Plácido Navas
Leonardo Salviati *Editors*

# Mitochondrial Diseases

## Theory, Diagnosis and Therapy

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Plácido Navas • Leonardo Salviati
Editors

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## Theory, Diagnosis and Therapy

*Editors*
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# Chapter 1
# Mtochonrial Neurology: A Tale of Two Genomes

**Salvatore DiMauro and Emanuele Barca**

**Abstract** The pre-molecular era of mitochondrial diseases included evidence of muscle morphology, histochemistry and electron microscopy, multiple results of mitochondrial biochemistry, and description of two classical mitochondrial diseases, Leigh syndrome and Luft disease.

The molecular era started in 1988, reporting pathogenic mutations in mtDNA, which opened a rich chapter of mitochondrial genetics, giving rise to maternally inherited (or sporadic) multisystem disorders, due to heteroplasmic (or homoplasmic) mtDNA mutations.

Mendelian inheritance of mitochondrial diseases is due to mutations in innumerable nuclear genes encoding subunits of the respiratory chain ("direct hits"), assembly proteins of respiratory chain complexes ("indirect hits"), proteins involved in mtRNA translation, proteins controlling the lipid bilayer of the inner mitochondrial membrane, or proteins regulating complex "mitochondrial dynamics."

We can add a group of variegated diseases due to problems of intergenomic signaling, leading to dysfunctional mtDNA maintenance (mtDNA depletion or multiple deletions).

We followed the history of mitochondrial diseases genetically but serious therapy begins to be realized, including mitochondrial donation in infants predestined to have mtDNA diseases.

## Acronyms

| | |
|---|---|
| AD | Alzheimer Disease |
| AHS | Alpers-Huttenlocher Syndrome. Described by Drs. Bernard Alperts and Peter Huttenlocher, this hepatocerebral syndrome belongs to the Intergenomic Signaling. |

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| | |
|---|---|
| AHSCT | Allogeneic Hematopoietic Stem el Transplant, introduced by Dr. Michio Hirano, i.e. bone marrow transplantation in patients with MNGIE syndrome. |
| AOA1 | Ataxia. Oculomotor Apraxia type 1is a form of secondary-CoQ10 deficiency belong to the Nuclear Genome |
| ARSAL | Autosomal Recessive Ataxia and Leukoencephalopathy belongs to the Nuclear Genome section. |
| Barth Syndrome | described by Dr. Peter Barth, and alterations of cardiolipin in this X-linked were reported by Dr. Michael Schlame (Schlame et al. 2003). It belongs to Defects of Lipid Milieu in Nuclear Genome. |
| CACT | Carnitine AcylCarnitineTranslocase. |
| CMT | Charcot-Marie-Tooth disease |
| COFS | CerebroOculoFacioSkeletal Syndrome, a rare presentation of mutations in *ATP12*, an indirect gene responsible for complex V deficiency {De Meirleir et al. 2004 #70}. |
| CPT | Carnitine Palmitoyl Tranferase |
| DIC | Dicarboxylate Carrier. |
| ETF | Electron-Transfer Favoprotein. |
| ETF-DH | Electron-Trabsfer Flavoprotein Dehydrogenase. |
| FBSN | Familial Bilateral Striatal Necrosis, a mtDNA disorder similar to Leigh syndrome. |
| GRACILE | Growth Retardation Aminociduria Cholestasis Iron overLoad nd Early dearh, introduced in 2002 by a Finnish scientist, Dr. Ilona Visapää. This fatal infantile encephalomyopathy belongs to the indirect hits in the Nuclear Genome. |
| HUPRA | HyperUricemia, Pulmonary hypertension, Renal failure, Alkalosis belongs to the Nucleat Genome section. |
| KSS | Kearns Sayre Syndrome, described in 1958 by Drs. Thomas Kearns and George Sayre and was attributed to single large-scale mtDNA deletions by Dr. Massimo Zeviani. |
| IMM | Inner Mitochondrial Membrane |
| IOSCA | Infantile Onset SpinoCerebellar Ataxia was introduced in 1994 by Dr. Tuula Koskinen {Koskinen et al. 1994 #145} and recessive mutations in *TWNK* were identified by Dr. Leena Peltonen in 2005 {Nikali et al. 2005 #146}. |
| LBSL | Leukoencephalopathy, Brainstem, Spinal cord and high brain Lactate. |
| LHON | Leber's Hereditary Optic Neuropathy, was described in 1871 by Dr. Theodor Leber {1871 #147} and the first mtDNA related mutation was discovered in 1988 by Douglas Wallace, |
| LS | Leigh Syndrome. The paradigmatic patient was described in 1951 by Dr. Denis Archibald Leigh. This most common pediatric encephalomyopathy, due to over 80 mutations, belongs to all three sections. |

