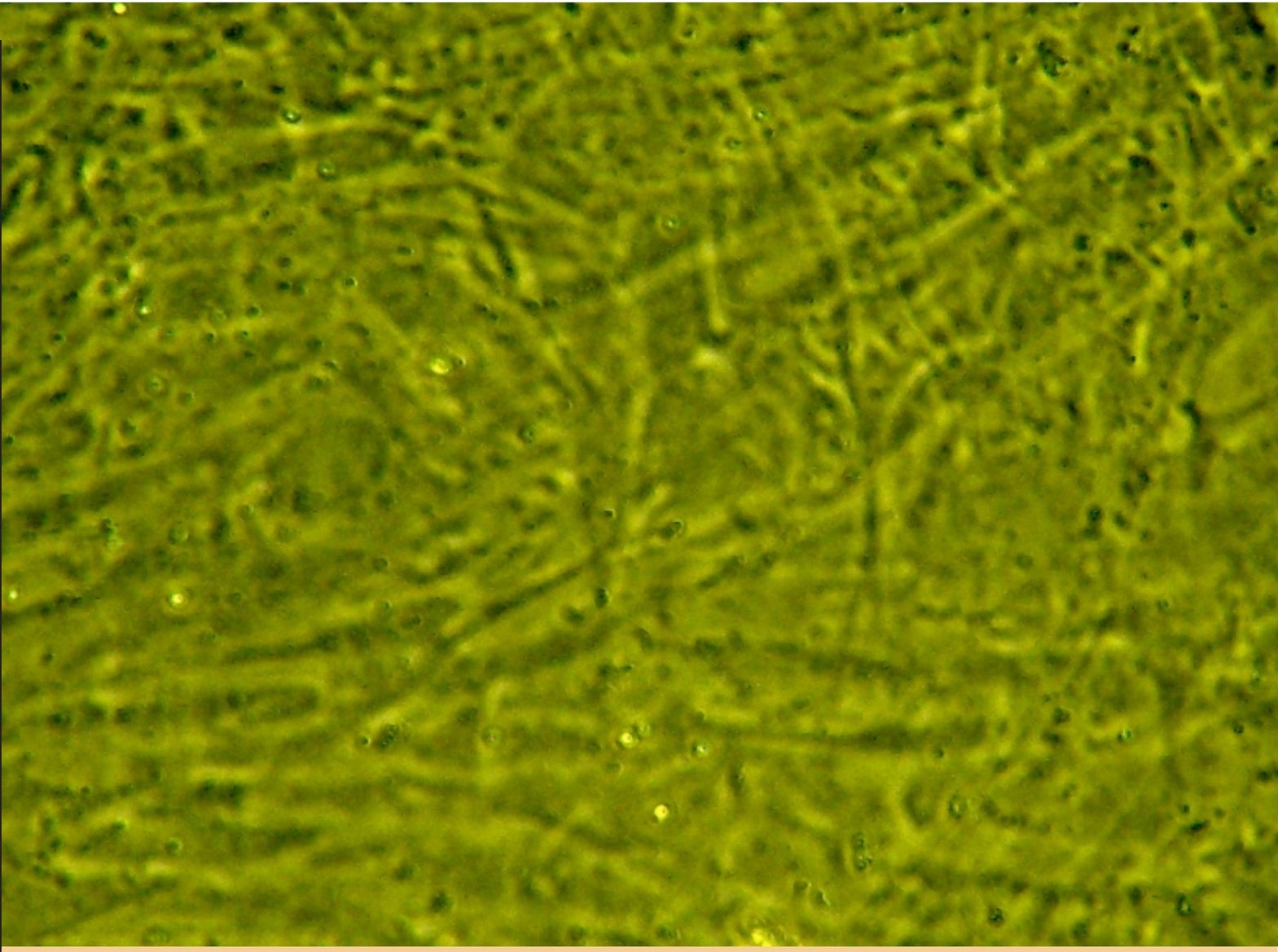

Maria Inês Arêlo Manso da Fonseca

# Functional Investigation of OXPHOS assembly factors in Leber's Hereditary Optic Neuropathy

Tese de Mestrado em Bioquímica, orientada pela Professora Doutora Manuela Grazina e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Julho 2016

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Universidade de Coimbra

**On the front of the page:**

Human fibroblasts in culture. Capture on an optical microscope (magnification 100x).

FACULDADE DE CIÊNCIAS
E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

# Functional investigation of OXPHOS assembly factors in Leber's Hereditary Optic Neuropathy

**Maria Inês Arêlo Manso da Fonseca**

Dissertation presented to the Faculty of Sciences and Technology of the University of Coimbra in order to obtain the MSc degree in Biochemistry. The work was performed at the Laboratory of Biochemical Genetics, Center for Neuroscience and Cell Biology, University of Coimbra and Faculty of Medicine, University of Coimbra, under the scientific supervision of Professor Manuela Grazina (Assistant Professor - Senior lecturer, Faculty of Medicine of the University of Coimbra; Researcher and Lab Director, Center for Neuroscience and Cell Biology, University of Coimbra).

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## ABSTRACT

Mitochondrial cytopathies comprise a heterogeneous group of multisystem disorders related to mitochondrial dysfunction and deficiency in ATP production. The tissues more susceptible to this impairment are those that require more energy for their maintenance and function, such as the nervous system having a higher number of mitochondria per cell. The energy dysfunction may occur in one or more complexes of the mitochondrial respiratory chain (MRC). Since MRC subunits are encoded by both nuclear genome (nDNA) and mitochondrial DNA (mtDNA), the pathology may be caused by diverse types of mutations in one or both genomes. Accordingly, mutations in mtDNA, although responsible for a significant number of pathologies, may exhibit incomplete "penetrance", suggesting the involvement of other factors such as mtDNA haplogroups, nDNA single nucleotide polymorphisms (SNPs), or alcohol, tobacco and other environmental factors.

Leber's hereditary optic neuropathy (LHON) is one of the most common mitochondrial cytopathies. It is mainly characterized by degeneration of retinal ganglion cells, causing blindness in young males. It is estimated that ~70% of LHON cases are due to the point mutation m.11778G>A that is responsible for encoding the ND4 Complex I (CI) subunit. However, mtDNA mutations do not always explain the incomplete "penetrance" verified.

Accordingly, it may be predicted that mutations in nDNA may play a synergistic role with mtDNA alterations for causing a severe biochemical defect. Moreover, modifications in nDNA coding for mitochondrial factors of major importance to the assembly, stability and maintenance of the MRC, may lead to an imbalance in oxidative phosphorylation (OXPHOS)-dependent energy production, compromising mitochondrial function. The assembly factors, despite not being part of MRC structure, play a crucial role in the correct complexes assembly for an adequate energy production.

Therefore, the present study aims to clarify the role of MRC assembly in LHON, pathology, related to the presence and absence of the m.11778G>A mutation. A genetic, structural, and functional set of analyses were performed.

The results show that, in patients harbouring the referred primary LHON mutation, the CI activity is highly reduced. The analysis of the MRC complexes' assembly status allowed to observe that patients 1 and 4 present impaired assembly of CI, and patient 4 also shows decreased assembled CV.

The genetic analysis of the assembly factors revealed a promising sequence variation only in patient 1, under characterization.

In conclusion, the present results are a promising and the impairment of the MRC assembly may be an important issue to consider in LHON, although there are other factors to be taken into account in the pathological mechanism underlying the disease.

**Key words:** Assembly factors; MRC complexes; Leber's Hereditary Optic Neuropathy; mtDNA; nDNA.

## RESUMO

As citopatias mitocondriais incluem um grupo heterogéneo de doenças multissistémicas associadas à disfunção mitocondrial, o que leva a uma insuficiência na produção de ATP. Os tecidos que requerem maiores níveis de energia para a sua manutenção e funcionamento, como o sistema nervoso, apresentam um maior número de mitocôndrias por célula, sendo os mais afetados. Os défices energéticos podem afetar um ou mais complexos da cadeira respiratória mitocondrial (MRC). Dado que as subunidades dos complexos da MRC são codificadas, tanto por DNA nuclear (nDNA), como por DNA mitocondrial (mtDNA), estas doenças podem ter origem em mutações localizadas em um ou nos dois genomas. Consequentemente, mutações no mtDNA embora responsáveis por um número significativo de patologias, podem exibir "penetrância incompleta", sugerindo que existem outros fatores envolvidos, tais como haplogrupos mitocondriais, polimorfismos (SNPs), consumo de álcool, tabaco e fatores ambientais.

A neuropatia ótica hereditária de Leber (LHON) é uma das citopatias mitocondriais mais frequentes. É caraterizada principalmente pela degenerescência das células ganglionares da retina (RGC), causando cegueira, principalmente em adultos jovens do sexo masculino. Estima-se que cerca de 70% dos casos de LHON sejam devidos à mutação m.11778G>A, que codifica a subunidade ND4 do complexo I (CI). Contudo, as mutações no mtDNA não explicam sempre a existência de "penetrância incompleta".

Assim, prevê-se que alterações no nDNA, em sinergia com mutações no mtDNA possam gerar um fenótipo bioquímico grave. Para além disso, modificações no nDNA, em fatores mitocondriais, podem levar à função alterada de proteínas importantes para a montagem, estabilidade e manutenção da MRC, comprometendo a função mitocondrial normal e a produção de energia na fosforilação oxidativa (OXPHOS).

Os fatores de *assembly*, embora não façam parte da estrutura final da MRC, fazem parte do grupo de fatores codificados pelo nDNA e têm um papel fundamental para que a montagem dos complexos seja eficaz, de modo a assegurar uma produção de energia adequada.

Assim, o presente estudo tem como objetivo clarificar o papel do *assembly* da MRC na patologia da LHON relacionada com a presença ou ausência da mutação m.11778G>A. Foi realizado um estudo genético, estrutural e funcional.

Os resultados mostram que, em doentes com a mutação primária, a atividade do CI é mais baixa. A análise do estado de *assembly* dos complexos da MRC permitiu

observar que os doentes 1 e 4 têm alteração do *assembly* do CI, e que o doente 4 apresenta também decréscimo no *assembly* do CV.

A análise genética do doente 1 revelou uma alteração muito promissora num dos fatores de *assembly*, que se encontra em caraterização.

Em conclusão, os resultados do presente trabalho são promissores e a falha no *assembly* da MRC parece ser um importante parâmetro a considerar na LHON, embora haja outros fatores a ter em conta nos mecanismos patogénicos subjacentes à manifestação da doença.

**Palavras-chave:** Fatores de *assembly*; Complexos da MRC; Neuropatia ótica hereditária de Leber; mtDNA; nDNA.

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### Chapter 1 – INTRODUCTION

# ABBREVIATIONS

**A**

| | |
|---|---|
| AMP | Adenosine monophosphate |
| ATP | Adenosine diphosphate |

**B**

| | |
|---|---|
| BN-PAGE | Blue native polyacrylamide gel electrophoresis |
| bp | Base pair |

**C**

| | |
|---|---|
| CI | Complex I |
| CII | Complex II |
| CIII | Complex IIII |
| CIV | Complex IV |
| CV | Complex V |
| CS | Citrate synthase |
| CoQ | Coenzyme Q |
| $CoQH_2$ | Ubiquinol |
| Cyt b | Cytochrome b |
| Cyt c | Cytochrome c |

**D**

| | |
|---|---|
| DDM | β-dodecyl maltoside |
| DNA | Deoxyribonucleic acid |

**E**

| | |
|---|---|
| ECL | Enhanced chemiluminescence |
| EDTA | Ethylenediaminetetraacetic acid |
| EM | Electron microscopy |
| ETC | Electron transport chain |

**F**

| | |
|---|---|
| $FADH_2$ | Flavine adenine dinucleotide |
| FBS | Fetal bovine serum |
| FILA | Fatal infantile lactic acidosis |

**G**

| | |
|---|---|
| G | Guanine |

**H**

| | |
|---|---|
| H | ‘Heavy’ strand |
| HBSS | Hank’s balanced salt solution |

**I**

| | |
|---|---|
| IVA | Ingenuity® Variant Analysis |

**K**

| | |
|---|---|
| KDa | Kilodalton |

**L**

| | |
|---|---|
| L | “Light” strand |
| LBG | Laboratório de Bioquímica Genética |
| LHON | Leber's hereditary optic neuropathy |
| LS | Leigh syndrome |

**M**

| | |
|---|---|
| MIM | Mitochondrial inner membrane |
| MOM | Mitochondrial outer membrane |
| Mo | Months old |
| MRC | Mitochondrial respiratory chain |
| MtDNA | Mitochondrial DNA |

**N**

| | |
|---|---|
| $NAD^+$ dinucleotide | Nicotinamide adenine |
| NADH | Reduced nicotinamide adenine dinucleotide hydrate |
| ND | NADH dehydrogenase subunit |
| nDNA | Nuclear DNA |
| NDUFA | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex |
| NDUFAF | NADH dehydrogenase (ubiquinone) assembly protein |
| NDUFS | NADH dehydrogenase (ubiquinone) Fe-S protein |
| NDUFV | NADH dehydrogenase (ubiquinone) flavoprotein |
| NGS | Next generation sequencing |

**O**

| | |
|---|---|
| OXPHOS | Oxidative phosphorylation |

**P**

| | |
|---|---|
| PBS | Phosphate buffered saline |
| PEO | Progressive external ophthalmoplegia |
| PDH | Pyruvate dehydrogenase |
| PVDF | Polyvinylidene fluoride |

**R**

| | |
|---|---|
| RFLP | Restriction fragment length polymorphism |
| RISP | Rieske iron–sulfur protein |
| RGC | Retinal ganglion cells |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |

**S**

| | |
|---|---|
| SCs | Supercomplexes |
| SDH | Succinate dehydrogenase |
| SEM | Standard error deviation |

**T**

| | |
|---|---|
| TCA | Tricarboxylic acids cycle |

**V**

| | |
|---|---|
| VDAC channel | Voltage-dependent anion |

**W**

| | |
|---|---|
| WB | Western blot |

**Y**

| | |
|---|---|
| Yo | Years old |

# CHAPTER 1

## GENERAL INTRODUCTION

# 1. INTRODUCTION

## 1.1. Mitochondria

### 1.1.1. Mitochondria Origin

Cells and their organelles are interconnected and dependent on each other to execute multiple reactions that ultimately result in processes that are essential to the mechanism underlying cell viability. One of those organelles is the mitochondrion, which is involved in a complex set of linked reactions, ubiquitous to most eukaryotic cells (Solomon *et al.,*2008; Alberts *et al.,*2013).

This idea of “cell inside a cell” is a clue on the origin of mitochondria, since the endosymbiotic theory is pointed as the most probable hypothesis. This theory suggests that α-proteobacteria were engulfed by larger cells and established a symbiotic relationship inside a primitive eukaryotic cell, about 2 billion years ago (Sagan, 1967; Yang *et al.,*1985). One of the main points of the hypothesis is based on the similarities of the mitochondria with bacterial ancestry, such as the circular genome and the ability to produce ATP (Gabaldón & Huynen, 2004; Friedman & Nunnari, 2014).

### 1.1.2. Structure and Function

Each eukaryotic cell has a variable number of mitochondria which exhibit a spherical or rod-shape. Electron microscopy studies performed by George Palade and Fritjof Sjöstrand (Palade, 1953; Sjostrand, 1953) revealed that mitochondria contain a double-membrane, constituted by the mitochondrial outer membrane (MOM) and a mitochondrial inner membrane (MIM). This membrane is creased into a series of internal folds named cristae, increasing the total surface area (Houshmand, 2003; Campbell *et al.,*2008).

The MOM is permeable to small molecules and ions, due to the presence of many voltage-dependent anion channels (VDAC). In contrast, the MIM is almost impermeable to ions and polar molecules, due to its lipid bilayer composed by high proportion of "double" phospholipid cardiolipin, which has four fatty acids rather than the usual two. There are also transport shuttles in the MIM that are responsible for the flux of ATP, pyruvate, and citrate (Solomon *et al.,*2008), among others.

These two membranes define two internal compartments: the intermembrane space, between the outer and the inner membranes; and the matrix, bounded by the

MIM, containing mitochondrial DNA (mtDNA), ribosomes, and many enzymes that catalyse some of the oxidative reactions (Krebs cycle, fatty acid β-oxidation, complex pyruvate dehydrogenase (PDH) and amino acids oxidation, but not glycolysis that takes place in cytosol) (Grazina, 2004). Some other functions mediated by mitochondria are the regulation of some aspects in calcium buffering, intracellular signalling, apoptosis, reactive oxygen species (ROS) production and detoxification, metabolism of lipids, cholesterol, steroids, and nucleotides (Chinnery, 2003; Lloyd & McGeehan, 2013; Shokolenko & Alexeyev, 2015; Quirós *et al.,*2016).

The majority of mitochondrial reactions occur in the matrix. Therefore, the mitochondrial respiratory chain (MRC) is strategically inserted in the MIM. The MRC harbours the oxidative phosphorylation (OXPHOS), which is the metabolic pathway, related to the main role of mitochondria, leading to ATP production. The MRC advantaged location potencies ATP production since the vast surface area created by the cristae provides space for thousands of MRC sets (Chinnery, 2003; Grazina, 2004; Solomon *et al.,*2008; Alberts *et al.,*2013). On average, there are 10 to 100.000 mitochondria per cell. However, in cells that are metabolically more active and therefore have high energy requirements, the amount of mitochondria is higher (Grazina, 2004; Schapira, 2006; Solomon *et al.,*2008). This organelle have a crucial role in the maintenance of cellular homeostasis; so, any defect in their function could have devastating consequences in the human body functioning (Schapira, 2006).

### 1.1.3. Human mitochondrial genome – mtDNA

Nass and Nass demonstrated the presence of DNA in mitochondria by electron microscopy, in 1963 (Nass & Nass, 1963). Further studies were performed in order to better understand the mtDNA organization and machinery, vital for understanding the etiology and pathogenicity of mitochondrial disorders. The total sequencing of human mtDNA was performed for the first time in 1981 by Anderson *et al.,* (Anderson *et al.,*1981) and reviewed in 1999 by Andrews (Andrews *et al.,*1999) [information cited from (Grazina, 2004)].

