media with either L-cysteine or N-acetylcysteine can reverse the mitochondrial defect in patients' cell lines. Mitochondrial complex assembly was assessed by BN-PAGE and oxygen consumption.




## RESULTS

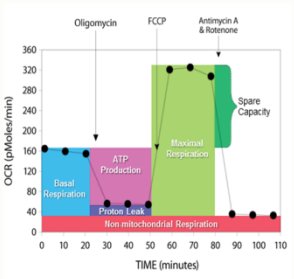



**Fig. 1**: Outline of the oxygen consumption measurement to assess mitochondrial respiratory function by using the Seahorse Bioscience XF°96 Extracellular Flux Analyser

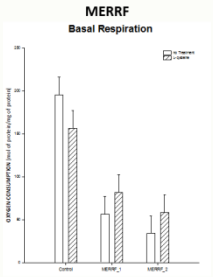



**Fig 2:** Oxygen consumption in fibroblasts before and after 9 days of supplementation with L-cysteine. The white and striped bars represent the cells with and without the supplementation respectively.

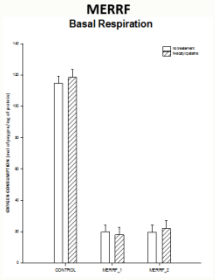



**Fig 3:** Oxygen consumption in fibroblasts with before and after 9 days of supplementation with N-acetylcysteine. The white and striped bars represent the cells with and without the supplementation respectively.

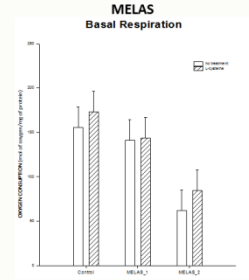



**Fig 4:** Oxygen consumption in fibroblasts with before and after 9 days of supplementation. The white bar represents the cells before the supplementation and the striped the supplemented ones.

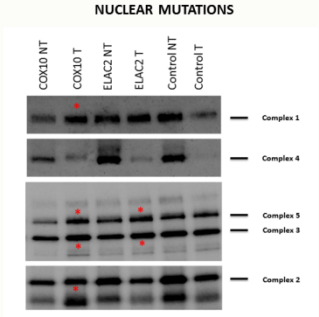



**Fig. 5**: Blue native PAGE of mitochondrial respiratory chain complexes before and after 14 days of treatment with L-cysteine. Increase in protein levels for certain complexes after treatment are highlighted with red stars. NT = not treated; T= treated with L-cysteine

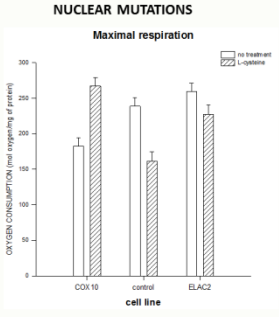



**Fig 6**: Oxygen consumption in fibroblasts with before and after 14 days of supplementation with L-cysteine. The white bars represent the cells before treatment with L-cysteine and the striped bars the cell lines after treatment, respectively.

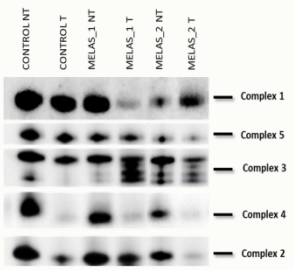



**Fig. 7**: Representative Blue native PAGE of mitochondrial respiratory chain complexes before and after 9days of treatment with L-cysteine in control and MELAS patients' cells.

## CONCLUSIONS

- Treatment with L-cysteine shows a slight improvement of mitochondrial respiration in some of the tested cell lines; the difference, however, is not statistically significant
- The slight increase of oxygen consumption is not necessarily mirrored by an increase in respiratory chain components protein level
- In addition, we observed that L-cysteine slows down the growth rate of the cells
- As consequence of these observations, we have just started testing N-acetylcysteine as alternative sulphur donor
- We hope that our experiments will elucidate whether L-cysteine or N-acetylcysteine may be used as treatment for some types of mitochondrial translation defects

Abstract for poster presentation at Mitochondrial Medicine: Developing new treatment for Mitochondrial Disease (Hinxton, Cambridge, UK, May 2016)

Title:

MIEF2 mutations impair mitochondrial fission in skeletal muscle

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Abstract:

Mitochondria undergo constant fission and fusion to maintain their function and biogenesis. Impairment of mitochondrial dynamics can result in altered mitochondrial DNA maintenance, reduced oxidative phosphorylation and has been correlated to a variety of mitochondrial disorders. DRP1 (DNM1L) has an important role in the division of mitochondria and peroxisomes. It is located in the cytoplasm, but in order to promote fission, DRP1 is recruited to the mitochondrial outer membrane. An autosomal dominant DRP1 mutation has been previously reported in a child with lethal infantile encephalopathy and defective mitochondrial peroxisomal fission. Recently, two new proteins MIEF1 and MIEF2 were discovered in the mitochondrial fission machinery and their role is to directly recruit DRP1 to

the mitochondrial surface. Both proteins are anchored in the mitochondrial outer membrane. Knock-down of *MIEF1/MIEF2* results in asymmetrical distribution of the network and in fused mitochondria.