| | |
|---|---|
| LSFC | Leigh Syndrome French Canadian, is a form of French Canadian COX-deficiency LS due to mutations in *LRPPRC,* encoding leucine-rich PPR containing protein {Mootha et al. 2003 #148}. |
| LTBL | Leucoencephalopathy, Thalamus, Brainstem involvement and high brain Lactate belongs to the Nuclear Genome section. |
| MAM | Mitochondria Associated Membrane, introduced by Dr. Eric Schon, expressing a complex membranous structure connection mitochondria wit endoplasmic reticulum. |
| MEGDEL | MethylGlutaconic aciduria type IV, Deafness, and Encephalopty Leigh-like syndrome. This unusual disease belongs to the defects of lipid milieu in the nuclear genome. |
| MELAS | Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes was introduced in 1984 by Dr. Darryl De Vivo {Pavlakis et al. 1984 #30} but the first and most common mtDNA mutation was reported by Dr. Yu-Ichi Goto in 1990. |
| MERRF | Myoclonus Epilepsy and Ragged-Red Fibers was introduced by Dr. Fukuhara, a Japanese clinician {Fukuhara et al. 1980 #149}. The first mtDNA mutation was reported by Dr. Douglas Wallace {Shoffner et al. 1990 #38}. |
| MILS | Maternally Inherited Leigh Syndrome refers to any mtDNA related LS |
| MIRAS | Mitochondrial Recessive Ataxia Syndrome, introduced in 2005 by Dr. Anu Suomalainen {Hakonen et al. 2005 #150}, belongs to the intergenomic signaling section. |
| MLASA | Myopathy, Lactic Acidosis and Sideroblastic Anemia and belongs to the mtRNA Translation Defects in the Nuclear Genome. |
| MNGIE | Mitochondrial NeuroGastroIntestinal Encephalomyopathy, introduced in 1987 by Dr. Attila Bardosi as Myo-Neuro-Gastrointestinal Encephalopathy {Bardosi et al. 1987 #151} but aptly modified by Dr. Michio Hirano. This devastating disease belong to the intergenome signaling. |
| MRT | Mitochondria Replacement Therapy |
| NAMDC | North American Mitochondrial Disease Consortium |
| NARP | Neuropathy Ataxia Retinitis Pigmentosa, introduced by Dr. Anita Harding in 1989 and was associated with MILS (NARP/MILS) in a special mtDNA disorder. |
| NNH | Navajo NeuroHepatopathy, introduced by Dr. Tuan Vu {Vu et al. 2001 #152}, belongs to the Intergenomic Signaling. |
| OMM | Outer Mitochondrial Membrane |
| PD | Parkinson Disease. |
| PDHC | Pyruvate Dehydrogenase Complex. |
| PEO | Progressive External Ophthalmoplegia belongs to all three sections. |
| POLG | polymerase gamma |

| | |
|---|---|
| RIRCD | Reversible Infantile Respiratory Chain Deficiency, introduced by Dr. Rita Horvath {Boczonadi et al. 2013 #153}. |
| RRF | Ragged-Red Fibers, was introduced in 1973 by Dr. W. King Engel, who described muscle fibers with accumulations of mitochondria staining purple with the modified Gomori trichrome. |
| SANDO | Sensory Ataxia Neuropathy Dysarthria an Ophthalmoparesis, introduced by Dr. G Van Goethem in 2003 {Van Goethem et al. 2001 #52} and it belongs to the Intergenomic Signaling. |
| SRNS | Steroid-Resistant Nephrotic Syndrome, ttributed to mutations in *COQ6*. |
| TAZ | Tafazzin, the crucial enzyme dealing with cardiolipin was so called after the discovery of the X-linked gene (*TAZ*) by Dr. Daniela Toniolo {Bione et al. 1996 #96}, who used the name of Tafazzi, a popular comic character in Italian TV. |
| TCA | TriCarboxylic Acid cycle. |
| TWNK | Twinkle, a helicase |
| WES | Whole Exome Sequencing |