Each mitochondrion contains about two to ten copies of mtDNA, designed as copy number (Houshmand, 2003; Grazina, 2004). The mtDNA is structurally organized as a circular, double-stranded molecule, with a remarkably economic sequence organization, comprising 37 genes into 16.6 kilobase pairs (Kbp) of nucleotides. From the 37 genes, 22 code for transfer RNAs (tRNA) and two for ribosomal RNAs (rRNA) (12S and 16S), for mitochondrial protein synthesis. The mitochondrial genome, also

encodes 13 polypeptides, all of which are subunits of the MRC complexes: seven of the 45 complex I (CI) subunits (ND1-3, ND4L, ND4-6); one of the 11 complex III (CIII) subunits (cytochrome b, cyt b); three of the 13 complex IV (CIV) subunits (COI-III); and two of the 15 complex V (CV) subunits (ATP6 and ATP8). Complex II (CII) is exclusively encoded by nuclear DNA (nDNA) (Shoubridge, 2001; García-Rodríguez, 2007; Pagliarini *et al.,*2008; Wallace, *et al.,*2014).

The two strands of human mtDNA present a heterogeneous nucleotide composition, being the 'heavy' strand (H) rich in guanine (G) and the 'light' strand (L) poor in G. The encoding material of 12 of the 13 protein-coding genes, both rRNAs, and14 of the 22 tRNAs are located in the H-strand. Whereas the mtDNA L-strand encodes a single protein (ND6) and eight tRNAs (Wallace & Fan, 2009; Pearce *et al.,*2013). The mtDNA undergoes semiautonomous regulation, since mtDNA replication and transcription depends on nDNA factors (i.e. DNA polymerase gamma (Polγ) – catalyses the human mtDNA replication) (Grazina, 2004).

The mtDNA replication is bi-directional and begins in the replication origin ($O_H$) of the H-strand, in the displacement loop (D-loop). This D-loop is a triple-stranded region, found in the major non-coding region (NCR) of many mitochondrial genomes, and formed by stable incorporation of a third short DNA strand known as 7S DNA (Nicholls & Minczuk, 2014). The D-loop also contains the promoters for H-and L-strand transcription. Both strands have opposite replication directions, whereas the H-strand forms towards clockwise until the end of the chain, allowing a new H-strand synthesis. The mtDNA has some particular characteristics contributing to its unique genetic features, namely the fact of this molecule being a compartmentalized extrachromosomal element: heteroplasmy and threshold effect, mitotic segregation, semi-autonomous replication, maternal inheritance, highly compact structure, high mutation rates.

### 1.1.4. Crosstalk between mtDNA and nDNA

Only a reduced number of the proteins involved in mitochondrial integrity and function is synthesized in mitochondria. During the evolution, approximately 90% of proteobacterial genes were gradually transferred to the nDNA. Mitochondria acquired new components and functions from the host cell, resulting in profound changes in both mitochondrial and nuclear genome and proteome (Wallace, 2009).

The majority of essential proteins to the mitochondria activity is encoded by nDNA and later imported into the mitochondria, after being synthesized in the cytosol.

Therefore, the continuously coordination of nucleus and mitochondria is crucial for the regulation of transcription and translation mechanisms, as well as the translocation and import of mitochondrial proteins (Pagliarini *et al.,*2008). Although there are major differences between mtDNA and nDNA, the mitochondrial function, and consequently cell homeostasis, depends on the bigenomic interaction, a crosstalk between these two physically separated genomes.

The only metabolic pathway in the cell under this dual (mtDNA and nDNA) control is the OXPHOS that occurs in the MRC (Chan, 2006). Moreover, all the remaining protein subunits of MRC, enzymes responsible for the import and import machinery, MRC assembly factors, as well as crucial factors for intramitochondrial transcription and translation are encoded by nDNA and transported to the mitochondria (Larsson *et al.,*1998; Shoubridge, 2001; Falkenberg *et al.,*2002).

## 1.2. Mitochondrial Respiratory Chain and Oxidative Phosphorylation

The OXPHOS is the final step of a succession of biochemical reactions to generate energy, in other words, it is the last stage of cellular respiration in eukaryotes and aerobic prokaryotes. It provides the major part of energy production, in ATP form, necessary for adequate cellular function, by coupling two sets of reactions: oxygen consumption and ATP synthesis (Fernández-Vizarra *et al.,*2009).

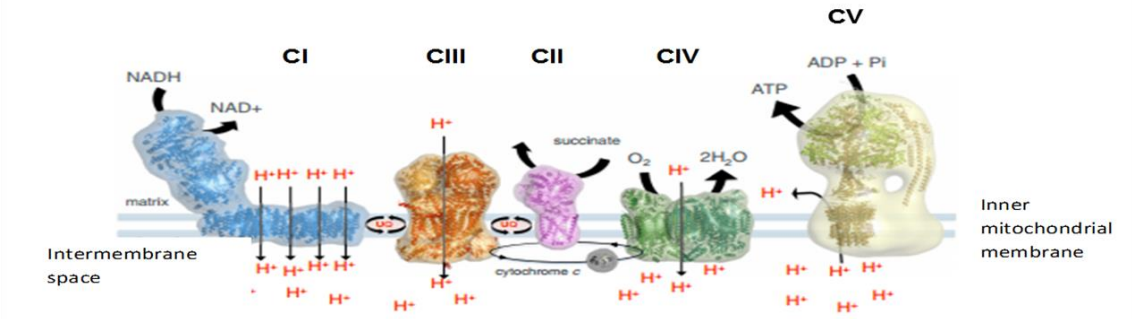
The MRC is composed by five hydrophobic and extremely organized complexes and two electron carriers (**Figure 1.1.**), coenzyme Q (CoQ), or ubiquinone, and cytochrome *c*, which facilitate the electron flux between complexes (Solomon *et al.,*2008; Rötig & Munnich, 2003). The first four complexes are responsible for the oxidation of products from glycolysis and beta-oxidation of fatty acids, in the respiration phase of OXPHOS. CI (NADH: ubiquinone oxidoreductase, EC 1.6.5.3) and CII (succinate: ubiquinone oxidoreductase, EC 1.3.5.1) accept electrons from NADH (reducing equivalents produced during glycolysis, fatty acid β-oxidation and Krebs cycle) and $FADH_2$ (reducing equivalents produced in fatty acid β-oxidation and in Krebs cycle), respectively. Then, ubiquinone accepts the electrons and become reduced, being the substrate of CIII (ubiquinol: cytochrome*c* oxidoreductase, EC 1.10.2.2). Electrons from reduced ubiquinone are accepted by CIII and transferred to cytochrome *c*. Then, CIV (cytochrome *c* oxidase, EC 1.9.3.1) accepts electrons from cytochrome *c*, and reduces molecular oxygen (final electron acceptor) to water. This sequential transfer of electrons, releases energy that is used to pump protons ($H^+$) from the

mitochondria matrix to the intermembrane space, creating a proton gradient, across the MIM (Wallace, 2010).

The CV (ATP synthase, EC 3.6.3.14) is involved in the final step of OXPHOS, the ATP production. The re-entry of the protons, placed in the intermembrane space, through the ATP synthase uses the energy generated by the electrochemical gradient to catalyse the ATP formation from inorganic phosphate and ADP. Therefore, the energy is stored in ATP bonds instead of being dissipated as heat (Dimauro & Schon, 2003; Zeviani & Di Donato, 2004; DiMauro & Hirano, 2005; Campbell *et al.,*2008).

The electrochemical potential generated by complexes I, III and IV, is also required for the transport of nuclear-encoded proteins across the inner membrane, including OXPHOS complexes subunits and assembly factors. The import machinery is needed not only for the biogenesis of the MRC, but it is also indispensable for the protein import machinery functioning (Dudek *et al.,*2013; Kulawiak *et al.,*2013).

The MRC is composed by, approximately, 100 different subunits, 13 of which are encoded by mitochondrial genes and the remaining are encoded by nuclear genome (Rötig & Munnich, 2003; Lloyd & McGeehan, 2013) (**Figure 1.1**). These complexes have some peculiarities at both structural and genetic levels. From a structural perspective, the majority of the MRC is contained in the MIM, as previously referred, so the subunits of the complexes are mostly composed of hydrophobic proteins. From a genetic standpoint, the OXPHOS system is unique as it is encoded by two physically and functionally separated genomes, the nDNA and the mtDNA (Zeviani & Di Donato, 2004; DiMauro & Hirano, 2005). Thus, it is believable that mutations in any gene essential to the integrity and function of MRC can change mitochondria structure and activity (Wong, 2010).




| Subunits | Complex I | Complex II | Complex III | Complex IV | Complex IV |
|---|---|---|---|---|---|
| mtDNA-encoded | 7 | - | 1 | 3 | 2 |
| nDNA- encoded | 37 | 4 | 10 | 11 | 17 |
| Assembly factors | 11 | 2 | 1 | 7 | 2 |

**Figure 1. 1. Schematic representation of the mitochondrial respiratory chain (MRC) complexes and the oxidative phosphorylation (OXPHOS) system.** Complex I (CI, NADH: ubiquinone oxidoreductase, EC 1.6.5.3); complex II (CII, succinate: ubiquinone oxidoreductase, EC 1.3.5.1); complex III (CIII, ubiquinol: cytochrome c oxidoreductase, EC 1.10.2.2); complex IV (CIV, cytochrome c oxidase, EC 1.9.3.1); and complex V (CV, ATP synthase, EC 3.6.3.14). Complexes I-IV are responsible for reduction of $O_2$ to water and CV for ATP synthesis (adapted from: Schon, DiMauro, & Hirano, 2012; Kühlbrandt, 2015).

### 1.2.1. Complex I

The mammalian CI is the largest complex of OXPHOS (**Figure1.2.**) with a molecular weight of, approximately 980 kDa (Fernández-Vizarra *et al.,*2009). It is composed by 45 subunits, of which seven are encoded by mtDNA (ND1, ND2, ND3, ND4, ND4L, ND5 and ND6), and the remaining 38 by nuclear DNA genes. This complex is the main electron entry point, since it catalyses the oxidation of reduced NADH by CoQ. It is also responsible for the creation of an electrochemical gradient, which is used to ATP production (Walker *et al.,*2003; Murray *et al.,*2003; Carroll *et al.,*2006).

Electron microscopy (EM) showed that CI has a 'L' shape, composed by two perpendicular arms: an hydrophobic "membrane arm", inserted in the MIM, which contains all the mtDNA-encoded subunits; and an hydrophilic "peripheral arm" that protrudes into the matrix, containing the nDNA-encoded subunits as well as Fe–S centres (Carroll *et al.,*2003; Janssen *et al.,*2006). This is a bigenomic encoded structure that harbours three functional modules: the P module or proton translocation that constitutes the majority of the "membrane" arm, including at least the seven mtDNA-encoded subunits; the N module that contains the dehydrogenase site,

responsible for the oxidation of NADH to $NAD^+$, includes nDNA-encoded subunits, such as NDUFV2, NDUFV1, and NDUFS1; the Q module contains the hydrogenase site responsible for the electron transfer to ubiquinone, comprising nDNA-encoded subunits, such as NDUFS2, NDUFS3, NDUFS7, and NDUFS8 (Nijtmans *et al.,*2007).

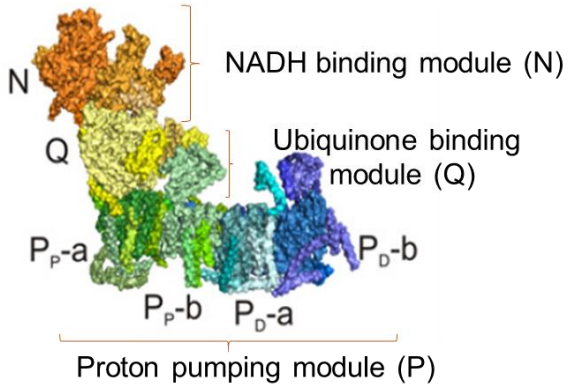



**Figure 1. 2. Schematic representation of mammalian CI structure** (adapted from Sánchez-Caballero *et al*., 2016). Legend: N - NADH binding module; Q - Ubiquinone binding module; $P_P$ - proximal half of the membrane integral P - module it composed by $P_P$-a and $P_P$-b; $P_D$ - distal part of the membrane arm of complex I it is composed by $P_D$-a and $P_D$-b.

### 1.2.2. Complex II

The complex II acts as a succinate dehydrogenase (SDH) catalysing the oxidation and dehydration of succinate to fumarate (**Figure 1.3.**). CII is one of the five MRC complexes, and is responsible for coupling the reduction of ubiquinone to ubiquinol ($CoQH_2$), that in turn directs electrons to CIII (Ernster & Dallner, 1995). It is the smallest MRC complex (123 kDa), and the only membrane-bound of the tricarboxylic acid (TCA) cycle. This complex is composed of four subunits, encoded by SDHA, SDHB, SDHC, and SDHD nuclear genes. The SDHA subunit contains a FAD moiety, whereas three Fe–S centers are bound to SDHB. These two hydrophilic subunits are linked to SDHC and SDHD, two small, hydrophobic polypeptides that contain a heme *b* moiety and anchor the complex to the MIM. The amino acid sequences of the flavin and Fe–S binding domains of CII are highly conserved, since it shows a high level of structure homology with α - proteobacteria. Contrariwise, the membrane domain is less conserved, although a four-helix bundle motif is ubiquitously present across species (Yankovskaya *et al.,*2003; Sun *et al.,*2005).

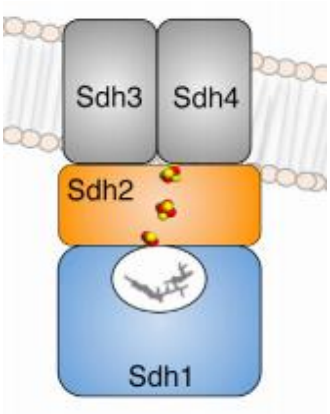



**Figure 1. 3. Schematic representation of CII structure** (adapted from Un Na *et al*., 2014). Legend: Sdh1, 2, 3, 4 - four subunit proteins that constitute the Succinate dehydrogenase complex.

### 1.2.3. Complex III

The complex III occupies the middle position of the MRC (**Figure 1.4.**) and couples the transfer of electrons from reduced CoQH2, to cytochrome c, generating a proton gradient across the MIM (Brandt & Trumpower, 1994; Peter Mitchell, 1976). CIII has a dimer structure where each monomer is composed of 11 different subunits (Iwata *et al.,*1998), of which only one is encoded by mtDNA, cytochrome b, (cyt b), located centrally in the trans-membrane region. Nuclear genes encode the remaining subunits which are the cytochrome c1, cyt c1, the Rieske-type $Fe_2S_2$ iron-sulphur cluster (RISP or UQCRFS1) and two hemes, bH and bL which together facilitate the proton-motive Q-cycle mechanism (Trumpower, 1990), two relatively large “core” subunits, Core 1 (UQCRC1) and Core 2 (UQCRC2). The exact function of the other smaller eight subunits (UQCRC1, UQCRC2, UQCRH, UQCRB, UQCRQ, Subunit 9, UQCR10 and UQCR11) remains to be established (Xia *et al.,*2013).

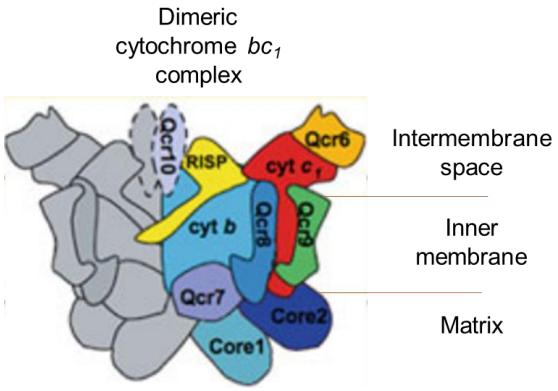



**Figure 1. 4. Schematic representation of CIII structure** (adapted from Ghezzi and Zeviani, 2012).

### 1.2.4. Complex IV

The complex IV is the last component of the electron transport chain (ETC), transfers electrons from reduced cytochrome c, a CIII product, to molecular oxygen (**Figure 1.5.**). This reaction is coupled to proton pumping across the MIM, from the mitochondrial matrix to the intermembrane space (Malmstroem, 1990; Ferguson-Miller & Babcock, 1996). The mammalian CIV is composed by three main areas, such as an outer region, that faces the inter-membrane space, an inner region, that faces the matrix side, and a large trans-membrane region. This complex is constituted by 13 subunits, of which three are mtDNA encoded, and are responsible to form the central core (MTCOI, MTCOII and MTCOIII), whereas the remaining subunits are nDNA encoded and constitutes the structural scaffold around the central core (Tsukihara *et al.,*1996). The complex also contains two iron sites (heme *a* and a3), two copper sites (CuA and CuB), as well as zinc and magnesium sites (Tsukihara *et al.,*1995). Electron and proton transfers are performed by the MTCO1 and MTCO2 subunits. The third mtDNA-encoded subunit, MTCO3, is part of the structural core, being possibly involved in proton pumping. The function of the ten nuclear-encoded subunits (COX4, COX5A, COX5B, COX6A, COX6B, COX6C, COX7A, COX7B, COX7C and COX8) is currently unknown, but they may play regulatory and stabilization roles (Arnold & Kadenbach, 1997). In the complete cycle, four electrons are transferred to molecular oxygen ($O_2$) from four molecules of cytochrome c, producing two molecules of water. A total of four protons are removed from the mitochondrial matrix of which two are translocated across the membrane.

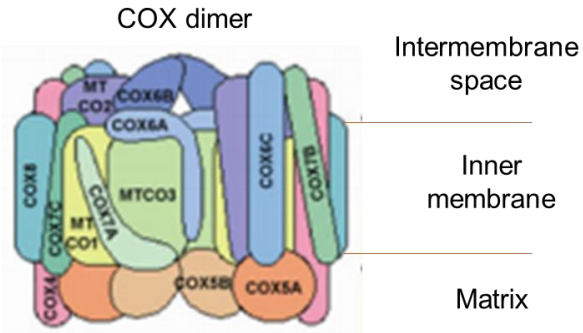



**Figure 1. 5. Schematic representation of CIV structure** (adapted from Ghezzi and Zeviani, 2012).

### 1.2.5. Complex V

The complex V is the last of the MRC complexes involved in OXPHOS, and is responsible for dissipate the proton electrochemical gradient generated by ETC in order to produce ATP (**Figure 1.6.**). Although ATP synthase does not contain the same number of subunits between bacteria to human, the function is conserved across species. Accordingly, the CV comprises a membrane-integrated subcomplex (F0), containing 9 MIM subunits that form a rotor-like structure harbouring a proton channel, and a soluble component (F1), containing 5 matrix subunits, that generates or hydrolyses ATP through the action of a rotational mechanism (Kucharczyk *et al.,*2009). These two functional domains are physically connected to each other by another two structures: a centrally located stalk and an external stator. Protons pass from the intermembrane space to the matrix through F0, which transfers the energy created by the proton electrochemical gradient through the stalk to F1, the catalytic ATP synthase domain, where ADP is phosphorylated to ATP (Devenish et al. 2008). All five subunits of F1 (a, b, g, d, e), and most of the F0 subunits (b c, d, e, f, g, OSCP and F6) are nuclear encoded. Only two proteins of F0, MT-ATP6 and 8, are encoded by mtDNA and connected to the stator. Dimeric and higher oligomeric forms of ATP synthase seem critical to maintain the shape of mitochondria by promoting the formation of the inner membrane cristae (Ghezzi & Zeviani, 2012; Rühle & Leister, 2015).