Whole exome sequencing identified a homozygous nonsense mutation (p.Q81*) in MIEF2 in a 15 year old boy from a consanguineous Jewish family. He developed progressive muscle weakness and exercise intolerance. His muscle biopsy revealed mitochondrial myopathy with numerous ragged red and COX negative fibers. Biochemical measurement of the respiratory chain enzymes in skeletal muscle detected combined deficiency of complex I and IV. Protein levels of some mtDNA encoded proteins were decreased. MtDNA copy number was normal and no deletions of the mtDNA were detected in muscle DNA. Immunoblotting revealed increased levels of proteins promoting mitochondrial fusion (MFN2) and decreased levels of the fission protein DRP1. Fibroblasts carrying the homozygous nonsense mutation in MIEF2 had elongated mitochondria, and showed significantly higher frequency of fusion events than controls after transfection with mtDsRed and mtPA-GFP codifying plasmids.

Our data suggest that mutations in MIEF2 cause increased fusion events, which result in imbalanced mitochondrial dynamics and lead to mitochondrial myopathy.

# *MIEF2 (Mid49)* mutations impair mitochondrial fission in skeletal muscle

**Marina Bartsakoulia[1],** Janbernd Kirschner[2], Angela Pyle[1], Sarah Grünert[3], Jennifer Duff[1], Helen Griffin[1], Patrick Chinnery[1,4], Veronica Eisner[5], Rita Horvath[1]

[1]Institute of Genetic Medicine, Newcastle University, Newcastle upon Tyne, UK; [2] Department of Neuropediatrics and Muscle Disorders and [3]Department of General Pediatrics, Adolescent Medicine and Neonatology, University Medical Center, Freiburg, Germany; [4]Department of Clinical Neurosciences, University of Cambridge, UK; [5]Department of Cellular and Molecular Biology, School of Biological Sciences, Pontificia Universidad Católica de Chile, Santiago, Chile

## BACKGROUND

Mitochondria undergo constant fission and fusion to maintain their function and biogenesis. Impairment of mitochondrial dynamics can result in altered mitochondrial DNA maintenance, reduced oxidative phosphorylation and has been correlated to a variety of mitochondrial disorders[1]. Drp1 has an important role in the division of mitochondrial and peroxisomes. It is located in the cytoplasm, but in order to promote fission, Drp1 is recruited to the mitochondrial outer membrane[2]. Recently, two new proteins MIEF1 and MIEF2, located in the mitochondrial outer membrane, were discovered to directly recruit Drp1 to the mitochondrial surface[3].

## METHODS

Whole exome sequencing identified a homozygous nonsense mutation (p.Q81*) in *MIEF2* in a 15 year old boy from a consanguineous Jewish family. At the age of 7 , he was diagnosed with mild proximal muscle weakness and increased levels of CK (1200U/l). Muscle biopsy revealed mitochondrial disorder. MtDNA copy number was increased compared to the control and no mtDNA deletions were detected. The clinical investigation revealed pure muscular phenotype with no other organ involvement. Measurement of oxygen consumption by seahorse in fibroblasts did not show any defect. However, immunoblotting illustrated significantly decreased expression levels of OXPHOS complexes and Drp1 both in the muscle and fibroblasts accompanied with increased protein expression levels of Mfn2 and OPA1. Furthermore, evaluation of fusion events by photoactivation indicated increased fusion events in the fibroblasts carrying the mutation compared to control fibroblasts.