## 1.1 Introduction

I am quite old and I have been interested in mitochondrial diseases since the start of my career under the mentorship of Dr. Lewis P. (Bud) Rowland, then Chairman of the Department of Neurology, University of Pennsylvania. In those times, I became familiar with Luft disease, the first *bona fide* mitochondrial disease, which described – in a masterful piece of clinical investigation – a young Swedish woman with non-thyroid hypermetabolism, proximal weakness, abnormal mitochondria by electron microscopy in her muscle biopsy, and the unusual finding of "loose coupled" oxidative phosphorylation in her isolated muscle mitochondria (Luft et al. 1962).

And I was lucky enough to confirm clinical features, muscle morphology and biochemical findings in a second young Jordanian woman, who had excess heat, perspiration, polyphagia, polydipsia, mild weakness, and radiator-like diffuse erythema in her legs (DiMauro et al. 1976).

Rare as it is, it remains a puzzle to identify the molecular genetic lesion in this unique Luft mitochondrial myopathy.

In 1951, Denis Archibald Leigh reported the first patient with a prototypical mitochondrial syndrome, an infant boy normal at birth and at 7 weeks manifesting developmental regression, poor feeding, optic atrophy, and limb spasticity. Postmortem neuropathological examination revealed bilateral symmetrical subacute necrotic lesion in the thalami, in brainstem, in the posterior columns of the spinal cord with relative sparing of the caudate and the lentiform nuclei (Leigh 1951).

This “subacute necrotizing encephalomyelopthy” was attributed to various mitochondrial biochemical errors and – later – to mutations in more than 75 genes in the two genomes (Lake et al. 2016).

In the 1970s, morphology was the definition of mitochondrial myopathies through the work of Milton Shy and Nicholas Gonatas describing at the electron microscopic level excessive number of mitochondria (“pleoconial myopathy”) and large mitochondria (“megaconial myopathy”) in muscle biopsies from children and adults (Shy et al. 1966).

A classical histochemical stain, using the modified Gomori trichrome assay, was introduced by W. King Engel (Engel and Cunningham 1963) to reveal abundant mitochondrial proliferation in the subsarcolemmal fibers, which were defined “ragged-red fibers” and became the acronym (RRF) for mitochondrial myopathy or – more in general – for mitochondrial encephalomyopathies.

In the same decade, there was growing interest in the biochemical identification of mitochondrial defects, including carnitine deficiency (Engel and Angelini 1973), carnitine palmitoyltranferase (CPT) deficiency (DiMauro and DiMauro 1973), cytochrome *c* oxidase (COX) deficiency (Willems et al. 1977; Minchom et al. 1983; Johnson et al. 1983), and pyruvate dehydrogenase (PDH) deficiency (Blass et al. 1970). In 1985, we proposed a biochemical classification of mitochondrial diseases into five major steps of mitochondrial metabolism (DiMauro et al. 1985) and shortly thereafter we described COX deficiency as one important cause of Leigh syndrome (DiMauro et al. 1987).

## 1.2 The Mitochondrial Genome

Thirty years after the report of the first pathogenic mutations in the mitochondrial genome (mtDNA) (Holt et al. 1988; Wallace et al. 1988), it seems appropriate to take stock of the extraordinary progress in mitochondrial genetics. Two billion years ago, eukaryotic cells were invaded by protobacteria that had adapted to an oxygen-rich atmosphere and were becoming permanent endosymbionts that we call mitochondria. The bacteria have brought into the cells their own DNA, which explains why all eukaryotic cells carry two genomes, their own nuclear DNA (nDNA) and mtDNA, a relic but not a fossil of the endosymbionts(Gray et al. 1999; Martijn et al. 2018).

In 1963, Nass and Nass reported the presence of extranuclear mitochondrial DNA in chick embryos(Nass and Nass 1963), and strictly maternal inheritance of mtDNA was confirmed in humans by Giles et al. in 1980 (Giles et al. 1980).

Mitochondria have innumerable functions subserved by ~1700 nuclear-encoded proteins, although a fundamental role of mitochondria is the provision of energy in the form of ATP.