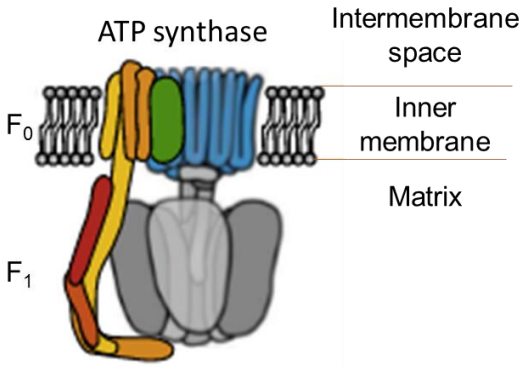



**Figure 1. 6. Schematic representation of CIV structure** (adapted from Fujikawa *et al.,* 2015). Legend: F1 - hydrophilic domain; F0 - hydrophobic domain.

### 1.3. Supercomplexes

In the last fifteen years, the organization of OXPHOS complexes into the MIM has been exhaustively discussed. However, the debate of physical and functional organization of the MRC complexes lasts for more than 100 years.

The first model of the arrangement of mitochondrial supercomplexes (SCs) in the MIM, named “solid-state model”, was proposed in 1947 by Keilin & Hartree. It defends that respiratory components were more or less rigidly held together in a framework that ensures their mutual accessibility and a consequent high catalytic activity (Keilin & Hartree, 1947).

On the other hand, in 1962, the achievement of Hatefi *et al.,*(Hatefi *et al.,*1962) demonstrated that purified respiratory complexes were functional, confirming the chemiosmotic model proposed by Mitchell one year earlier (Mitchell, 1961), and giving rise to the classical or “fluid-state model”. This model defends the existence of two mobile electron carriers, CoQ and cytochrome *c*, which are responsible for the enzymatic complexes connection (Green & Tzagoloff, 1966).

In 1963, Chance et al. introduced the concept of the oxysome, and the “solid-state model” became widely accepted (Chance *et al.,*1963).

In the 70’s, Hackenbrock (Hackenbrock, 1977) stated that mitochondrial complexes of MRC were not static entities in the MIM, having a high degree of free movements. Nevertheless, in 2000, Schägger and Pfeiffer refuted the idea of random diffusion of complexes and electron transfer based on random collisions of isolated complexes in mitochondrion cristae. These two researchers published the first blue

native polyacrylamide gel electrophoresis (BN-PAGE) analysis of digitonin-solubilized yeast and bovine preparations, where they found multiple co-migrating complexes renamed SCs. Although in a somewhat similar way to the oxysome described by Chance *et al.,*the SCs lacked the ATP synthase (H Schägger & Pfeiffer, 2000). In mammals, these dynamic complexes may aggregate in different stoichiometric combinations to form supramolecular associations in which the individual complexes of the respiratory chain interact with each other (Lenaz & Genova, 2007; Acín-Pérez *et al.,*2008; Jha *et al*.,2016). The real existence of SCs has been questioned. In order to prove their physical existence, a wide range of experiments, such as EM, has been playing a crucial role in revealing the interaction of OXPHOS complexes within SCs (Dudkina *et al.,*2005; Schäfer *et al.,*2006; Heinemeyer 2007). The performance of flux control assays proved that MRC acts as a single unit. Furthermore, point mutations in genes encoding one of the OXPHOS complex subunits were demonstrated to affect the stability of another complex (Blanchi *et al.,*2004; Carroll *et al.,*2006). So far, the BN-PAGE was the procedure most commonly used to resolve and characterize the OXPHOS system organization, similarly to what Schägger and Pfeiffer discussed. That methodology allows the separation of proteins according to their molecular mass and/or size, since it preserves their native structure (Schägger & Pfeiffer, 2000; Eubel *et al.,*2004; Lenaz *et al.,*2010; Perales-Clemente *et al.,*2010). Respiratory SCs have been found in organisms belonging to different kingdoms of eukaryotes, and despite their phylogenetic distances, all of them have in common the supramolecular organization of the OXPHOS system. Despite the variation in results depends on BN-PAGE conditions, mitochondria from different species seem to be organized into SCs composed by a CI monomer ($I_1$), a CIII dimmer ($III_2$), and up to four CIV ($IV_{0-4}$) components. Based on their organization, SCs can be divided into three main groups accordingly to $I_1$, $III_2$ and $IV_{0-4}$ assembly: $I_1III_2$; $III_2IV_{1-2}$; $I_1III_2IV_{1-4}$. The SCs composed by $I_1III_2$ are the most abundant in plants (Eubel *et al.,*2003), $III_2IV_2$ in fungi (Boumans *et al.,*1998; Kouřil *et al.,*2007) and $I_1III_2IV_{1-4}$ in mammals (H Schägger & Pfeiffer, 2000). However, the coexistence of the SCs unit composed by at least $I_1III_2IV_{1-4}$, together with free $III_2$ and $IV_{1-4}$, has been exhaustively discussed (Moreno-Lastres *et al.,*2012). On the other hand, the association of CII and CV with SCs remains controversial (Moreno-Lastres *et al.,*2012).

As a consequence of their organization in supercomplexes, individual OXPHOS complexes demonstrate a structural interdependence (Moreno-Lastres *et al.,*2012). Although the exact functional relevance of these SCs is not yet fully proven, it has been suggested that association of MRC complexes in SCs may offer structural and

functional advantages, such as the prevention of their destabilization and degradation, the improvement of electron transport efficiency and substrate directing, and the decrease of electron or proton leaks, by reducing the distance of diffusion between complexes (Moreno-Lastres *et al.,*2012). The formation of SCs is also suggested to be important in preventing the imbalance of ROS formation, since decrease of respiratory SCs correlates with higher ROS accumulation (Lenaz & Genova, 2009; Moreno-Loshuertos & Enríquez, 2016).

### 1.4. MRC assembly factors

The MRC complexes assembly factors **(Table I.i.)** are proteins involved in the organization of CI, CII, CIII, CIV, and CV, not being part of the final structure of the enzyme. Some of them are probably also involved in supercomplexes assembly (Moreno-Loshuertos & Enríquez, 2016).

**Table I. i. Description of human MRC assembly factors.** This table summarizes the main assembly factors and their crucial contribute to the assembly pathway of MRC complexes I-V.

| Assembly factors | Function |
|---|---|
| **Complex I** | |
| **NDUFAF1 (MIM 606934) (NADH: ubiquinone oxireductase complex assembly factor 1)** | Interacts transiently with early arm membrane intermediates, in the initial assembly of the separate ND2 subcomplex (Perales-Clemente *et al.,*2010). Mutations in this factor, heterozygous or homozygous missense or nonsense, are associated with cardioencephalomyopathy, lactic acidosis. Knockdown of NDUFAF1 results in dramatic decreased levels of CI activity and assembly (Vogel *et al.,*2005; Dunning *et al.,*2007). |
| **ECSIT (MIM 608388) (Evolutionary conserved signalling intermediate in Toll pathway)** | Required for stabilization of NDUFAF1 (CI-specific assembly chaperone). Its absence, in humans, results in the reduction of the NDUFAF1 levels. Therefore, mutations in the gene leads to impaired CI assembly and activity, accumulation of intermediates, and consequently mitochondrial dysfunction (Vogel *et al.,*2007). |
| **NDUFAF2 (MIM 609653) (NADH: ubiquinone oxireductase complex assembly factor 2)** | NDUFAF2 participates in the insertion of the N module into CI. The absence of this protein does not prevent the fully assembled of CI, so it is predicted that NDUFAF2 stabilize CI late intermediates or |

| Assembly factors | Function |
|---|---|
| | allow the incorporation of late subunits into the complex. Mutations in this factor, are associated with progressive encephalopathy, Leigh syndrome (Ogilvie *et al.,*2005; Saada *et al.,*2012; Rhein *et al.,*2016). |
| **NDUFAF3 (MIM 612911) (NADH: ubiquinone oxireductase complex assembly factor 3)**<br><br>**NDUFAF4 (MIM 611776) (NADH: ubiquinone oxireductase complex assembly factor 4)** | NDUFAF3 and NDUFAF4 interact with each other, since the knock down of NDUFAF3 leads to the reduction of NDUFAF4 amount, and vice-versa. They most likely have an important involvement in the anchoring of subcomplex ND1 to the mitochondrial membrane, which means that they are essential in early assembly stages of CI (Perales-Clemente *et al.,*2010). Mutations in NDUFAF3 were associated with fatal neonatal mitochondrial disorders (Saada *et al.,*2009). Lethal infantile mitochondrial disease and antenatal cardiomyopathy, were associated to NDUFAF4 mutations (Saada *et al.,*2008). |
| **NDUFAF5 (MIM 61236) (NADH: ubiquinone oxireductase complex assembly factor 5)** | Encodes a matrix mitochondrial protein and is required for CI early stages of assembly. A mutation in this gene results in reduction of mature levels and activity of mitochondrial CI. This factor may also be involved in the incorporation of ND1 into the membrane. It is predicted that this protein methylates the CI subunit NDUFB3, since it has an S-adenosymethionine-dependent methyltransferase (SAM) domain (Sugiana *et al.,*2008; Saada *et al.,*2012; Rhein *et al.,*2016) (1.http://www.ncbi.nlm.nih.gov/gene/79133). |
| **NDUFAF6 (MIM 612392) (NADH: ubiquinone oxireductase complex assembly factor 6)** | The encoded protein plays an important role in the assembly of CI, stabilizing the subcomplexes that interact with the membrane subunit ND1, of the peripheral arm. A homozygous missense mutation in a conserved residue was associated with Leigh's syndrome with isolated CI deficiency (Pagliarini *et al.,*2008). Early CI assembly defects result in rapid turnover of the ND1 subunit (2.http://www.ncbi.nlm.nih.gov/gene/137682). |
| **NDUFAF7 (MIM 615898)**<br>**(NADH: ubiquinone oxireductase complex assembly factor 7)** | NDUFAF7, a protein of the 7β-strand methyltransferase family that dimethylates Arg-85 in the NDUFS2 at the mitochondria matrix, during CI assembly. This methylation is required for efficient CI assembly (Rhein *et al.,*2013; Zurita Rendon *et al.,*2014). |

| Assembly factors | Function |
|---|---|
| **NUBPL (MIM 613621) (Nucleotide-binding protein-like protein)/ ind1 (Iron-sulphur protein requires for NADH dehydrogenase)** | NUBPL, in humans, facilitates the incorporation of Fe-S cluster centers into CI membrane arm. This protein is an assembly factor for CI. Mutations in NUBPL cause decreased activity of CI and abnormal mitochondrial morphology assembly, due to the inappropriate assembly of the CI peripheral arm (Sheftel *et al.,*2009; Bych *et al.,*2008; Calvo *et al.,*2010; Saada *et al.,*2012). |
| **FOXRED1 (MIM 613622)**<br>**(FAD-dependent oxidoreductase containing-domain protein 1)** | FOXRED1, contains an FAD-dependent oxidoreductase domain, and encodes a factor involved in the mid-late stages of CI assembly. It is known that mutations in this gene results in CI deficiency, infantile onset encephalomyopathy and Leigh syndrome. These facts make FOXRED1 crucial for correct CI biogenesis. FOXRED1 was found to co-immunoprecipitate with NDUFS3, NDUFA10 and NDUFA5, CI subunits, and to have a role in the amino acids metabolism. Perhaps FOXRED1 has a role in glutathione metabolism and a protective effect against oxidative stress in CI (Calvo *et al.,*2010; Formosa *et al.,*2015; Lemire, 2015; Zurita Rendón *et al.,*2016). |
| **ACAD9 (MIM 611103)**<br>**(FADH2 dependent Acyl-CoA dehydrogenase family member 9)** | ACAD9 is an essential assembly factor for CI biogenesis by the interaction with the MIM and other assembly factors. Knockdown of *ACAD9* affect the long-chain fatty acid oxidation, as well as the amount of NDUFAF1, ECSIT, since this protein was discovered to co-migrate with them, and consequently CI holocomplex. Mutations in *ACAD9* are associated with infantile hypertrophic cardiomyopathy, encephalopathy and lactic acidosis. (Nouws *et al*., 2010; Haack *et al*., 2010; Gerards *et al*., 2011;Schiff *et al*., 2015). |
| **Complex II** | |
| **SDHAF1 (MIM 612848) (Succinate dehydrogenase assembly factor 1)** | SDHAF1 is a small protein that could have a role in the insertion or retention of the FE-S in the CII. Mutations in SDHAF1 are associated with a drastic decrease of CII activity and amount in muscle and fibroblasts. Leuko encephalopathy with the accumulation of lactate and succinate in the white matter, constitute some clinical manifestations reported in infants (Ghezzi *et al.,*2009). |

| Assembly factors | Function |
|---|---|
| **SDHAF2 (MIM 601650) (Succinate dehydrogenase assembly factor 2)** | This chaperone is responsible for the incorporation of FAD into SDHA. Mutations in this gene have been reported in two families with hereditary multiple head and neck paragangliomas (H. Hao *et al.,*2009). |
| **Complex III** | |
| **BCS1L (MIM 603647) (BCS1 homolog, ubiquinol-cytochrome c reductase complex chaperone)** | BCS1L encodes a member of the AAA family of ATPases that is necessary for the fully assembly of CIII in the mitochondria. All BCS1L mutations disrupted the assembly of CIII, however those in ATPase domain are associated with more severe phenotype. These have been reported associated with severe clinical representations like neonatal proximal tubulopathy, hepatopathy, encephalopathy, and isolated progressive infantile encephalopathy (Hinson *et al.,*2007; Petruzzella *et al.,*1998). |
| **Complex IV** | |
| **SURF1 (MIM 185620) (surfeit 1)** | SURF1 possible plays a role in the formation of the early assembly stages of COX. Almost all *SURF1* mutations cause complete absence of SURF1, and they are the most frequent cause of Leigh Syndrome (Zhu *et al.,*1998; Kovářová *et al.,*2016). |
| **SCO1 (MIM 603644) (cytochrome c oxidase assembly protein 1)**<br><br>**SCO2 (MIM 604272) (cytochrome c oxidase assembly protein 2)** | SCO1 and SCO2 are COX assembly genes, whose role is to enable the incorporation of MTCO1 and MTCO2 into the CIV. These very early steps also require additional assembly factors like COX 17 and COX11 (Papadopoulou *et al.,*1999; Stiburek *et al.,*2009).<br><br>*SCO2* mutations originate heart hypertrophy, Leigh Syndrome and spinal muscular atrophy. Whereas *SCO1* originate severe keto acidosis and deficiency in muscle and liver (Papadopoulou *et al.,*1999; Stiburek *et al.,*2009). |
| **COX10 (MIM 602125) (heme A: farnesyltransferase cytochrome c oxidase assembly factor) and** | COX 10 and COX 15 are two enzymes involved in terminal steps of the biosynthesis of hemes *a* and *a*3. In both, rare missense mutations were found, resulting in partial COX deficiency. On the other hand, complete loss of enzymatic activity is not compatible with postnatal life. Hypertrophic cardiomyopathy, hypotonia, |

| Assembly factors | Function |
|---|---|
| **COX15 (MIM 603646) (cytochrome c oxidase assembly homolog)** | metabolic acidosis, sensorial deafness, encephalopathy with proximal renal tubulopathy. This is a spectrum of conditions associated with mutations in COX 10, to which the Leigh syndrome (LS) also belongs. In turn, mutations in COX 15 can cause fatal infantile hypertrophic cardiomyopathy, as well as rapidly progressive or protracted LS (Ghezzi & Zeviani, 2012). |
| **COX11 (MIM 602125) (cytochrome c oxidase copper chaperone)**<br><br>**COX17 (MIM 603646) (cytochrome c oxidase copper chaperone)**<br><br>**COX19 (MIM 610429) (cytochrome c oxidase assembly factor)** | This group of three proteins are encoded by genes responsible for the maturation and insertion of the copper prosthetic groups in COX biogenesis. COX 11, are involved in the synthesis of heme *a;* COX 17 is responsible for copper recruitment; and COX19 for copper translocation to mitochondria. mutations in this genes are not reported in humans, however, COX 17, and COX19 are candidates for CIV deficiency (Ghezzi & Zeviani, 2012). |
| **Complex V** | |
| **ATPAF1 (MIM 608917) or Atp11p (ATP synthase mitochondrial F1 complex assembly factor 1)** | ATPAF1 is an assembly factor for the F (1)-ATPases in human mitochondria. This gene is highly conserved among the eukaryotes and are expressed in a large range of different cells type (Wang, *et al.,*2001). |
| **ATPAF2 (MIM 608918) or Atp11p (ATP synthase mitochondrial F1 complex assembly factor 2)** | ATPAF2 is an assembly factor for the F (1)-ATPases in human mitochondria. This gene is highly conserved among the eukaryotes and is expressed in a large range of different cells type. Using mouse models was possible to conclude that interstitial genomic deletion within chromosome 17p11.2 causes Smith-Magenis syndrome (SMS) (Wang *et al.,*2001; Bi, 2002). |

### 1.4.1. The mechanism of Complex I assembly

For a long time, CI was poorly understood in terms of its complete assembly pathway and function. The first evidence indicating the existence of assembly intermediates for CI and their characterization was possible due to the study of patients with mutations in subunits or assembly factors of CI (Antonicka *et al.,*2003). With that evidence, researchers were able to conclude/hypothesize that CI is dynamic and that the assembly of its intermediates is sequential and not simultaneous.