## RESULTS

### ❖ FIBROBLASTS

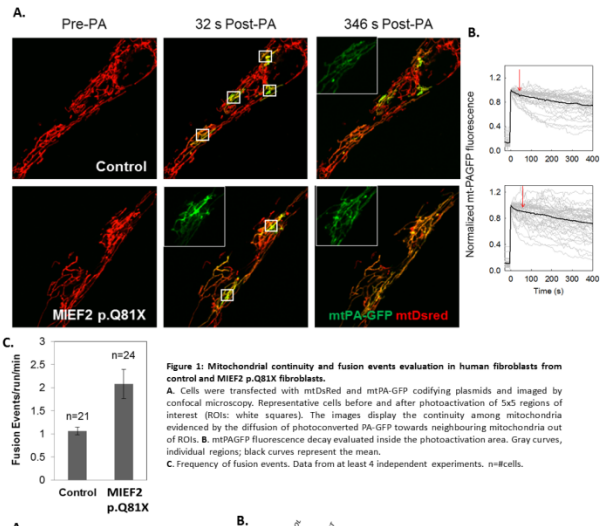


**Figure 1: Mitochondrial continuity and fusion events evaluation in human fibroblasts from control and MIEF2 p.Q81X fibroblasts.**
**A.** Cells were transfected with mtDsRed and mtPA-GFP codifying plasmids and imaged by confocal microscopy. Representative cells before and after photoactivation of 5x5 regions of interest (ROIs: white squares). The images display the continuity among mitochondria evidenced by the diffusion of photoconverted PA-GFP towards neighbouring mitochondria out of ROIs. **B.** mtPAGFP fluorescence decay evaluated inside the photoactivation area. Gray curves, individual regions; black curves represent the mean.
**C.** Frequency of fusion events. Data from at least 4 independent experiments. n=#cells.

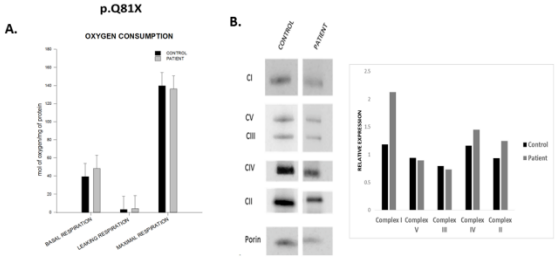


**Figure 2: Oxygen consumption and BN-PAGE in control and MIEF2 p.Q81X primary fibroblasts.**
**A.** Oxygen consumption in fibroblasts. Black and grey bars represent the mean values of control and patient respectively. Corrected oxygen consumption by the non mitochondrial respiration and mg of protein is represented as basal respiration, leaking respiration and maximal capacity respiration. **B.** Quantification of Blue native PAGE in fibroblasts detected increased levels of Complex I, IV and V.

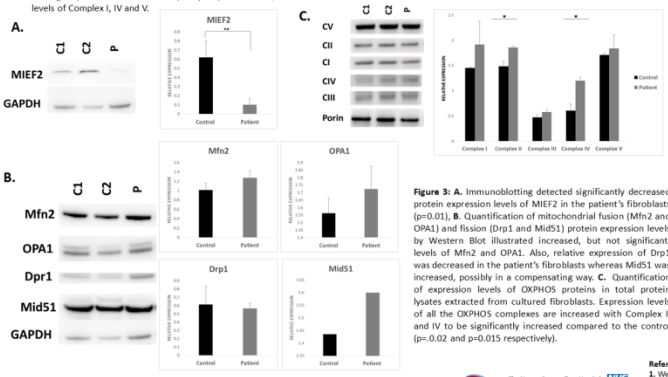


**Figure 3: A.** Immunoblotting detected significantly decreased protein expression levels of MIEF2 in the patient's fibroblasts (p=0.01), **B.** Quantification of mitochondrial fusion (Mfn2 and OPA1) and fission (Drp1 and Mid51) protein expression levels by Western Blot illustrated increased, but not significant, levels of Mfn2 and OPA1. Also, relative expression of Drp1 was decreased in the patient's fibroblasts whereas Mid51 was increased, possibly in a compensating way. **C.** Quantification of expression levels of OXPHOS proteins in total protein lysates extracted from cultured fibroblasts. Expression levels of all the OXPHOS complexes are increased with Complex II and IV to be significantly increased compared to the control (p=.0.02 and p=0.015 respectively).

### ❖ MUSCLE BIOPSY

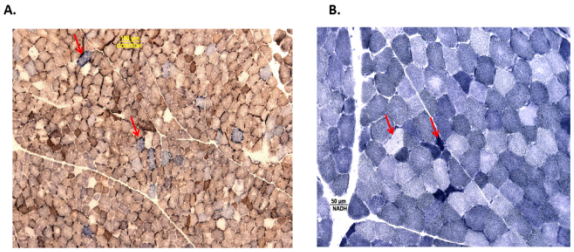


**Figure 4: A.** Simultaneous staining of muscle fibers for cytochrome oxidase (COX) and succinic dehydrogenase (SDH). Light blue muscle fibers arrows illustrate COX-deficient fibers. **B.** Nicotinamide adenine dinucleotide (NADH) staining of muscle fibers. Dark blue muscle fibers represent increased mitochondrial proliferation and pale muscle fibers illustrate deficient muscle fibers.