An oversimplified scheme of mitochondrial metabolism shows the points of entry of metabolic fuels: carbohydrates through pyruvate transport and fatty acids through the carnitine/acylcarnitine shuttle (Fig. 1.1).

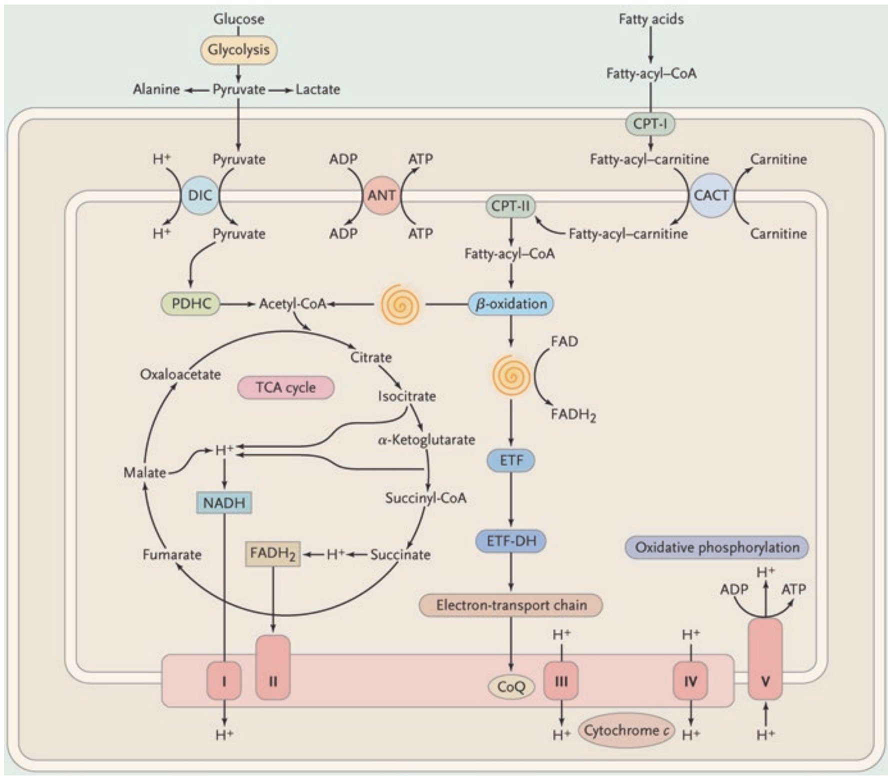


**Fig. 1.1 Schematic view of mitochondrial metabolism.** The spirals represent the cyclic reactions of the beta-oxidation pathway resulting in the formation of acetyl-CaA and the reduction of flavoprotein. Acronyms are explained in the Appendix

The oxidation of pyruvate by the pyruvate dehydrogenase complex (PDHC) and of fatty acids though the beta-oxidation "spirals" results in acetyl -CoA, which is further oxidized in the Krebs cycle. Electrons resulting from the dehydrogenases of the Krebs cycle and from the beta-oxidation pathway flow down the electron carrier chain, which is embedded in the inner mitochondrial membrane (IMM) and is composed of four protein complexes (I to IV) and of two simple electron carriers, Coenzyme $Q_{10}$ ($CoQ_{10}$) and cytochrome ***c***. Together with the horizontal flow of electrons, there is a vertical flow of protons from the mitochondrial matrix through he IMM into the intermembrane space. This results in an electrochemical proton gradient that drives ATP synthase (complex V), a magnificent rotor enzyme, which uses reverse flow of protons to convert ADP to ATP. The electron transport chain together with ATP synthase (jointly known as "respiratory chain") is the only "business end" (ATP producing) of mitochondrial metabolism and the only mitochondrial pathway containing 13 proteins encoded by mtDNA (all the rest are encoded by nDNA). Figure 1.2.