The assembly process of CI subunits is very difficult to characterize, not only due to its large size and numerous subunits, but also due to the bigenomic contribution and the lack of detailed crystal structure (Mimaki *et al.,*2012). In order to better understand the CI underlying assembly mechanisms, researchers have been using different models to study the eukaryotic formation of mature CI. For example, the comparison of CI between the bovine and the *Yarrowialipolytica* species, allows the prediction of a more detailed model of the assembly intermediates that compose CI and its assembly patterns (Fernández-Vizarra *et al.,*2009).

Currently, the favoured model of CI assembly mechanism is based on the independent assembly of the membrane and the peripheral arm, via sequential insertion of subcomplexes that join together to form the characteristic L shaped structure (Vartak *et al.,*2015). As aforesaid, the membrane arm includes the P module and the peripheral arm includes the N and Q modules.

The N module is composed, at least, by two subassemblies that join together: one composed by NDUFV1 and NDUFV2 (Ugalde *et al.,*2004); and other composed by NDUFS1 and NDUFA2 (Guarani *et al.,*2014). The N module also has, at least, another four subunits that does not make part of the N module-subassembly, being only inserted in the final steps of the pathway: NDUFS6 and NDUFA12 which are located in the interface between N/Q module, NDUFS4 and NDUFV3 (Pereira *et al.,*2013; Kmita *et al.,*2015). The Q module is composed, at least, by nine subunits NDUFS2, NDUFS3, NDUFA5, NDUFS7, NDUFS8, NDUFA6, NDUFA9, NDUFB1 and NDUFA7. The initial step of the Q module assembly is composed by NDUFS2, NDUFS3, NDUFA5 binding to NDUFS7 and NDUFS8, subunits containing Fe-S clusters, are added in a sequential step. The next step comprises the subassembly formed by the assembly factors NDUFA3 and NDUFAF4. It is predicted that this subassembly has the role of stabilizing the Q module or even to allow the association of Q and P modules, since it is not integrated in the membrane *(Sánchez-Caballero et al.,*2016). The exact time point when the remaining four subunits (NDUFA6, NDUFA9, NDUFB1 and NDUFA7), are incorporated into the module, is not clear yet.

The P module, which is integrated in the membrane, is composed by two subassembly parts: the P proximal ($P_P$) module and the P distal ($P_D$) module. The first half of the membrane arm, $P_P$, is also composed by two parts: the $P_P$–a, which comprises ND1, NDUFA8, NDUFA3, NDUFA13 and NDUFA1 subunits (Vinothkumar *et al.,*2014); and the $P_P$–b, which includes ND2, ND3, ND4L, ND6, NDUFC1, NDUFC2, NDUFA10 and NDUFA11subunits (Sánchez-Caballero *et al.,*2016).

The attachment of the Q-module with the transmembrane subunits occur through the assembly of the Q-module preformed, NDUFAF3 and NDUFAF4, with the ND1 subunit of the $P_P$–a. Then, the remaining four subunits NDUFA8, NDUFA3, NDUFA13 and NDUFA1 are inserted. Recently, it was proposed that there are entry points of the mtDNA-encoded subunits in the CI assembly (Perales-Clemente *et al.,*2010). Through this study, it was possible to define the first entry point for ND1, which is the core subunit of $P_P$–a, described as a subcomplex with approximately 400 kDa, and also the second entry point for ND2, the major subunit of $P_P$–b. The last one contains other mtDNA-encoded subunits, such as ND3, ND4L and ND6, and some nDNA-encoded subunits, as NDUFV1, NDUFV2, NDUFA10 and NDUFA11. In terms of assembly order, it is speculated that ND3 is incorporated before NDL4, and it is known that NDUFA10 assembly only takes place in later stages of the mechanism (Hoefs *et al.,*2011; Hornig-Do *et al.,*2012). Taken together, these findings allow to conclude that ND1 and ND2 belong to two different subcomplexes that progress differently, and also that ND1 and ND2 after attach to peripheral-arm subunits, forming an intermediate that is anchored to the MIM (Perales-Clemente *et al.,*2010).

The other part of the P module, $P_D$, is composed of two mitochondrial encoded subunits, ND4 and ND5. Similarly, to what happen in the $P_P$, it is possible to define two different subassemblies, the $P_D$–a and the $P_D$–b. It is presumed, based on previous research, that $P_D$–a subassembly is constituted by the mitochondrial encoded subunit ND4, which will bind to the nuclear encoded subassembly, NDUFB5, NDUFB6, NDUFB10 and NDUFB11. Therefore, the core subunit of $P_D$–a is ND4, whereas the core subunit of $P_D$–b is ND5. The last one also has in its composition NDUFB3 and NDUFB9 that are probably attached to NDUFB8 and NDUFB7. However, the exactly entry time point of NDUFB2 and NDUFB4 remain unknown (Hoefs *et al.,*2011; Guarani *et al.,*2014). After the description of the assembly of subassemblies, the focus will be on the next phase which is the assembly of N, Q/$P_P$–a, $P_P$–b, $P_D$–a and $P_D$–b. Based on previous human CI studies, it was possible to: infer that first, the central part of the membrane arm expands and the preassembled submodule $P_P$-b interacts with the submodule $P_D$–a, forming the $P_P$–b/$P_D$–a intermediate. This intermediate has approximately 680kDa and contains ND2 and ND4, which are membrane embedded subunits, functioning as the assembly basis of CI. Before the formation of the Q/P subassembly, NDUFS5, NDUFA10 and NDUFB4 are attached into the $P_P$–b/$P_D$–a intermediate (Heide *et al.,*2012; Formosa *et al.,*2015; Guarani *et al.,*2014). At this time point, this intermediate is ready to interact with Q/$P_P$–a and $P_D$–b in order to originate the Q/P subassembly with approximately 830 kDa. The NDUFA9 is added in this step,

being part of the interface between Q and N modules. The next step of the assembly is the integration of the N module in the distal part of the peripheral arm, followed by the incorporation of NDUFS6 and NDUFA12, N/Q interface subunits, and the outgoing of the assembly factors. The CI assembly involves at least 11 assembly factors (**Figure 1.7**) (Sánchez-Caballero *et al.,*2016).

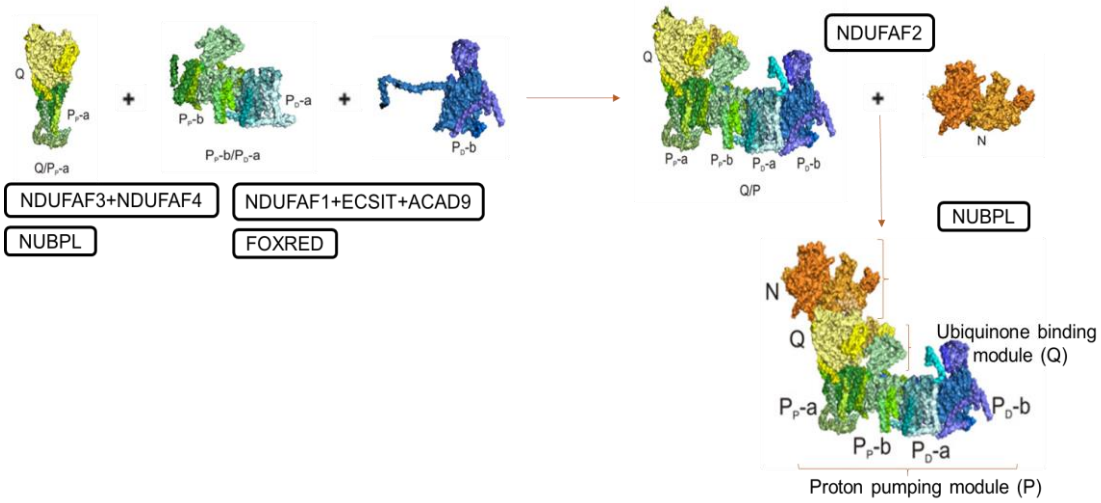



**Figure 1. 7. Schematic representation of the mechanism of Complex I assembly.** (adapted from Sánchez-Caballero *et al*., 2016).

### 1.4.2. The mechanism of Complex II assembly

The current model for CII assembly is based on the evidence that heme *b* play a role in the assembly and stability of CII (Hagerhall and Hederstedt 1996; Nakamura *et al.,* 1996)**.** The membrane - bound subassembly is formed upon the heme *b* binding to SdhD, this subassembly joins after to the SdhC subunit (Lenaz and Genova 2010). The heme *b* is also responsible for link the pre-formed subassemblies SdhA + SdhB, the hydrophilic module, to the SdhC + SdhD, membrane anchor subunits. SDHAF1 (Ghezzi *et al.,*2009) and SDHAF2 (Hao *et al.,*2009) are essential for CII assembly (**Figure 1.8**).

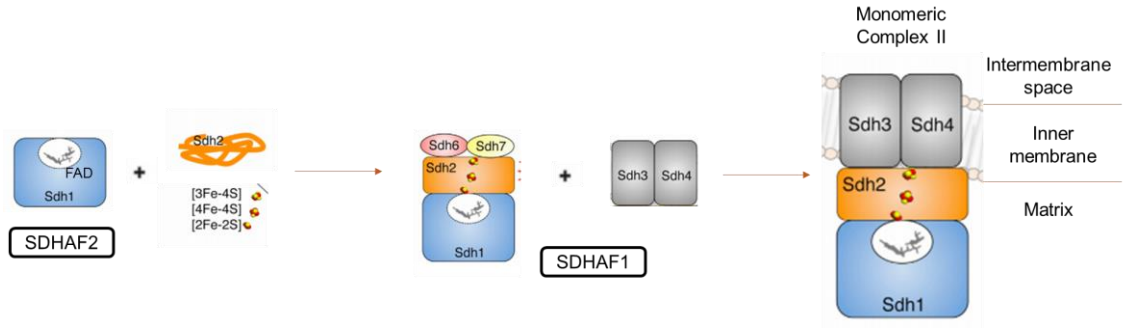



**Figure 1. 8**. **Schematic representation of the mechanism of Complex II assembly.** (adapted from Un Na *et al*., 2014).

### 1.4.3. The mechanism of Complex III assembly

The mammalian CIII assembly was outlined from studies in *S.ceravisiae*. Recently, through 2D-BN-PAGE analysis of yeast CIII mutant has been possible to describe a new pathway for the CIII assembly (**Figure 1.9**). It was described the existence of an initial subassembly, containing the cyt b + Qcr7 + Qcr8 subunits, then later incorporate a second subassembly composed by Core 1 + Core 2 + cyt c1, in order to form a 500 kDa dimerized sub-complex. The Qcr6, Qcr9, RISP and Qcr10 subunits are sequentially added to form the final structure of the enzymatically active CIII (Fernández-Vizarra *et al.,*2009; Zara *et al.,*2009).

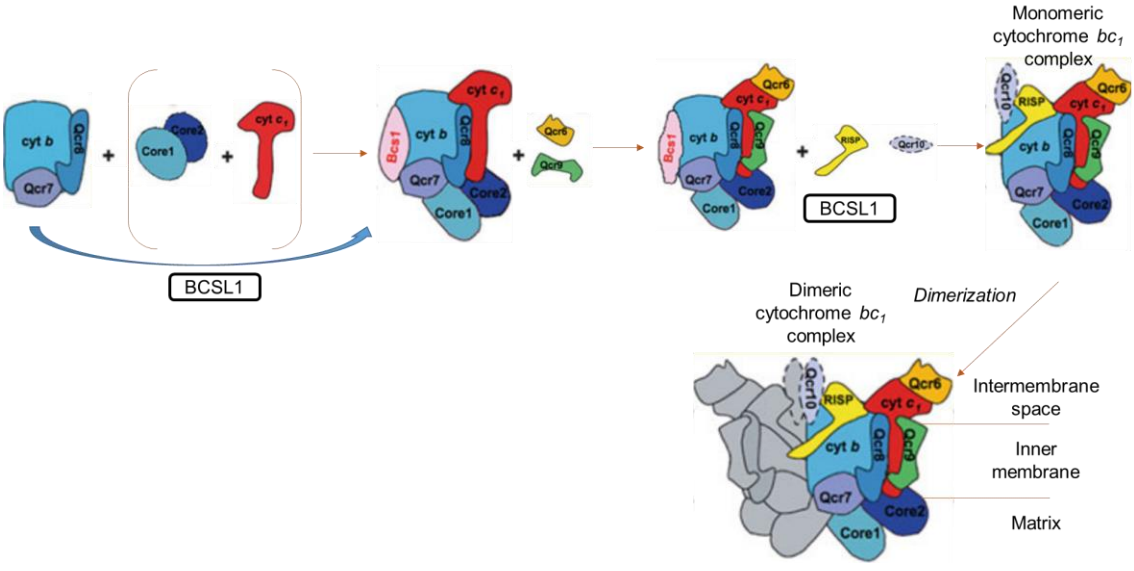



**Figure 1. 9. Schematic representation of the mechanism of Complex III assembly.** (adapted from Ghezzi and Zeviani, 2012).

### 1.4.4. The mechanism of Complex IV assembly

This complex assembly begins with the insertion of a newly synthesized MTCO1 into the MIM. Then, subunits COX4 and COX5A are incorporated in order to give rise to the second assembly intermediate. The heme *a* insertion occurs just after the formation of the first intermediate or during the formation of the second one, and proceeds together with the insertion of CuB and heme *a3* into MTCO1. The formation of the MTCO2-associated CuA center is followed by the incorporation of MTCO2 into the second intermediate. Next, the MTCO3 subunit and smaller nuclear encoded subunits, including COX5b and COX8 are sequentially incorporated, forming the third intermediate. The resulting intermediate leads to the formation of the almost complete CIV (Williams *et al.,*2004; Stiburek *et al.,*2005). The addition of a few remaining subunits, including COX6A, COX6B, COX7A and COX7B, all converging on the surface of the complex core, results in the formation of a holocomplex monomer (Ghezzi & Zeviani, 2012). Finally, monomeric COX dimerizes in an active structure that contains the cytochrome c (cyt c) binding site, where two molecules of cyt c cooperatively bind at the interface between the two COX monomers formed by contacts among MTCO1, COX6A, COX6B, and COX5B (Lee et al. 2001). **Figure 1.10** illustrates the mechanism hypothesis for the assembly of CIV.

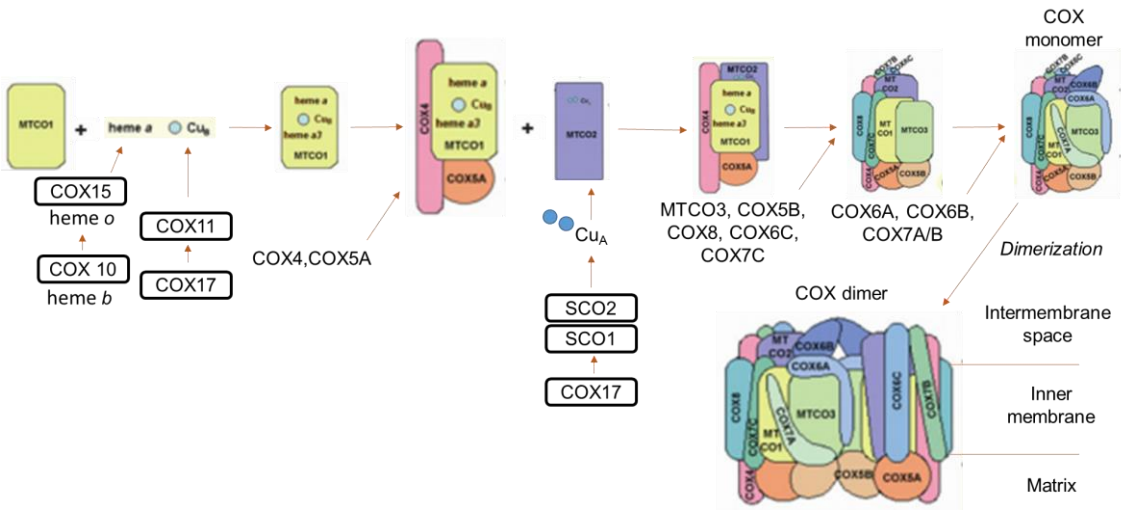



**Figure 1. 10. Schematic representation of the mechanism of Complex IV assembly.** (Adapted from Ghezzi and Zeviani, 2012).

### 1.4.5. The mechanism of Complex V assembly

It is already known that CV components, F0 and F1, assemble in an independent way. The F1 assembly, composed by the central stalk γδε and $\alpha_3\beta_3$ hexamer, is carried out, in mammalian mitochondria by ATPAF1 and the ATPAF2. These two assembly factors bind to the β- and α- subunit, respectively, forming the initial intermediate F1. Then this intermediate interacts with the pre-assembled F0 c-ring, forming an assembly intermediate of CV. The two mtDNA encoded subunits pre-assembly with the stator subunits, forming a second intermediator. CV fully assembled (**Figure 1.11**) results of the assembly of, at least, these two intermediates (Rak *et al.,*2011). The final steps in mammalian CV biogenesis include the formation of dimers (H Schägger & Pfeiffer, 2000), and the formation of higher order oligomers (V1–V4) (Krause, Reifschneider *et al.,*2005).

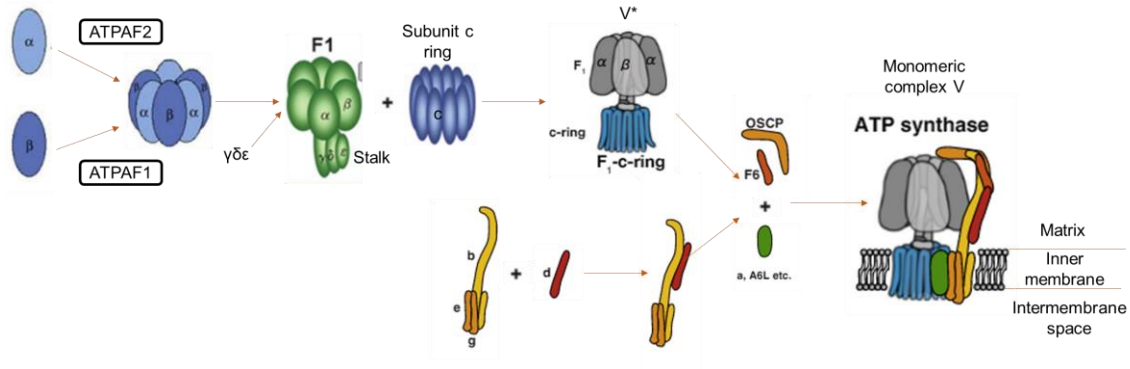



**Figure 1. 11. Schematic representation of the mechanism of Complex V assembly.** (Adapted from Fujikawa *et al.,*2015).