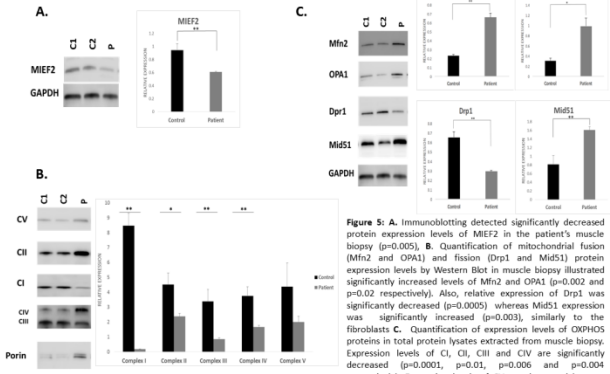


**Figure 5: A.** Immunoblotting detected significantly decreased protein expression levels of MIEF2 in the patient's muscle biopsy (p=0.005), **B.** Quantification of mitochondrial fusion (Mfn2 and OPA1) and fission (Drp1 and Mid51) protein expression levels by Western Blot in muscle biopsy illustrated significantly increased levels of Mfn2 and OPA1 (p=0.002 and p=0.02 respectively). Also, relative expression of Drp1 was significantly decreased (p=0.0005) whereas Mid51 expression was significantly increased (p=0.003), similarly to the fibroblasts **C.** Quantification of expression levels of OXPHOS proteins in total protein lysates extracted from muscle biopsy. Expression levels of CI, CII, CIII and CIV are significantly decreased (p=0.0001, p=0.01, p=0.006 and p=0.004 respectively). Expression levels of CV are decreased but not significantly.

## CONCLUSIONS

- ❑ Fibroblasts carrying the homozygous mutation p.Q81* in *MIEF2* carried elongated mitochondria with increased mtDNA copy number (data not shown) and presented significantly increased fusion events compared to the control fibroblasts indicating that MIEF2 plays a vital role in mitochondrial dynamics.
- ❑ Increased levels of oxygen consumption in the patient's fibroblasts accompanied with increased protein expression levels of the OXPHOS complexes suggest a compensatory mechanism in the fibroblasts.
- ❑ Significantly increased levels of proteins involved in mitochondrial fusion (Mfn2 and OPA1) and significantly decreased levels of Drp1 expression levels in the muscle biopsy, suggest that reduced levels of *MIEF2* result in imbalanced mitochondrial dynamics .
- ❑ In conclusion, mutations in *MIEF2* affect the mitochondrial fission in the skeletal muscle and lead to mitochondrial myopathy.

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**3.** Palmer, C. S., Osellame, L. D., Laine, D., Koutsopoulos, O. S., Frazier, A. E., and Ryan, M. T. (2011) MID49 and MID51, new components of the mitochondrial fission machinery. EMBO Rep. 12, 565–573

Abstract for poster presentation in UMDF Mitochondrial Medicine (Seattle, USA, June 2016)

Title:

Cysteine supplementation improves mitochondrial respiratory chain function in a subgroup of mitochondrial translation deficiencies

Authors:

Marina Bartsakoulia[1], Juliane S Müller[1], Aurora Gomez-Duran[1,2], Patrick Yu Wai Man[1,3,4], Veronika Boczonadi[1]and Rita Horvath[1]