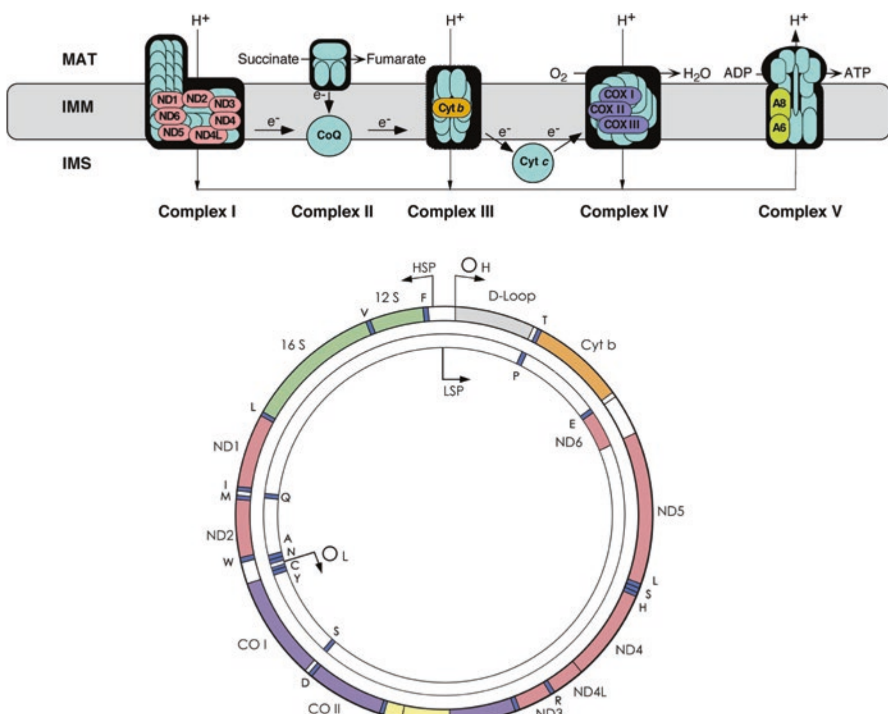


**Fig. 1.2 The respiratory chain and the mitochondrial DNA.** Genes and corresponding gene products are similarly color-coded. Subunits of cytochrome *c* oxidase are labeled CO in he mtDNA scheme and COX in the respiratory chain rendition. The 22 tRNA genes are denoted by a one-letter amino acid nomenclature. 12S and 16S denote ribosomal RNAs (rRNAs). $O_H$ and $O_L$ are the origin of heavy and light strand replication. HSP and LSP are the promoters of heavy- and light-stranded transcription

The mtDNA, a small double-stranded circular molecule (16,569 base pairs) is a piddling amount of DNA compared to the 3 billion base pairs of nDNA. In addition to the 13 protein-coding genes, mtDNA contains 2 ribosomal RNAs (rRNAs), and 22 transfer RNAs (tRNAs) but no introns.

In 1981, the group of Sanger published the complete sequence of human mtDNA (Anderson et al. 1981) and corrected small errors in 1999 (Andrews et al. 1999).

Over the past 30 years, the mtDNA has become crowded with pathogenic point mutations (~300 at latest count) to which must added hundreds of large-scale deletions (Fig. 1.3).

For the diagnosis of mtDNA related diseases, three rules of “mtDNA genetics” serve useful to the clinician.

*Maternal inheritance* is the first diagnostic clue since symptoms are transmitted from the mother to all her children, boys and girls, and no father is involved. Careful observation of the maternal lineage gives interesting data but this “rule of thumb” suggests not to ignore soft signs, such as headache, diabetes mellitus, short stature,