## 1.5. Mitochondrial Respiratory Chain Disorders

Mitochondrial cytopathies are a heterogeneous group of multisystem disorders which are related to the ATP production system involving MRC deficiency (Rodenburg, 2011). The tissues more susceptible to impaired ATP production are those who require more energy for their maintenance and function, with higher number of mitochondria per cell, such as the nervous system, cardiac conduction system, muscle, liver, pancreas and kidney (Wallace *et al.,*1992; McClelland *et al.,*2015). Accordingly, many patients with mitochondrial disease display a cluster of clinical features that can originate clinical syndrome such as Kearns-Sayer syndrome, mitochondrial encephalopathy with lactic acidosis, and stroke-like episodes (MELAS), myoclonic

epilepsy with ragged-red fibers (MERF), dominant autossomal optic atrophy (DOA), Leigh Syndrome (LS) and Leber´s Hereditary Optic Neuropathy (LHON).

These diseases may occur due to dysfunctions in at least one of the MRC complexes, which can be caused by diverse types of mutations in mtDNA and/or nDNA, possibly in a bigenomic mode (Ugalde *et al.,*2004). Mutations in nDNA, may play a synergistic role with mutations in mtDNA generating a severe biochemical defect (Potluri *et al.,*2009). Accordingly, mutations in mtDNA, although responsible for a significant number of pathologies often exhibit incomplete "penetrance", suggesting the involvement of other factors such as mtDNA haplotypes, nDNA single nucleotide polymorphisms (SNPs), alcohol, tobacco and environmental factors. Emphasizing that about 1,500 proteins encoded by nDNA are targeted to the mitochondria, mutations in mtDNA may not be the only cause of mitochondrial cytopathies. Moreover, defects in nDNA encoding mitochondrial factors of major importance to the assembly, stability and maintenance of the MRC (assembly factors, replication and transcription factors and other proteins involved in mitochondrial dynamics and molecular transportation) lead to an imbalance in OXPHOS-dependent energy production that compromise mitochondrial function (Wallace *et al*.,2010; Wong, 2010).

An important example of mitochondrial cytopathies is the group of hereditary optic neuropathies, which represents a significant cause of chronic visual impairment affecting approximately 1:45,000 individuals (Mascialino *et al.,*2012). From a clinical perspective, these neuropathies are mainly characterized by the involvement of central nervous system, skeletal muscle or both. The selective death of retinal ganglion cells (RGCs), as a consequence of mitochondrial dysfunction, is one of the causes of retinopathy, ocular motility disorders, or optic neuropathy. The most common mitochondrial optic neuropathy is LHON, mainly associated with CI deficiency. In addition to RGCs dysfunction, organs like heart, liver, pancreas, eye and kidney may also be affected, which culminates with a large range of different clinical manifestations (Votruba, 2004; Yu-Wai-Man *et al.,*2011; McClelland *et al.,*2015).

Deficiency in CI is the most common enzymatic defect in both children and adults representing about one-third of all cases of OXPHOS disorders, though most of the CI defects remain indeterminate at genetic and molecular levels. Given the complexity of the enzyme, a large number of yet unknown nuclear proteins are likely to be involved in its biogenesis. Deficiency in CI shows an extreme genetic heterogeneity and may be caused by a gene mutation in both nDNA or mtDNA. There are no obvious genotype-phenotype correlations, and inference of the underlying basis from the clinical or biochemical presentation is very difficult. Depending on which genome is

affected, mutations associated to CI can be divided into two groups: those affecting mtDNA-encoded ND genes and those affecting CI-related nDNA-encoded genes (Zeviani *et al.,*2009). Thus, mutations in any of these factors may lead to this complex deficiency and disease.

However, it is predicted that the majority of cases are caused by mutations in nuclear-encoded genes.

Currently, there are some mutations in genes coding for CI identified as potentially pathogenic and so far responsible for CI dysfunction and, consequently, MRC impairment, leading to the onset of a pathological phenotype (Wallace *et al.,*2000). In addition to LHON, the clinical manifestations caused by CI deficiency range from lethal neonatal disease to adult-onset neurodegenerative disorders. The most frequent phenotypic manifestations include Parkinson's disease, macrocephaly with progressive leukodystrophy, encephalopathy, hypertrophic cardiomyopathy, myopathy, liver disease, fatal infantile lactic acidosis (FILA), LS (Schapira, 2007; Sharma & Bai, 2009).

The genomic crosstalk is thought to prevent the accumulation of nuclear or mitochondrial proteins, since the accumulation of immature or defective complexes may contribute to severe mitochondrial disorders through an increased ROS production during the OXPHOS (Koopman *et al.,*2012). Furthermore, CI is also responsible for the main production of ROS in the mitochondrial matrix, which may cause damage to mitochondria and other cell components. The ROS result of a small percentage of electrons that prematurely leaks to oxygen, that if not completely reduced and not effectively neutralized by antioxidant defences, lead to oxidative stress and lesions of important biomolecules, such as proteins, lipids and nucleotides (Grazina, 2004; Kim *et al.,*2007).

### 1.5.1. Leber's Hereditary Optic Neuropathy – LHON

The LHON (OMIM #535000) is a rare mitochondrial neurodegenerative maternally inherited disease, firstly described in 1871 by the ophthalmologist Theodor Leber (Leber, 1871). It is mainly characterized by an acute or subacute painless and bilateral loss of central vision. Dyschromatopsia (fading of colors), central scotoma and severe reduction of visual acuity, also characterize the disease phenotype (Carelli *et al.,*2004; Kirkman *et al.,*2009; Koilkonda & Guy, 2011; Sadun *et al.,*2011; Yu-Wai-Man *et al.,*2009, 2011)**.** These symptoms may appear simultaneously in both eyes or affect them with a time lapse from days to months, and rarely years (Yen *et al.,*2006;

Koilkonda & Guy, 2011). Almost 80% of the cases occur in young males and the onset of visual loss is between 15 and 35 years old (Yo) (Yen *et al.,*2006).

#### 1.5.1.1. Clinical Features of LHON

The optic nerve transmits visual information from the retina to the visual cortex. In humans, optic nerve emerges from the RGCs and has about 1.2 million axons. After leaving the eye, these axons become myelinated and transmit visual information to the thalamus (Carelli *et al.,*2004). The first portion of the RGC axon is demyelinated. Since the demyelinated region has a very low conduct velocity it requires more energy; so, in this area there are higher number of mitochondria (relevant to the signal transmission). On the other hand, the second portion of the RGC axon is myelinated and presents higher energy efficiency due to saltatory conduction. The loss of RGCs leads to optic neuropathy, as it has been shown in the case of LHON (Carelli *et al.,*2004; Amati-Bonneau *et al.,*2009).

In most of LHON cases, the examination of the ocular fundus revealed a triad of symptoms like circumpapillary telangiectatic microangiopathy, swelling of the optic nerve head (pseudoedema), and vascular tortuosity (Schatz *et al.,*1984; Carelli *et al.,*2004). The **Figure 1.12.,** represents a typically normal and a LHON patient eye fundus. In **Figure 1.12. (B),** it is visible the optic disc, a white region that results from the RGCs loss.

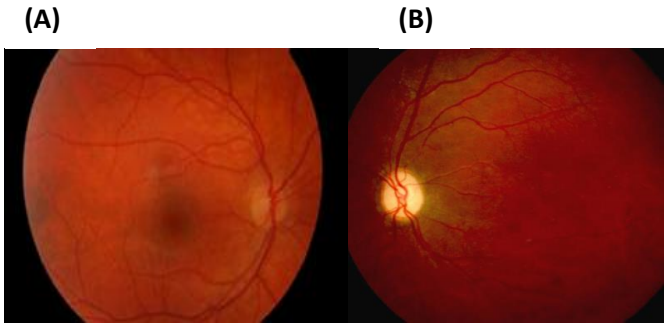



**Figure 1. 12. Eye fundus' images** representing the normal eye fundus **(A)** (adapted from Piotrowska *et al.,*2015); and example of optical atrophy **(B)** (Grazina *et al.,*2007).

#### 1.5.1.2. Epidemiology

In 2003, Man *et al*. published a study with a population of the North East England where the estimated prevalence of visual impairment as a result of LHON was 3.22 per 100,000 habitants, increasing to 7.11 per 100,000 habitants if only adult men are considered. The estimated prevalence of visual impairment as a consequence of a primary LHON mtDNA mutation was 11.82 per 100,000 habitants (Man *et al.,*2003).

At the end of 2004, another study from a Finish population reports the prevalence of visual failure as a result of LHON to be 2.06 per 100,000 habitants. The prevalence of individuals presenting a primary mutation associated with LHON was 10.69 per 100,000 habitants (Puomila *et al.,*2007).

It was reported that LHON in a Danish population has a prevalence was 1.9 per 100,000 habitants. The gender proportion for male in this population was 2.9 per 100,000 (Rosenberg *et al.,*2016).

#### 1.5.1.3. Genetic and Biochemical causes

In 1988, Wallace *et al.,* associated LHON with genetic alterations, by describing a point mutation in the *MTND4* gene, m.11778G>A, which resulted in a substitution of an arginine for a histidine, at position 340 (p. R340H) in the subunit ND4 of CI (Wallace *et al.,*1988). Presently, LHON is associated with three point mutations in mtDNA. Besides the mutation described by Wallace *et al.,* other two mutations have been frequently observed in these patients, m.3460G>A and m.14484T>C. The mutation m.3460G>T results in the exchange of an alanine for a threonine, at the position 52 (p. A52T) in the ND1 subunit of CI. The mutation m.14484T>C results in a substitution of a methionine for a valine, at the position 64 (p.M64V) in the subunit ND6 of the same complex. All these changes in amino acids result in missense mutations in three different subunits of CI (Bi, 2002; Carelli, 2002; Carelli *et al.,*2004; Giordano *et al.,*2014). These three mutations, described in **Table I.ii**, are considered the primary LHON mutations, since they are present in 90% to 95% of LHON patients, with mutations identified (Yu-Wai-Man *et al.,*2011). The most common point mutation is m.11778G>A, which accounts for 70% of all cases (Newman, 2005). The m.14484T>C and m.3460G>T mutations account for approximately 14% and 13%, respectively (Fraser *et al.,*2010; Yu-Wai-Man *et al.,*2011).

**Table I. ii. LHON top three mutations.** This table briefly describe the primary pathogenic mutations associated with LHON phenotype (adapted form (Carelli *et al.,*2004) and 3.www.mitomap.org/MITOMAP/MutationsLHON). Het- Heteroplasmy; Hom- Homoplasmy.

| Mutation | Gene | Substituted amino acid | Het | Hom | Phenotype | Features | References |
|---|---|---|---|---|---|---|---|
| m.3460G>A | *ND1* | A52T | Yes | Yes | Most severe | Lower rates of recover | Huoponene *et al.,*(1991); Howell *et al.,*(1991) |
| m.11778G>A | *ND4* | R340H | Yes | Yes | Most common | Interme-diate rates of recover | Wallace *et al.,*(1988) |
| m.14484T>C | *ND6* | M64V | Yes | Yes | Milder | High rates of recover | Mackey and Howell (1992); Johns et al. (1992) |

Unlike the majority of mitochondrial diseases that are frequently associated with heteroplasmic mtDNA mutations, LHON is often caused by homoplasmic primary point mutations, which are present in both symptomatic patients and asymptomatic carriers (Wallace *et al.,*1988; Grazina *et al.,*2007). Reports have shown that heteroplasmy cases have lower prevalence (Piotrowska *et al.,*2015).

The severity of biochemical impairments occurring in LHON depends on the CI activity. Cells carrying one of the three most common primary mutations associated with LHON showed severe decrease of CI activity for m.3460G>A, intermediate for m.11778G>A and milder for m.14484T>C (Michael D. Brown *et al.,*2000). In conclusion, m.3460G>A is related with the most severe phenotype and m.14484T>C with the milder one, being visual recovery more probable in the last case (Tońska *et al.,*2010).

The mtDNA haplogroups are genetic backgrounds with specific mitochondrial polymorphisms that are segregated together, which also seems to be important factors in LHON phenotype evolution. The m.11778G>A and m.14484T>C have a strong association with the European haplogroup J, whose combination of mtDNA polymorphisms typical from 2 subgroups of this haplogroup was shown to increase the risk of visual loss. On the other hand, m.3460G>A is known to increase the risk of vision loss when associated with haplogroup K (Piotrowska *et al.,*2015). There is also evidence of low penetrance when the three previously mentioned mutations are present in subjects from the haplogroup H (Howell *et al.,*2003). Taken together, these evidences indicate that specific mtDNA polymorphisms are modulating LHON phenotype (Piotrowska *et al.,*2015).

Considering the bigenomic interaction, some studies have been reported with the aim of identifying nuclear genes involved in the variable penetrance of mtDNA mutations in LHON. In 2010, it was identified a region on chromosome 3 (3q26.2-3q28) that may be related to visual loss in patients carrying the primary mutations associated to LHON, including *SLC7A14*, *MFN1*, *MRPL47*, *MCCC1*, *ADR* and *OPA1* genes (Phasukkijwatana *et al.,*2010). The *OPA1* gene has received more attention. This gene, which encodes a dynamin-related protein target to mitochondria, presents four mutations including missense and frameshift types, associating mitochondria with RGCs pathophysiology (Delettre *et al.,*2000). Variations in the *MAPT* that is sited on chromosome 17, encoding a tau protein, are not only associated with various neurodegenerative diseases like Alzheimer's, but also with impairment in MRC function. Hence, it is thought that alterations in this gene may increase the risk of blindness in carriers of LHON mutations. However, the relationship between these genetic factors (either Ndna and/or mtDNA, related) was not yet clarified (Hudson *et al.,*2011).

Interestingly, LHON presents gender differences in outcome, since 50% of men and only 10% of women carrying one of the top three primary mutations develop visual loss. This higher predominance of LHON phenotype in males cannot be explained by the mitochondrial inheritance. Summing up, to develop a LHON phenotype it the presence of one of the three top mutations is required. However, some carriers of top mutations do not develop LHON phenotype, proving that this is necessary but not a determinant factor (Yen *et al.,*2006; Piotrowska *et al.,*2015). These evidences reinforce the incomplete "penetrance" of mtDNA mutations, suggesting that there are other genetic mitochondrial or nuclear factors related to epigenetic regulation, and even environmental factors that may be involved in the alteration on the phenotypic expression of LHON (Kerrison *et al.,*2000; Yen *et al.,*2006).

Therefore, a possible explanation for major incidence of LHON in males than females could be related with an interaction between an X-linked locus and a specific recessive LHON mutation leading to a phenotype of blindness. However, there is still no evidence supporting this hypothesis. Another explanation for the gender differences in clinical LHON manifestation is attributed to hormonal factors, since female hormones may play a protective role. Some studies proved that 17β-estradiol administration reverses the excessive production of ROS that consequently leads to apoptosis, associated to LHON mutations. Since the RGCs have a high number of estrogen receptors, it could highlight a strategic target in the LHON treatment (Piotrowska *et al.,*2015).

The influence of environmental factors in LHON “penetrance”, triggering the pathological features in previously unaffected carriers of mutation, remains controversial. Tobacco smoking and alcohol consumption are the most probable risk factors, since recent studies demonstrate that they are important trigger factors for typical and late-onset LHON (Dimitriadis *et al.,*2014). The exposure to a variety of less common toxics, non-controlled diabetes, and pharmaceutical agents (as ethambutol) can also interfere with mitochondrial metabolism (Carelli *et al.,*2004; Dimitriadis *et al.,*2014; Sadun *et al.,*2003, 2011). **Figure 1.13** summarizes the hypothetical pathways that may explain the optic nerve degeneration occurring in LHON patients.

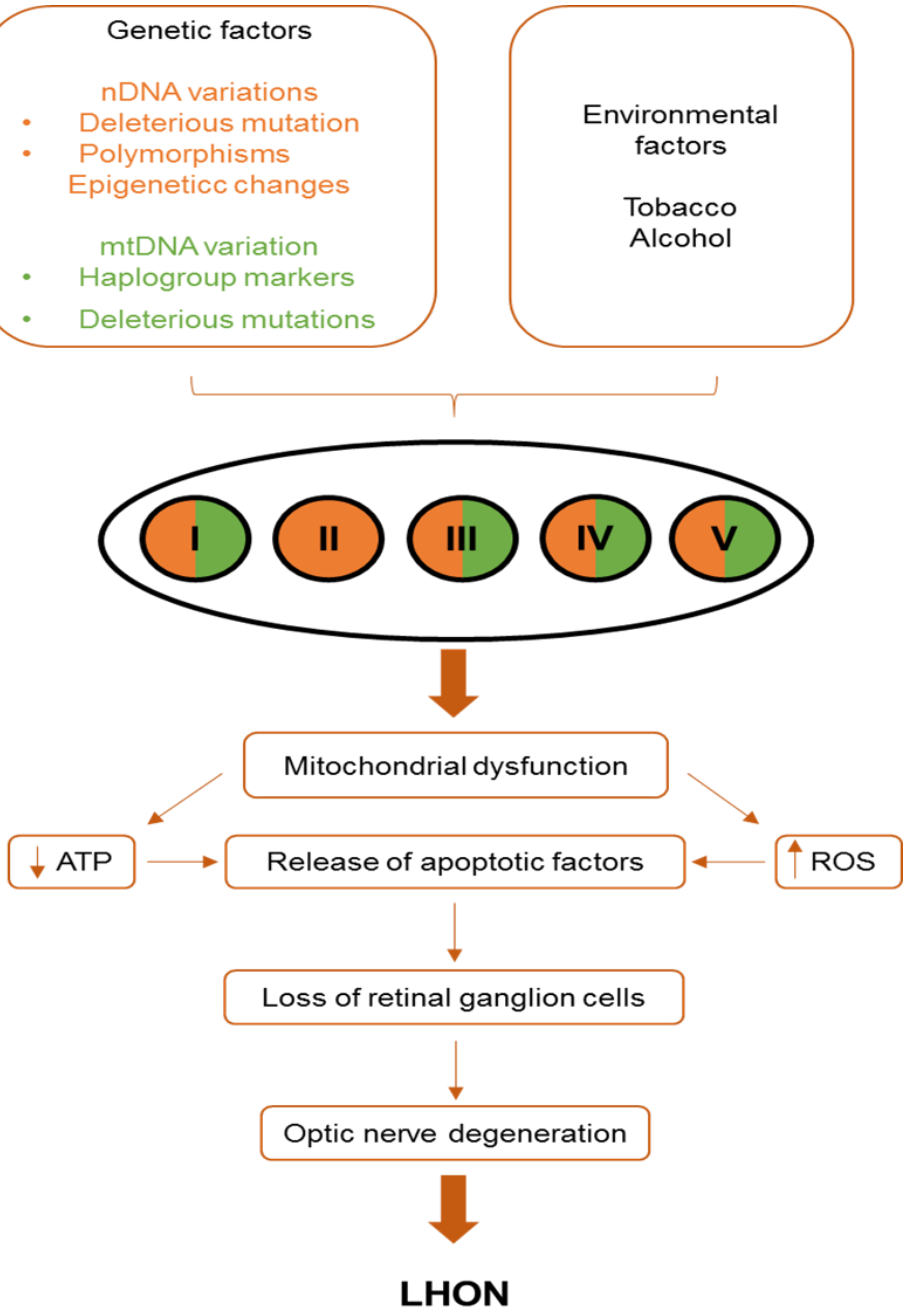



**Figure 1. 13. Schematic representation of the possible mechanism leading to the optic nerve degeneration occurring in LHON** (Adapted from Koilkonda & Guy, 2011).