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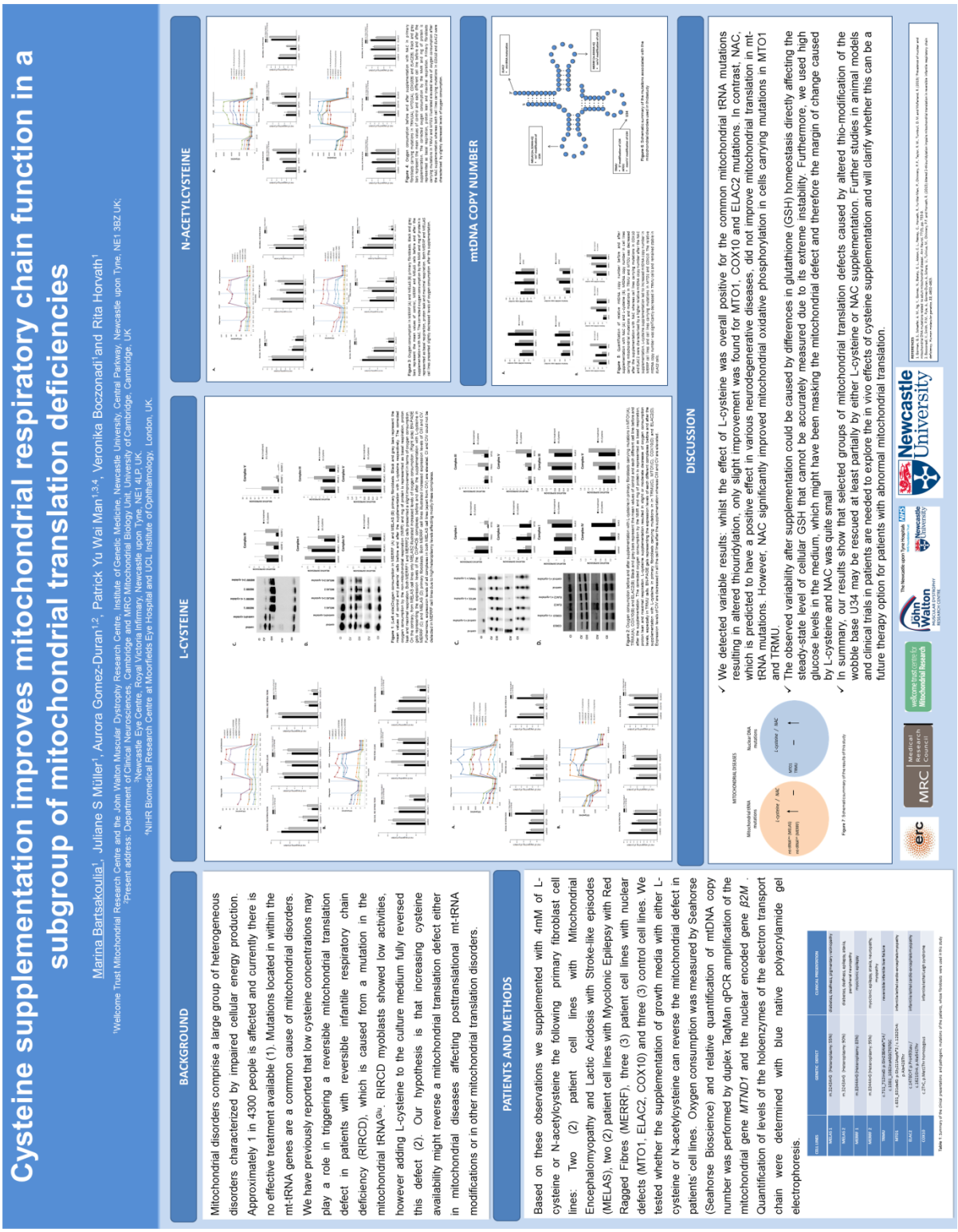
[4]NIHR Biomedical Research Centre at Moorfields Eye Hospital and UCL Institute of Ophthalmology, London, UK.

Abstract:

Mitochondrial encephalomyopathies are severe, relentlessly progressive conditions and there are very few effective therapies available to date. We have previously suggested that in two rare forms of reversible mitochondrial disease (reversible infantile respiratory chain deficiency and reversible infantile hepatopathy) supplementation with L-cysteine can improve mitochondrial protein synthesis. This beneficial effect is most likely due to improved tRNA modification, since cysteine is required for the 2-thiouridylation of mitochondrial tRNAs. Although reversible mitochondrial diseases are rare, an altered 2-thiouridylation has been shown to contribute to common mitochondrial tRNA mutations, such as m.3243A>G in mt-tRNALeu (MELAS) and m.8344A>G in mt-tRNALys (MERRF). Here we show that supplementation with L-cysteine, but not with N-acetyl-cysteine partially rescues the mitochondrial translation defect in vitro in fibroblasts of patients with MELAS

and MERRF. In contrast, N-acetyl-cysteine had a beneficial effect on mitochondrial translation in TRMU and MTO1 deficient fibroblasts. In summary, our results suggest that Lcysteine or N-acetyl-cysteine supplementation may be a potential treatment for selected subgroups of patients with mitochondrial translation deficiencies. Further studies are needed to explore the full potential of cysteine supplementation as a treatment for patients with mitochondrial disease.



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