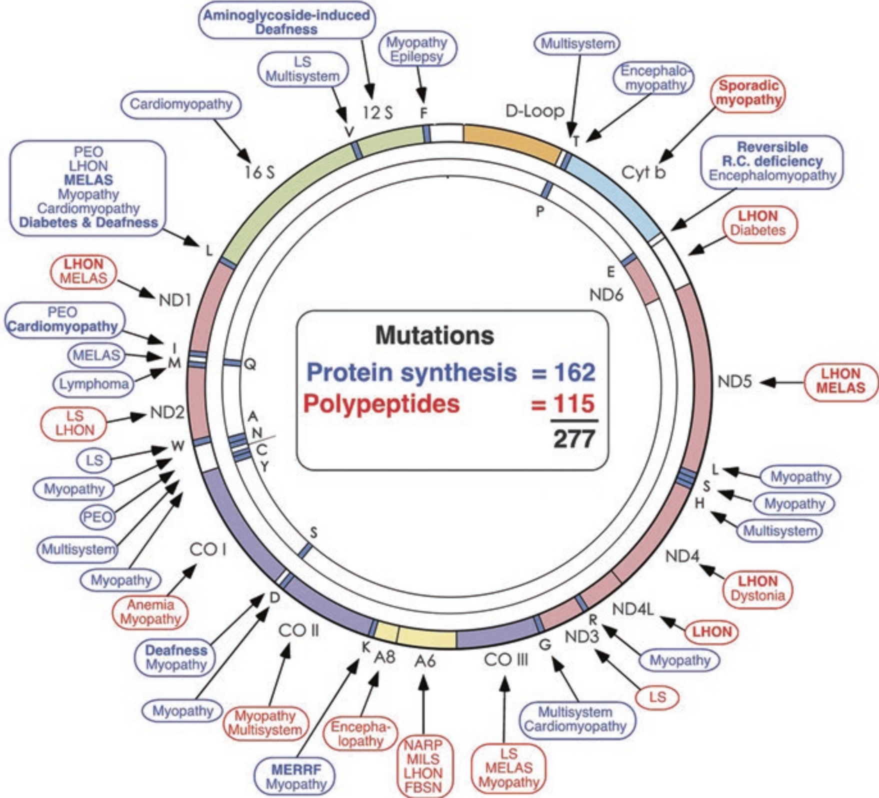


**Fig. 1.3 Morbidity map of mitochondrial DNA**. Disorders caused by mutations in protein-coding genes are shown in red. Disorders cause by mutations in genes controlling protein synthesis are shown in blue. All acronyms are listed in the Appendix

exercise intolerance, in maternal relatives. Often, on the other hand, is likely that an asymptotic mother may carry a deleterious mtDNA point mutation and generate a severely affected girl (e.g. with Leigh syndrome) and a normally healthy girl (Levy et al. 2014).

Family history clearly distinguishes maternally inherited mtDNA point mutations from sporadically occurring single mtDNA large-scale deletions and a large survey of patients with Kearns-Sayre syndrome (KSS, see below) has revealed the vast majority of sporadic patients (Chinnery et al. 2004).

*Polyplasmy, homoplasmy, heteroplasmy* is the second rule of mtNA genetics. Each cell contains hundreds or thousands of mitochondria and each mitochondrion harbors multiple copies of mtDNA. Normal cells hold "identical" copies of mtDNA (normal homoplasmy), but in patients with mtDNA mutations we observe a stochastic mixture of normal and deleterious mtDNAs (heteroplasmy). A crucial corollary of heteroplasmy is that a critical number of mutant mtDNAs is needed for the respiratory chain to suffer and for disease to manifest ("threshold effect").

Mitochondria are ubiquitous organelles, important in all tissues (excluding mature erythrocytes) and it is easy to understand that pathogenic mtDNA mutations cause multisystemic diseases, including brain, peripheral nerves, skeletal muscle, heart, gastrointestinal system in various ways. Typically, these are the phenotypes of MELAS, MERRF, NARP, and KSS (see below). However, there are rare cases in which individual tissues may be affected, for example skeletal muscle with myopathy due to mutations in the *cytb* gene (Andreu et al. 1999). In this situation, we postulated that the mutation load of mutant *cyt b* was disproportionally high in skeletal muscle than in other tissues, such as the mutation load surpassed the threshold effect. But typically mtDNA related diseases are a heterogeneous group of clinical disorders. In rare instances, homoplasmic mutant mtDNA mutations are known to cause diseases, such as Leber hereditary optic neuropathy (LHON) (see below).

*Mitotic segregation* is the third rule of mitochondrial genetics. As cells divide, they pass on to next generation various mutation loads of mtDNA (wild-type and mutant) and, thus, both the genotype and the phenotype may vary in time.

Here we come to describe – very briefly – the main phenotypes of mtDNA related disease.

KSS, PEO, and Pearson syndrome are three phenotypes due to single large-scale mtDNA deletions.

KSS was described by Kearns and Sayre from the Mayo clinic in nine sporadic patients with the triad of progressive external ophthalmoplegia (PEO), retinitis pigmentosa, and heart block (Kearns and Sayre 1958).