## 1.6. Blue-Native Polyacrylamide Gel Electrophoresis (BN-PAGE)

Initiated in the early 1990s by Schagger and colleagues, Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) has emerged as the favoured technique to examine the assembly and abundance of OXPHOS complexes within the

MIM (H Schägger & von Jagow, 1991; Hermann Schägger *et al*.,1996). This method has higher resolution than sucrose density gradient centrifugation or gel filtration. The hydrophobic proteins and membrane protein complexes can be isolated by mild solubilization with non-ionic detergents. A negative charge is imposed by the binding of the Coomassie brilliant blue G-250 dye to hydrophobic domains on the proteins surface, which also reduces the problem of protein aggregation (Schagger *et al*.,1994). The Coomassie introduces a negative charge-shift that enhances the migration of the proteins in a gradient native gel system (Schägger & von Jagow, 1991). Solubilization of membrane proteins with nonionic detergents is often poor at low ionic strength; so 6-aminocaproic acid, that helps keeping the protein complexes intact upon solubilization and electrophoresis, should be used in substitution of salts, which may lead to precipitation of Coomassie dye and stained protein (Schagger and von Jagow 1991; Schagger *et al*.,1994). The solubilization conditions must be stringent enough to disrupt lipid-lipid interactions, while mild enough to preserve protein interactions within complexes as well as some structural lipid-protein interactions. Therefore, only mild detergents such as n-Dodecyl maltoside (Schägger & von Jagow, 1991), Triton X-100 (Poetsch *et al*.,2000), and digitonin (Schägger & Pfeiffer, 2000) are suitable for the analysis of membrane protein complexes (Krause, 2006).

Electrophoresis under native conditions subsequently allows an optimal separation and resolution of protein complexes according to their molecular mass, i.e in their native form, by the use of a correct gradient of a polyacrylamide. Normally a polyacrylamide gradient of 5%-15% is used (Nijtmans *et al*.,2002; Calvaruso *et al*., 2008; Wittig & Schägger, 2008).

The supramolecular assemblies that are removed after unidimensional BN-PAGE can additionally be analyzed by two-dimensional BN-PAGE/ SDS-PAGE, in order to obtain more information about the assembly state of the affected complex, the nature of the dysfunction and the specific affected subunit (Calvaruso *et al*.,2008; Nijtmans *et al.*,2002).

The separated proteins, complexes I, II, III, IV and V, can then be detected in the gel by Comassie brilliant blue staining or by staining monoclonal antibodies when transferred to nitrocellulose membrane/PVDF after electrophoresis.

## AIMS OF THE THESIS

Since LHON is one of the most common mitochondrial cytopathies and presents typical clinical manifestations, it is necessary to better understand the pathogenic mechanisms, underlying the disease, that remain poorly known.

The present study aimed to highlight the molecular mechanisms behind the MRC assembly in LHON, providing new insights on the MRC basic research that can be used as additional diagnosis tools for clinicians. Therefore, the main goals of the work were to:

- Analyse the status of the activity of each MRC complex through a biochemical approach;
- Contribute to the implementation of BN-Page in LBG;
- Study the assembly of MRC complexes in fibroblasts from LHON patients (with and without the m.11778 G>A mutation);
- Search for pathogenic alterations in assembly factors of MRC through the exome analysis;
- Investigate the correlation between genotype and phenotype data in LHON.

# CHAPTER 2

## EXPERIMENTAL WORK

# INVESTIGATION OF MRC COMPLEXES IN FOUR LHON PATIENTS – HETEROGENEITY OF GENETIC BACKGROUND

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## 1. INTRODUCTION

Mitochondria are organelles from eukaryotic cells whose major function is to produce ATP through oxidative phosphorylation (OXPHOS). This metabolic pathway is carried by mitochondrial respiratory chain (MRC) complexes in mitochondrial inner membrane (MIM) which is organized in five enzymatic complexes The first four complexes and two electron carriers are responsible for the electrons transference from the reducing equivalents (NADH and $FADH_2$) to the final acceptor ($O_2$) (Rötig & Munnich, 2003; Campbell *et al.,*2008; Mimaki *et al.,*2012). The energy released by this sequential transference of electrons is used to create a $H^+$ gradient across the MIM (Wallace, 2010) and to the $H^+$ re-entry into mitochondrial matrix through complex V (CV), which uses the energy of the electrochemical gradient to catalyse the formation of ATP (Dimauro & Schon, 2003; Zeviani & Di Donato, 2004; DiMauro & Hirano, 2005; Campbell *et al.,*2008).

The MRC presents peculiarities at structural and genetic levels. From a structural perspective, the majority of the MRC is contained in the MIM. On the genetic level MRC is unique as it is encoded by two physically and functionally separated genomes, the nuclear genome (nDNA) and the mitochondrial genome (mtDNA) (Zeviani & Di Donato, 2004; DiMauro & Hirano, 2005). Among the approximately 100 different subunits that compose the complexes of MRC, 13 of them are encoded by the mtDNA, being the remaining ones encoded by the nDNA (Rötig & Munnich, 2003; Lloyd & McGeehan, 2013). Thus, it is expected that mutations in any gene essential to the integrity and function of MRC may impair mitochondria activity (Wong, 2010). Besides complexes subunits, the nDNA also encodes proteins that despite not being structural elements in the final MRC complexes have a crucial role in their correct assembly mechanism, namely the assembly factors. Pathogenic mutations in assembly

factors have been already described in mitochondrial cytopathies, such as LS. Mitochondrial cytopathies, or OXPHOS disorders, are a heterogeneous group of disorders characterized by different biochemical, genetics and clinical features. The majority of the mitochondrial cytopathies are associated with the impairment of at least one of the MRC complexes, which are due to primary mtDNA mutations as well as nDNA defects affecting key components of the mitochondrial machinery (i.e., complex subunits, assembly factors and others). The optic neuropathies, such as Leber's hereditary optic neuropathy (LHON) and autosomal-dominant optic atrophy (DOA), are mitochondrial cytopathies characterized by visual failure, which can represent a severe impact on the quality of patients' life (Khanna & Saneto, 2013).

LHON (OMIM #535000) is a rare, neurodegenerative, maternally inherited, mitochondrial disease, firstly described in 1871 by Theodor Leber (Leber, 1871). It is mainly characterized by an acute or subacute painless, bilateral loss of central vision and also by dyschromatopsia (fading of colors), central scotoma and severe reduction of visual acuity (Carelli *et al.,*2004; Kirkman *et al.,*2009; Koilkonda & Guy, 2011; Sadun *et al.,*2011; Yu-Wai-Man *et al.,*2009, 2011)**.** The symptoms affect simultaneously both eyes or with a time lapse from days to months, and rarely years (Yen *et al.,*2006; Koilkonda & Guy, 2011). Young males represent almost 80% of the cases and the onset of visual loss is between 15 and 35 years old (Yo) (Yen *et al.,*2006).

In the 80's, Wallace *et al*. described a point mutation associated with LHON affecting the CI, m.11778G>A (Wallace *et al.,*1988). Presently, this disorder is associated with three primary point mutations in mtDNA genes encoding CI subunits, the top mutations, present in 90% to 95% of LHON patients (Yu-Wai-Man *et al.,*2011): *MTND1* m.3460G>A, *MTND6* m.14484T>C, and the previously referred *MTND4* m.11778G>A that accounts for 70% of all cases being the most common (Newman, 2005). The presence of one of the three top mutations is required to present LHON phenotype, however some carriers do not develop the phenotype, proving that it is not a determinant factor (Yen *et al.,*2006; Piotrowska *et al.,*2015). As an evidence of the incomplete "penetrance" of mtDNA mutations, it suggests other factors as genetics, epigenetics, and even environmental factors as possibly being involved in the different phenotypic expressions of LHON (Kerrison *et al*.,2000; M.-Y. Yen *et al*.,2006). However, to fully understand this incomplete "penetrance" further investigation is necessary, for instance gender bias, age of onset or sequential loss of vision in both eyes.

Altogether, these evidences lead to the hypothesis of a crosstalk between alterations in the assembly of MRC complexes and mutations in MRC assembly

factors, which may be related to the disclosure of LHON phenotype, as well as its severity. This severity depends mainly on the CI activity, whose subunits are encoded by both mtDNA and nDNA. However, there are already mutations in other complexes subunits described as associated with LHON phenotype. In order to clarify this question, LHON patients with a primary mutation will be compared with individuals suspected of LHON diagnosis that do not carry a primary mutation. The present study aims to found correlations between genetics and functional impairment of MRC assembly in LHON.

## 2. METHODS

### 2.1. Biological Samples

The study population included four patients (Caucasians; one female and three males; onset age: patient 1 – 8 mo; patient 2 – 55 Yo; patient 3 – 30 Yo; and patient 4 – 40 Yo), followed at the Neurology Unit of the Centro Hospitalar e Universitário de Coimbra, suspected of LHON. Patients 1 and 2 have the LHON primary m.11778 G>A mutation. At baseline, a neurologist completed a medical history with the patient and the caregiver and conducted a general physical, neurological and psychiatric examination. A control population of two healthy Portuguese individuals without clinical evidence of a mitochondrial disorder (Caucasians; 1 female and 1 male; 11 Yo and 16 Yo, respectively), was included for setting reference values in our sample. This study was conducted during the diagnostic investigation of the genetic cause of the disease and informed consent was obtained from the participants or their legal representatives, as recommended by the local Ethics Committee, following the Tenets of the Helsinki Declaration.

### 2.2. Culture of human primary fibroblasts

Skin biopsies were collected in the hospital under local anesthesia and immediately placed in sterile tubes, containing Hanks balanced salt solution (HBSS) (saline solution without calcium and magnesium). Fibroblasts were grown in complete medium: Ham's F-10 (Gibco, Life Technologies) supplemented with 20% FBS (Gibco, Life Technologies), 4 Mm GlutaMAX (Gibco, Life Technologies), 2.5 mM sodium pyruvate (Gibco, Life Technologies), 65 µg $mL^{-1}$ uridine (Sigma-Aldrich, 250 U $mL^{-1}$ penicillin (Gibco, Life Technologies), 50 µg $mL^{-1}$ streptomycin (Gibco, Life Technologies), 50 µg $mL^{-1}$ kanamycin (Sigma-Aldrich), 2.5 µg $mL^{-1}$ amphotericin B

(Gibco, Life Technologies), 2% Ultroser G (Gibco, Life Technologies). Incubation took place at 37 ºC, 5% $CO_2$ and adequate humidity levels in 25 $cm^2$ flasks. Fibroblasts were used for biochemical analysis only upon reaching proper confluence. All handling was performed in aseptic conditions in a laminar flow camera using sterile instruments and reagents.

### 2.3. Total DNA extraction and DNA quantification

Total DNA was extracted from fibroblasts using standard methods (Sambrook *et al.,*1987; Moore *et al.,*1997) and quantified by UV spectrophotometry (λ = 260 nm). The quality of the samples was assessed by evaluation of the absorbance ratio at 260/280 nm using the NanoDrop™ 1000 (Thermo Fisher Scientific, Inc.).

### 2.4. Genetics Analysis

#### 2.4.1. Polymerase chain reaction and Sequencing

The target sequences containing LHON mtDNA mutations (m.3376G>A, m.3460G>A, m.3635G>A, m.3697G>A, m.3700G>A, m.3733G>A, m.4171C>A, m.10663T>C, m.11778G>A, m.14482C>A, m.14482C>G, m.14484T>C, m.14495A>G, m.14502T>C, m.14568C>T), to evaluate the LHON associated variants, were amplified by Polymerase Chain Reaction (PCR) technique using specific primers (Landsverk *et al.,*2012). The PCR technique was performed on GeneAmp® PCR System 9700 (Applied Biosystems), BiometraTProfessional Family, and C1000™ Termalcycle (Bio-Rad) thermocyclers. The reaction was performed in a total volume of 25 µl containing 100 ng/µl of total cellular DNA, 0.2 µM of each primer (life Tecnologies, Carlsband, United States of America) sequence of primers in (Landsverk *et al.,*2012), 0.09 mM MgCl2 (Thermo Scientific), 0.12 mM dNTPs (GE Healthcare Life Sciences, Upssala, Sweden), 0.04 U Taq Polymerase (Thermo Scientific, Fermentas), 1x complete Taq DNA polymerase Buffer (Thermos Scientific) and MilliQ water. The cycling conditions were an initial denaturation at 95 °C for 5 minutes followed by 35 cycles at 95 °C for 45 seconds, 50-60 °C for 45 seconds, 72 °C for 30 seconds and a final extension step of 72 °C for 5 minutes. In order to confirm the presence of the amplification product, it was run on an agarose gel stained with ethidium bromide. Detection of bands was carried out using the ChemiDoc $XRS^+$ Imaging System (Bio-Rad).

Automated sequencing analysis was performed according to the manufacturer's instructions (3130 ABI Prism sequencing system), using BigDye® Terminator Ready Reaction Mix v3.1 (Life Technologies) and specific primers for target genes. All

sequences were analyzed using Sequencing Analysis v5.4® and SeqScape v2.5® software (Applied Biosystems, Foster City, Calif., USA), by comparison with a reference sequence obtained from the GenBank database.

### 2.4.2. Next Generation Sequencing

Next Generation Sequencing (NGS) analysis was performed previously, in the scope of the project (PTDC/DTP-EPI/0929/2012) according to the following procedure: exome sequencing was done using the Nextera Exome Capture methodology - Illumina Nextera Rapid Capture Exome v1.2, PE100 from Illumina, totalizing around 200 thousand exons, followed by sequencing accomplished using the Illumina Hiseq2500 equipment. After obtaining the raw data, CLC Bio Genomics Workbench 8.0 was used for performing quality control, trimming alignment to reference and variant calling. Data analysis was performed using the software Ingenuity® Variant Analysis (IVA).

## 2.5. Protein Analysis

### 2.5.1. Spectrophotometric bioanalytical evaluation

Human primary fibroblasts were pelleted from six T25 culture flasks, washed twice with PBS prior to resuspension in the same buffer and storage at -80 ºC for 8 days. Ended this standardized period of time, samples were submitted to two freeze-thaw cycles. The spectrophotometric determination of the catalytic activity of the MRC complexes (I-V) and segments was performed as described elsewhere using a SLM AMINCO DW2000 UV-VIS spectrophotometer (M. M. Grazina, 2012). The determination of the activity requires the addition of a reaction substrate and an inhibitor, as follows: Complex I - NADH and ubiquinone, rotenone (λ=340-380 nm); Complex II - succinate and ubiquinone, malonate (λ=600-750 nm); Complex III - ubiquinol, antimicine A (λ=600-750 nm); Complex IV - reduced cytochrome c, cyanide, KCN, (λ=550-540 nm); Complex V - ADP, (λ=412-600 nm). This assay also includes a measuring of the combined activity of segments I+III and II+III, helpful in detecting a deficiency in ubiquinone. The activity of CIII depends not only on the capacity of CI or CII to oxidize their substrates and transfer them to ubiquinone, but also on the quantity of this mobile electron transporter. The enzymatic activities of all respiratory complexes and segments were normalized for total protein mass, determined by the Bradford method, at λ= 595 nm, and citrate synthase activity for normalizing variations in mitochondrial content. All measurements were performed at 37 ºC using a temperature-controlled cuvette jacket. A deficiency is considered when activity is below

40% of the reference mean value normalized to citrate synthase (M. M. Grazina, 2012). Values are presented as means±SEM.

### 2.5.2. Preparation of mitochondria - enriched fractions from cultured cells

Samples were processed according to the protocol previously described (Calvaruso, *et al.,*2008). Briefly, human primary fibroblasts were grown to confluence, harvested from the culture flasks and centrifuged at 800xg at 4 ºC for 5 minutes. The supernatant was removed and the pellet was washed twice with cold PBS and centrifuged at 287xg at 4 ºC for 5 minutes. After sedimentation of the mitochondria crude, the supernatant was discarded and the cell-pellet resuspended in cold PBS to a concentration of ~106 cells/100 µL. Then the total samples were solubilized by incubation with digitonin (4 mg/mL in PBS) at 4 ºC for 10 minutes. Following centrifugation at 12,000xg at 4 ºC for 10 minutes, the supernatant was removed and the pellet snap freezed in liquid nitrogen and stored at -80 ºC.

### 2.5.3. Protein Quantification in mitochondrial - enriched fractions

The crude mitochondrial fraction was resuspended in 100 µL of ACBT (75 mM Tris–glycine, 1.5 M aminocaproic acid, adjusted to pH 7.0 with HCl at 4 ºC). After the addition of 20 µL of 10% β-dodecyl maltoside, the cell suspension was left on ice for 10 minutes. The cells were centrifuged at 12,000xg at 4 ºC for 30 minutes, the mitochondrial fraction was transferred to a new eppendorf and the total protein was quantified by Bradford assay, as referred earlier by spectrophotometry (SPECTRmax Plus 384, Molecular Devices). Finally, 10 µL of BN-Sample buffer [750 Mm aminocaproic acid, 50 mM Tris–Glycine/HCl, pH 7.0, 0.5 mM EDTA and 5% Coomassie brilliant blue G250 (Serva blue G, Serva)] were added (Calvaruso, *et al.,*2008).

### 2.5.4. Blue Native – Page and Western Blot

This experimental protocol was performed as described (Calvaruso, *et al.,*2008). For all the samples, 30 µg of total protein was loaded in the gel. One anode buffer (50 mM Tris-glycine, pH 7.0) and two cathode buffers, deep blue cathode buffer A (15 mM Tris-glycine, 50 mM tricine, 0.02% Coomassie Blue G-250, pH 7.0) and cathode buffer B (15 mM Tris-glycine, 50 mM tricine, pH 7.0), were used. A molecular weight marker (NativeMARK Unstained Protein Standard, Life Technologies) and

samples were loaded into polyacrylamide gels (4-16% Mini-PROTEAN TGX Precast gel, Bio-Rad). First, proteins migrate at 60 V, 4 ºC, until the running front reached 1/3 of the total running length, with blue cathode buffer A. After that, the cathode buffer A was replaced by cathode buffer B, the run extends for 4 h at 80 V, 4 ºC. Proteins were transferred to a PVDF membrane (Hybond P 0.5 µm, Amersham) for 2 h at 200 mA, 4 ºC. Blocking was carried out using 5% skimmed milk in TBS-T (50 mM Tris, 150 mM NaCl, 0.1% Tween-20) for 1 h, at room temperature. Afterwards, the membrane was incubated with monoclonal primary antibodies [Complex I (CI) subunit NDUFA9 (Mouse mAb, Abcam, 1:1000, ab14713); Complex II (CII) subunit SDHA (Mouse mAb, Abcam, 1:5000, ab14715); Complex III (CIII) subunit UQCRC2 (Mouse mAb, Abcam, 1:1000, ab14745); Complex IV (CIV) subunit COX IV (Mouse mAb, Abcam, 1:1000, ab14744); and Complex V (CV) subunit ATP5A (Mouse mAb, Abcam, 1:1000, ab14748)] overnight, at 4 ºC. After washing membranes in TBS-T, they were incubated with secondary antibody (α- mouse, Abcam, 1:5000), for 60 min at room temperature. After electrophoretic separation of proteins by BN-PAGE and western blot, MRC complexes were visualized and the qualitative and quantitative analysis was performed. Optic densitometry analysis of bands corresponding to complexes I-V from fibroblasts samples was performed. In order to guaranty the assembly pattern quality, controls' samples were used to comparison with the patients' samples. Subsequently, detection was carried out using a chemiluminescence substrate (Clarity Western ECL Substrate, Bio-Rad), through the ChemiDoc$^{TM}$XRS$^{+}$ System (Bio-Rad). Protein band intensities were calculated by Quantity One® 1-D software (Bio-Rad) from at least 3 independent experiments, with values expressed as means±SEM. Quantification of BN-PAGE MRC complexes was performed in relation to CII levels.

### 2.6. Statistical analysis

Statistical analysis of the results was performed using GraphPadPrism®Software v.6 (San Diego, California, USA), www.graphpad.com. After the investigation of Gaussian distribution, the non-parametric Mann-Whitney test was selected. Significance is considered when $p < 0.05$ (* for $0.050 \geq p > 0.010$, ** for $0.010 \geq p > 0.001$ and *** for $p \leq 0.001$).

## 3. RESULTS

### 3.1. Genetic mtDNA analysis in fibroblasts

From the genetic analysis of the mtDNA it was possible to verify that patient 1 and patient 2 present the m.11778G>A mutation in homoplasmy located at the *MTND4* gene. The other two patients of this study, 3 and 4, do not present any primary mutation associated to LHON. The patients' mtDNA sequencing also revealed polymorphic alterations. The present results showed the existence of two patients with the primary mutation m.11778G>A and the other two without any primary mutations, allowing a comparison between the two groups in study, both compared to controls.

### 3.2. Study of the MRC complexes activity

The graphics in **Figure 2.1.,** illustrate the enzymatic activity of each complex in the four patients (**A-D**).

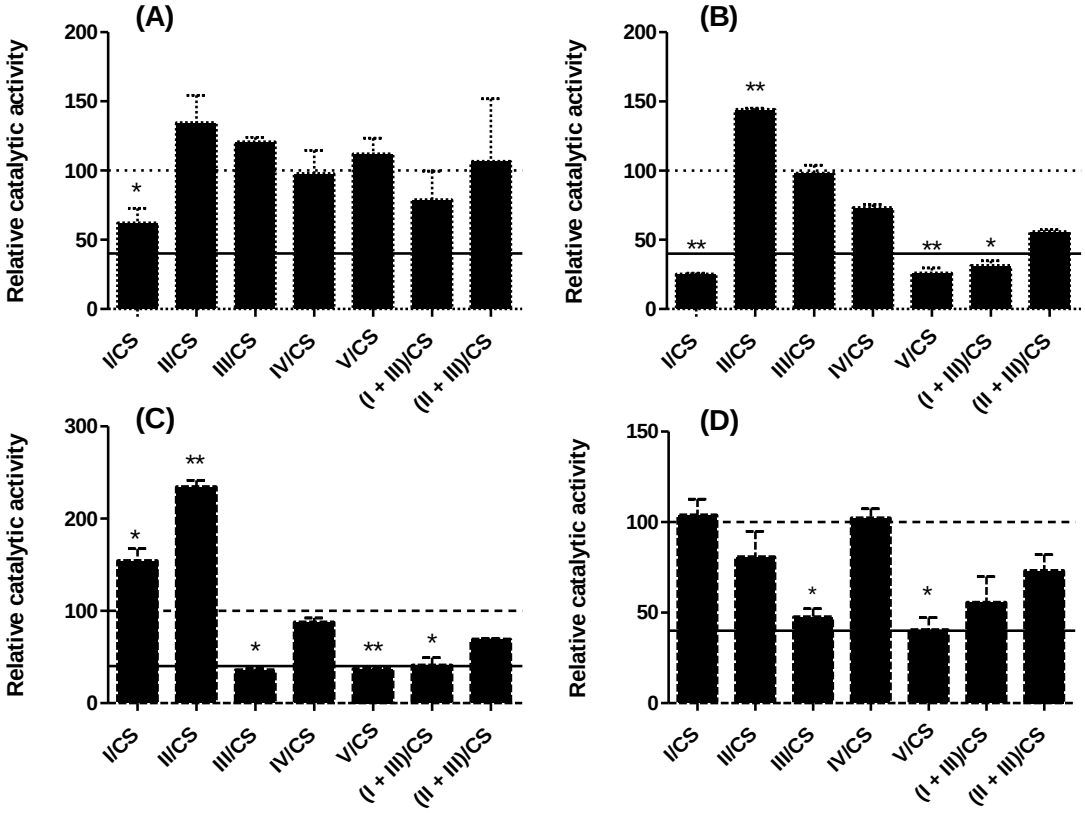



**Figure 2. 1. Spectrophotometric determination of the MRC catalytic activities in fibroblasts. (A)** Patient 1. **(B)** Patient 2. **(C)** Patient 3. **(D)** Patient 4. Values were normalized to citrate synthase (CS) and are presented as the mean of at least three experiments (Control 1: n=5; Control 2: n=5; Control 3: n=4 (III/CS: n=3); Patient 1: n=3; Patient 2: n=3; Patient 3: n=3; Patient 4: n=3, with the correspondent error bars. Values of the controls were standardized to

100% and are presented as a black dashed line; the individuals in study are represented as black bars; the black line represents the threshold for differences below 40%. Statistical significance of results compared to controls: * $p<0.05$, ** $0.010 \geq p > 0.001$; Mann Whitney test.

The analysis of the MRC activity shows that patients 1 and 2 present a significantly decreased of CI (respectively, p= 0.0376 and p= 0.0098). Patient 3 presents a significantly increase of CI (p= 0.0198). Also, the activity of complex II was significantly reduced in both patients 2 and 3 (p= 0.0098).

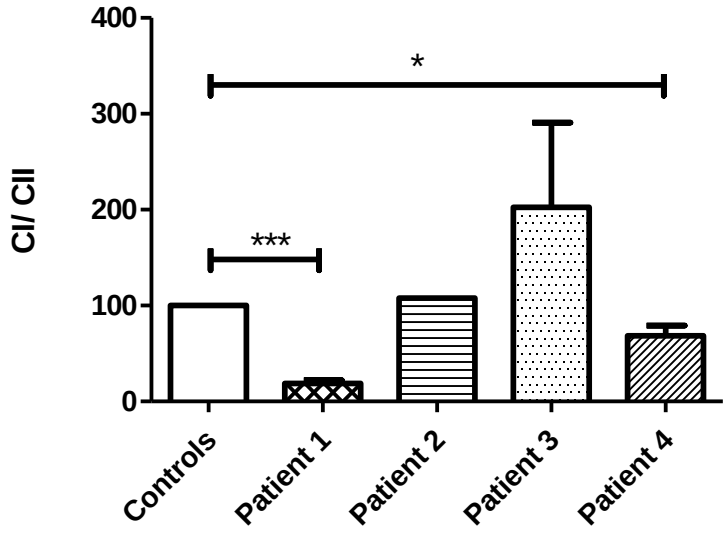
Complex III presented a shift in the activity, being decreased in patients 1 and 4 and significantly reduced in patient 3 (p= 0.0314). The CIV showed a normal activity in all patients. From the CV activity analysis, a significant decrease in both patients 2 and 3 (both with p= 0.0098), and 4 (p= 0.0198) was noticed.

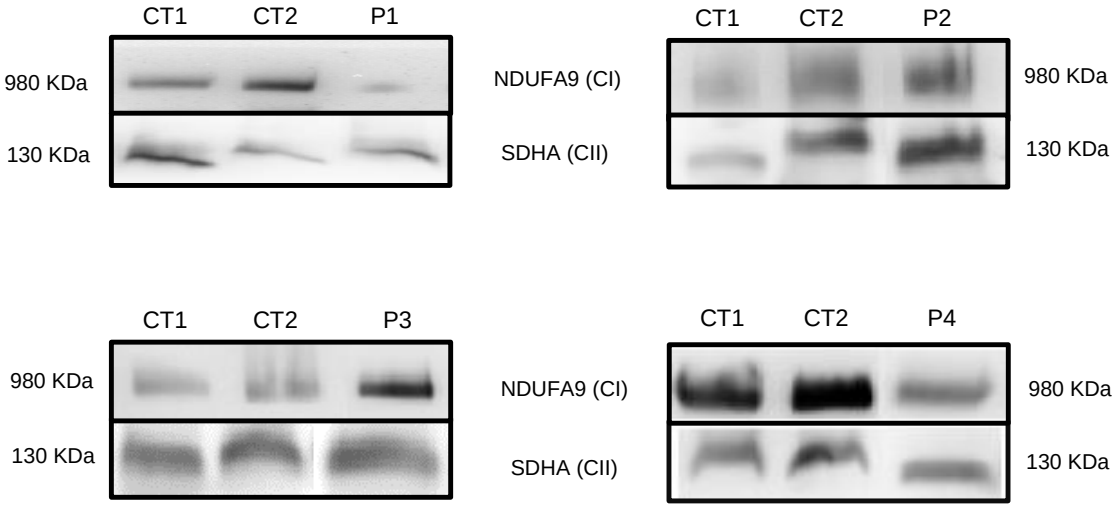
The examination of the activity for CI+CIII revealed a statistically significant decrease in activity for patients 2 and 3 (p= 0.0314), whereas for CII+CIII combined activity it was not detected any significant alteration.

### 3.3. Study of MRC complexes assembly

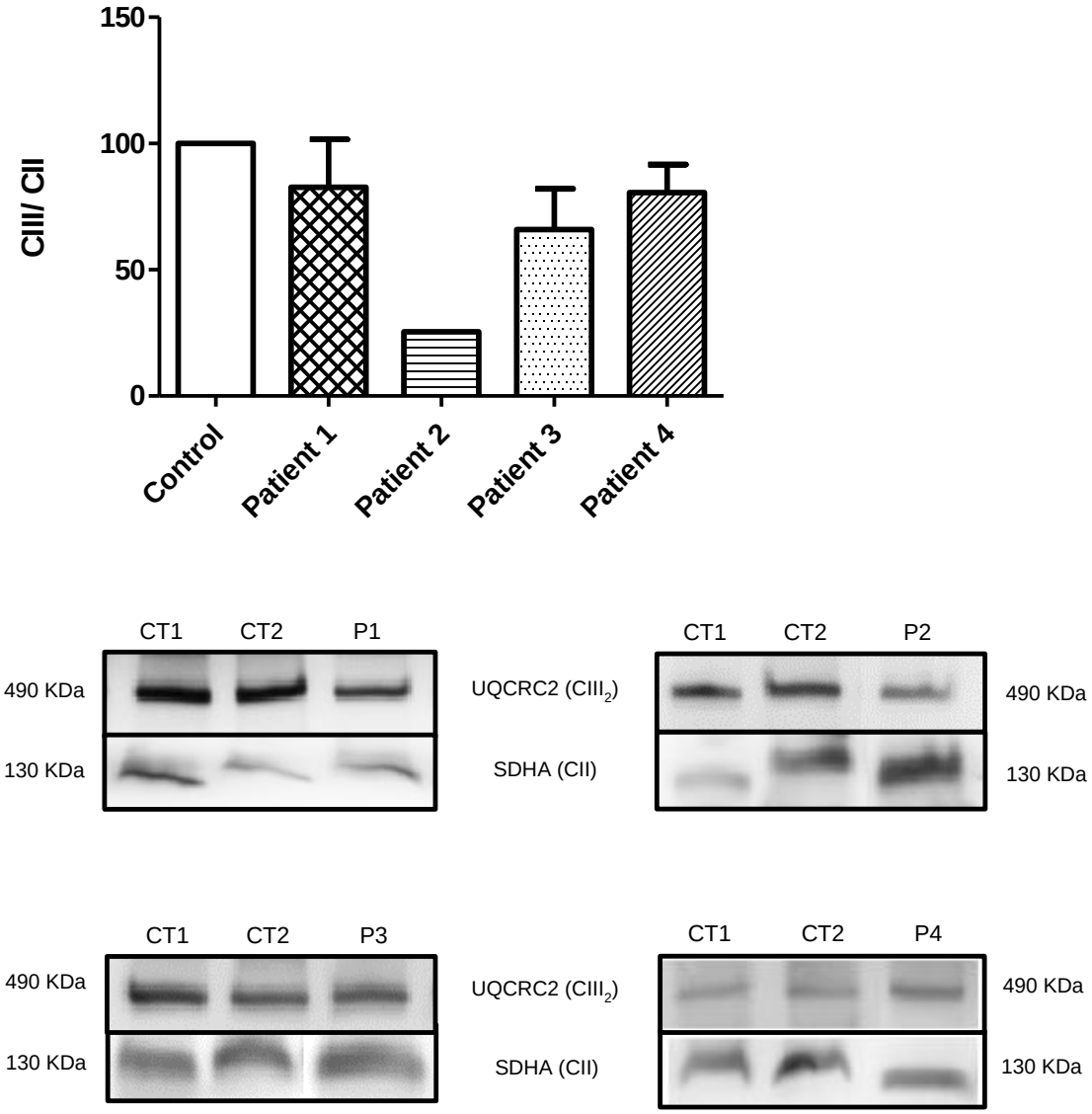
The **Figure 2.2.,** shows the results for the assembly of MRC complexes in the patients with clinical evidence of LHON. It is possible to notice that the CI significantly decreased (**Figure 2.2. (A)**) in patients 1 and patient 4 (respectively, p= 0.0005 and p= 0.0188), indicating an assembly defect of CI in those patients. The quantification of CIII and CIV revealed that, although diminished, it is no statistical significant (**Figure 2.2. (B)** and **(C),** respectively). Finally, concerning CV, it was possible to find a statistically significant decrease for patient 4 (p= 0.0027) (**Figure 2.2 (D)**).

**(A)**

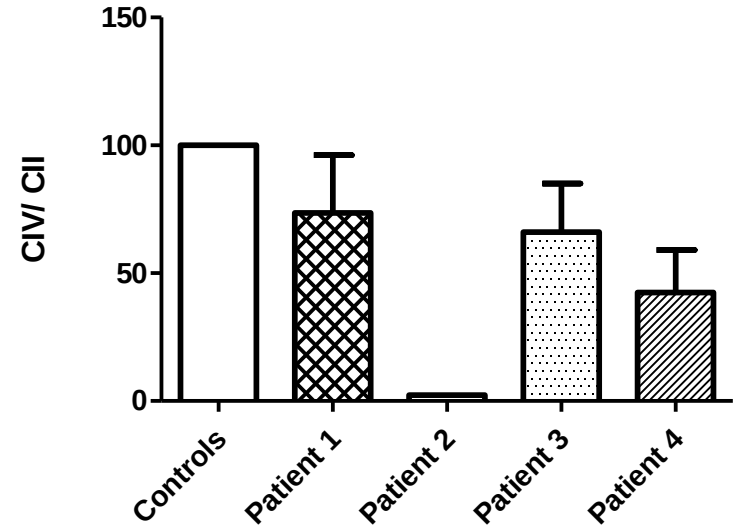

CI/ CII
400
300
200
100
0
*
***
Controls
Patient 1
Patient 2
Patient 3
Patient 4

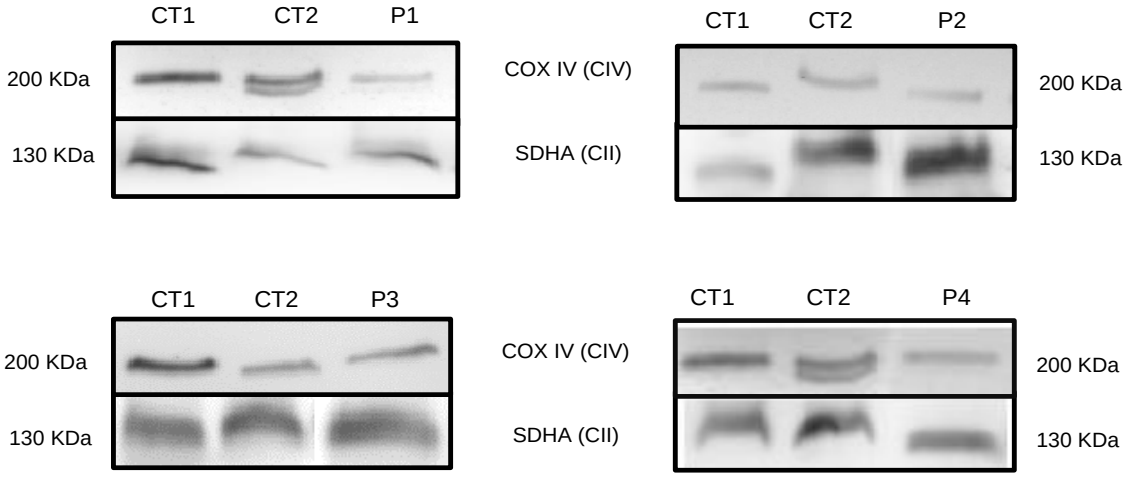

CT1
CT2
P1
980 KDa
130 KDa
NDUFA9 (CI)
SDHA (CII)
CT1
CT2
P2
980 KDa
130 KDa
CT1
CT2
P3
980 KDa
130 KDa
NDUFA9 (CI)
SDHA (CII)
CT1
CT2
P4
980 KDa
130 KDa

**(B)**


150
100
50
0
CIII/ CII
Control
Patient 1
Patient 2
Patient 3
Patient 4
CT1
CT2
P1
490 KDa
130 KDa
UQCRC2 (CIII2)
SDHA (CII)
CT1
CT2
P2
490 KDa
130 KDa
CT1
CT2
P3
490 KDa
130 KDa
UQCRC2 (CIII2)
SDHA (CII)
CT1
CT2
P4
490 KDa
130 KDa

**(C)**


150
100
50
0
CIV/ CII
Controls
Patient 1
Patient 2
Patient 3
Patient 4


CT1
CT2
P1
200 KDa
130 KDa
COX IV (CIV)
SDHA (CII)
CT1
CT2
P2
200 KDa
130 KDa
CT1
CT2
P3
200 KDa
130 KDa
COX IV (CIV)
SDHA (CII)
CT1
CT2
P4
200 KDa
130 KDa

**(D)**

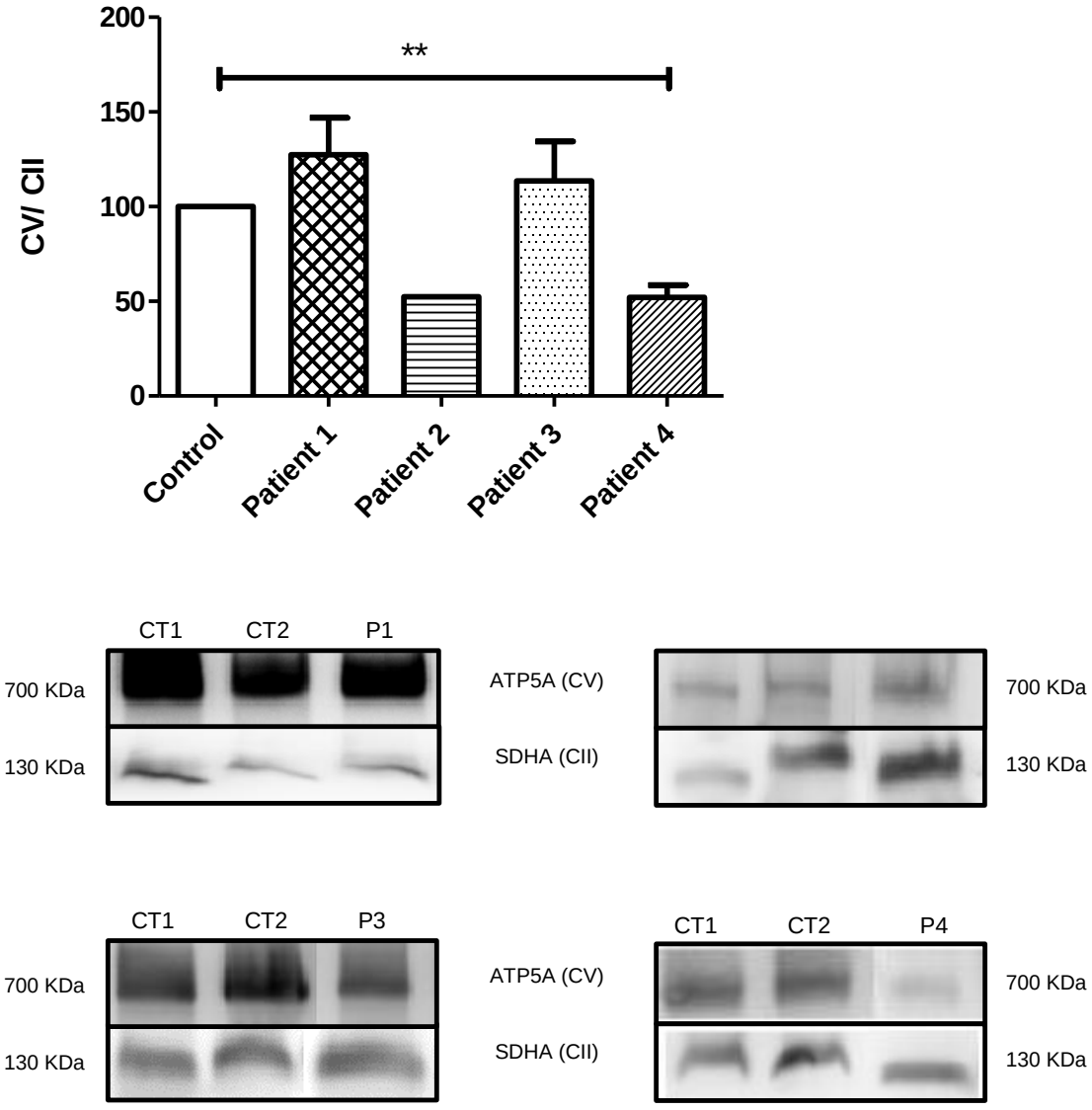



**Figure 2. 2. Qualitative and quantitative study of MRC complexes by BN-PAGE.** Representative blots after perform BN-PAGE with fibroblasts samples (from controls and LHON patients with and without the m.11778 LHON mutation). Each complex was identified with the antibody and the corresponding molecular weight marker. BN-PAGE analysis of NDUFA9 **(A)**, UQCRC2 **(B)**, COX IV **(C)**, and ATP5A **(D)** in patients 1, 2, 3, and 4 and in controls n = 3; except for patient 2, n=1. Data are expressed as means ± SEM; * p< 0.05, ** 0.010 ≥ p > 0.001, *** p≤ 0.001, Mann Whitney test.

### 3.4. Exome Analysis for Assembly factors – Next Generation Sequencing

In order to identify alterations in MRC assembly factors, NGS technique was performed to analyse the patients' exome. Due to time limitations only the genes

coding for MRC assembly factors were selected for analysis, but the results are in study, although one patient may have a promising alteration.

## 4. DISCUSSION

In the mitochondrial cytopathies, it is very difficult to find a genotype-phenotype association in patients, because they represent a multisystemic and heterogeneous group of diseases. As previously referred, LHON is the most common illness caused by MRC defects with an estimated prevalence of 1: 45,000 in Europe (Mascialino *et al.,*2012). It was estimated that about 95% of the LHON genetic cases are due to a mtDNA primary mutation, from which 70% of the total cases are due to the *ND4* (m.11778G>A) point mutation (Man *et al.,*2003; Yu-Wai-Man *et al.,*2011).

However, as previously stated, only 13 of the ~100 of the different subunits of MRC are encoded by mitochondrial genes, being the remaining encoded by nuclear genes (Rötig & Munnich, 2003; Lloyd & McGeehan, 2013). The pathogenic mutations may occur in both nDNA and mtDNA-encoded CI subunits, or even in nDNA-encoded assembly factors (Zeviani & Di Donato, 2004; DiMauro & Hirano, 2005). Thus, it has been reported that pathogenic alterations may occur in both genomes and even in genes essential to the integrity and function of MCR, which may alter the mitochondrial structure and MRC complexes' activity (Wong, 2010). Given the higher number of nDNA coded subunits, the probability to find a mutation in one nuclear gene is much higher.

The sample of this study include four patients with clinical diagnosis of LHON, two harboring the primary LHON m.11778G>A mutation and other two patients without any associated LHON primary mutation. This study proposes the analysis of the MRC assembly factors in both groups, compared to controls and evaluating the MRC complexes activity.

Concerning the enzymatic activity of MRC complexes, it is evident that for patients 1 and 2, with the m.11778G>A mutation, the CI activity is significantly decreased (**Figure 2.1 (A)**). Results previously described in literature (Yu-Wai-Man, 2016) for this mutation referred a decrease in CI activity in 0% to 50% control activity, which is in accordance with the data obtained in the present study. Therefore, the obtained results were in agreement with the expected results, since they are in accordance with data previously reported for this mutation. This result can be explained having in mind the effects of the m.11778G>A in CI activity, since it is one of the 14 core subunits of the CI. From previous studies, it was shown that the point mutation

m.11778G>A (*MTND4* gene), results in a substitution of an arginine for a histidine, at position 340 (p. R340H) (Wallace *et al.,*1988), which results in a missense mutation. Both amino acids have the same properties, basic side chains positively charged at neutral pH or near; they are classified as basic polar amino acids, so apparently there would be no damage effect for CI. However, it is important to refer that contrariwise to R, H has an imidazole ring in its chemical structure that will probably enhance the loss of stability of the connections established with the surrounded subunits. On the other hand, evolutionary conservation analysis shows that mtDNA ND4 Arg340 residue is highly conserved throughout different eukaryotes. Therefore, the probability of an amino acid alteration at this position being damaging is high. In 1994, Degli Esposti and his colleagues reported that this specific amino acid substitution leads to the affinity alteration of CI for the ubiquinone substrate and induce rotenone resistance in mitochondria of LHON patients, so they have proposed that this alteration may reflect a substantial loss in the energy conserving function of CI (Esposti *et al.,*1994).

From previous research, it was already known that CI, together with CIII and CIV, maintain the proton gradient, which means that they act as proton pump being the CI the major one followed by CIII, which receives electrons from CII, thought ubiquinone. The CII transfers electrons from succinate directly to ubiquinone, but it does not contribute to the proton gradient. The driving force created by the electron transfer between complexes behind ATP-generation in the MRC via ATP synthase, leads to a proton pumping. Accordingly, dysfunctions in one of that complexes could result in a damaging impact for the MRC, and it could also conduct to oxidative stress, due to ROS overproduction (Federico *et al.,*2012).

Thus, taking into account that the CI is the major proton pump, when it is dysfunctional, it jeopardizes the correct MRC functioning. The statistical analysis of CII activity for patients 1 and 2, reveals a significant increase of the activity, which may arise from a compensatory mechanism ( Yen *et al*.,1996). In order to rescue the low input of electrons through CI, the MRC adapts to the decreased activity of CI by increasing the activity of CII, allowing an equilibrated input of electrons. The CIII activity also increases to ensure that there is a constant electron flow through the downstream complexes of the MRC. The relatively high activity of CIII, in those two patients, may also contribute to the higher efficiency of electron transport from all the available substrates, which are imputed mainly at CII. The increase in CII activity could be the result of a higher complex components expression or, most likely, *via* postranslational modification (Cimen *et al.,*2010).

In patient 2, the decreased activity of the (I+III) segment could be explained by the CI activity of 24,96% related to the controls. However, the mtDNA mutation in ND4 subunit does not explain the huge decrease in the CV activity of this patient. A reasonable explanation could be the existence of an alteration in one core subunit of this complex. It is predicted that an alteration in an essential subunit to the correct function of the complex could lead to a decrease in its activity. However, an alteration in a structural subunit could also, contribute to the incorrect assembly, for example at the dimerization level, originating a fragmented complex, which consequently leads to a reduction of the activity.

As previously described, patients 3 and 4 do not show any mitochondrial or nuclear relevant variation in the studied genes. However, patient 3 presents a statistically significant decrease in CIII activity. This complex occupies the middle position of the MRC and is the second major proton pump, so it is also important to the electrons' input into the MRC. Similarly, to what occurs for the other complexes, with the exception of CII, it has subunits encoded by both nDNA (1) and mtDNA (10). Three of the 11 subunits contain the catalytic centers: cyt b, cyt c1 and UQCRFS1. The exact function of the other eight supernumerary subunits remains to be established (Xia *et al.,*2013). So, sequence variations in catalytic center proteins may likely be an explanation to the 35.7% of activity, compared to controls. The segment (I+III) is also decreased, which can be explained with the CIII decrease, although there is a significant increase in CI activity it is not sufficient to rescue the CIII deficit.

Similarly, to the results for patient 2, patients 3 and 4 presented a significant reduction in CV activity. The explanation could be again the existence of other alterations, likely affecting protein subunits that were not analysed, contributing to the results. For instance, in 1995, Lamminen and colleagues published a case report of a boy presenting LHON phenotype associated with a *MTATP6* mutation that causes biochemical defect in OXPHOS (Lamminen *et al.,*1995).

Some of the obtained values for the MRC activity remain without explanation. Therefore, searching in exome for pathogenic sequence variations of nDNA encoded subunits would be very helpful for the understanding of the results.

The evaluation of BN-PAGE results may indicate assembly defects in MRC complexes, which demonstrates that it is very useful tool for laboratory diagnosis supporting the clinics. This is a basic technique with a focus on elucidating biochemical defects in patients with OXPHOS deficiencies, and requires small quantities of biological material.

There is a broad range of traditional methods performed to identify biochemical defects in OXPHOS function in patient's cells or tissues, such like spectrophotometry, polarography, or histochemical analysis. However, these approaches have some limitations and none of these techniques directly addresses the process by which individual structural subunits are assembled into a mature holoenzyme. In contrast, differences in both the assembly of OXPHOS complexes and their absolute content can be readily assessed by BN-PAGE, followed by conventional immunoblotting (Williams *et al*.,2001; Leary *et al*.,2004).

Nevertheless, electrophoretic separation of the complexes has some crucial tasks that should be taken in account. First, the fact that OXPHOS complexes are integral membrane proteins, they are very hydrophobic, and consequently require the addition of detergents to keep them in solution. The concentration and type of detergent used is a critical step for the outcome of native electrophoresis experiments. Second, they are multi-subunit complexes, so there is risk of partial dissociation during the electrophoresis process. Third, it is believed that the complexes are not single entities but they are organized in higher order structures called SCs (Schägger & Pfeiffer, 2000).

As previously referred this technique combines the mild properties of DDM, Triton-X and digitonin in addiction to with the use of the anionic dye Coomassie brilliant blue G250. The OXPHOS protein complexes can be visualized using dye coomassie. Although this staining is sufficient to observe protein bands from muscle or lymphocytes tissues it is not suitable for the present study, once the amount and purity of the fibroblast mitochondrial fraction is not sufficient to identify OXPHOS complexes. So, it is necessary to perform western blotting, in order to detect the individual complexes. This procedure, which involves antibody labelling, is much more specific and trustworthy than comassie staining (Calvaruso et al., 2008).

However, possibly due to the lack of epitope availability in the native complexes some subunits are poorly detected (Calvaruso *et al*.,2008; Nijtmans *et al*.,2002), which represents a setback, since for some complexes the stain is very mild, e.g. for CIV. Another difficulty was the fact that some complexes mask neighbor complexes band, e.g. for CV. Therefore, a cocktail with all the antibodies could not be used. In order to overcome this problem, complexes were labeled in pairs. The first to be marked were those with weaker tag, like CI and CIV, followed by CII and CV, and finally CIII.

After electrophoretic separation of proteins by BN-PAGE and Coomassie staining, MRC complexes can be visualized and analyzed both in terms of quality and

quantity. The qualitative evaluation of MRC complexes was performed by comparing the migration patterns of patients and controls' samples. The relative quantification of OXPHOS complexes was performed using CII as a normalizer. Although its use may be controversial, it is widely used in studies of samples showing mitochondrial dysfunction (Leman *et al*.,2015; Cherm Lim *et al*.,2016; Cruz-bermúdez *et al*.,2016). Therefore, its use allows the discussion of the present results and comparison with studies already published. Furthermore, this complex is encoded only by nDNA and although may exist mutations in genes that encode proteins involved in its assembly, they are rare in humans.

For all the reasons presented above, the implementation of this protocol in the laboratory was a long and difficult journey, since it was the first time that it was fully performed at LBG. However, and once established the optimal conditions for the protocol performing, this straightforward technique may provide considerable mechanistic insight into the molecular genetic basis of human diseases caused by mitochondrial dysfunction. It can be used as a complementary technique in clinical diagnosis. Accordingly, this was an important achievement of this thesis.

Regarding this and through quantitative analysis of CI assembly it was possible to verify that for patients 1 and 2, although having the same mitochondrial mutation, and the same profile activity for the first four MRC complexes, there are significantly assembly differences between them. The explanation may be related with the presence of an alteration in one assembly factor, which could play a role in CI assembly of patient 1.

Therefore, this result allows to suggest that mtDNA mutation by itself is not the cause of assembly impairment, being the variant found in assembly factor the most reasonable explanation for the results.

The results of CI assembly for patient 4, indicate a possible alteration in one structural subunit crucial to the maintenance of the complex integrity, since the activity of CI for this patient is normal. This patient also presents a low amount of CV enzyme. So, it is predicted that the variant is most likely in a subunit that is not only important for the correct assembly, but also for the correct catalytic functioning of CV, since both assembly and activity are significantly decreased in this patient when compared to controls. As previously described, there are mutations, already described in MITOMAP, in *MTATP6* associated with LHON phenotype (Lamminen *et al*.,1995). In these studies, a decrease of CV activity was also demonstrated. However, assembly studies were not preformed, but ATP6 protein has a role in stabilization of the monomeric structure

along with ATP8, besides the proton channel function, which suggests that the mutation could probably have a role in assembly destabilization (Lamminen *et al*., 1995).

This functional/structural assay is a very important tool for helping to check the complexes I-V assembly pattern. The achieved differences in MRC activity results between patients prove once more that establishment of genotype-phenotype correlations is very complex and difficult. However, and besides the relevant scientific impact of the present results, further investigation will be needed, being the first step the more detailed exome analysis for pathogenic variants in assembly factors and in nuclear encoded subunits of MRC complexes.

The NGS results are already available, but it was not possible to perform a detailed analysis due to time limitations. Full sequencing and analysis of the mtDNA is already ongoing in the laboratory.

## 5. CONCLUSIONS

The results presented are original and promising, since the hypothesis of the involvement of the MRC assembly impairment in LHON has not been described before.

Furthermore, the data from the present work represent a step forward in the knowledge of the complex mechanisms underlying mitochondrial cytopathies.

The exome analysis data will be a major step for establishing a genotype-phenotype correlation and for clarifying the molecular genetics defects underlying LHON, including the bigenomic hypothesis.

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