KSS is characterized by the triad of PEO (ptosis and limitations of eye movements), pigmentary retinopathy, and cardiac conduction block. Additional signs are ataxia, cognitive decline, peripheral neuropathy, cardiomyopathy (sometimes leading to heart transplantation), and rare epilepsy associated with hypoparathyroidism. Muscle biopsy in KSS shows abundant RRF. In 1988, Massimo Zeviani and coworkers clearly documented the presence of different large-scale mtDNA deletions in seven patients with KSS (Zeviani et al. 1988).

All tissues can be affected in KSS but a skeletal muscle is predominantly involved in PEO, with presentation in young or middle age of ptosis and ophtahalmoplegia, with variable proximal limb weakness and RRF in muscle biopsies.

On the other hand, Pearson syndrome is a severe infantile disease, with large-scale mtDNA deletions affecting the bone marrow with sideroblastic anemia, the exocrine pancreas, the liver, and kidneys with growth retardation and death early in life (Rotig et al. 1989). However, a few patients with Pearson syndrome survive and will go though a well period but are predestined to develop KSS later in life, becoming the typical examples of “mitotic segregation”.

MELAS (mitochondrial encephalomyopahy, lactic acidosis and stroke-like episodes) is among the most common expression of point mutations in mtDNA. The first case was described in 1984 (Pavlakis et al. 1984) and reported in detail 15 years later (Hirano et al. 1992). By far the most common mutation was identified in MELAS by Goto and coworkers as m.3243A>G in 1990 (Goto et al. 1990). However, several point mutations in the same or in other genes were associated with MELAS.

Clinical presentation of MELAS includes: stroke-like events before age 40; encephalopathy with seizures or dementia; normal early development; recurrent migrainous headache; recurrent vomiting; lactic acidosis and RRF in muscle biopsy. Typically, stroke lesions are not confined to vascular territories and usually affect occipital, temporal, and parietal lobes.

MELAS has a heterogeneous phenotype in carrying mothers, manifesting with diabetes mellitus, neurosensory hearing loss, cardiomyophaty, GI pseudoobstruction, and psychiatric disturbances.

Leber hereditary optic neuropathy (LHON) is the most common mtDNA related disease and its first homoplasmic mutation in complex I (*ND4*, m.11778G>A) was identified in a large maternal pedigree by Wallace in 1988 (Wallace et al. 1988). Two other mutations in *ND1* (m.3460G>A) and *ND6* (14,484T>C), close in homoplasmy, were associated with LHON, which is characterized clinically by acute or subacute bilateral or unilateral vision loss in young adults, but predominantly affecting men than women.

The retinal ganglion cells are most vulnerable in LHON. It is well known that axons have a high energy demand, especially in the distal portion (Maresca et al. 2013; Sheng 2017), moreover retinal ganglion cells are more vulnerable because of the architecture: the axons of the ganglion cells of inner retina converge to form the optic nerve that is unmyelinated before crossing the lamina cribrosa but is thereafter heavily myelinated (Carelli et al. 2017). This leads to an unbalanced distribution of mitochondria along the axons, where many unmyelinated fibers are richer in mitochondria compared with heavily myelinated fibers that form the retrobulbar optic nerve. However, no genetic explanation is given for the male vulnerability, although epigenetic factors seem to play a role, namely estrogen favors women over men (Carelli et al. 2017).

The third maternally inherited syndrome (MERRF, myoclonus epilepsy and ragged-red fibers) was attributed to a mutation (m.8344A>G) in the gene $tRNA^{Lys}$ by the group of Doug Wallace (Shoffner et al. 1990) but two different mutations were identified in the same gene.

The typical phenotype of MERRF includes – in order of frequency – myoclonic epilepsy, ataxia, myopathic weakness, peripheral neuropathy, mental retardation, and hearing loss (Mancuso et al. 2013). One additional feature of MERRF is the presence of multiple, often disfiguring, lipomas predominantly localized in the neck area. Although related to mutation in the $tRNA^{Lys}$, the pathogenesis of these lipomas remains unclear.

Various degrees of m.8344A>G heteroplasmy (and threshold effects) affect different tissues and sometime high mutation loads cause a severe infantile disease. For example, we reported Leigh syndrome in a historical family with m.8433A>G mutation (Santorelli et al. 1998) and Leigh syndrome in two little girls who died in infancy, but their mother was well until she was in her 30s, when she developed typical MERRF with the m.8663G>A mutation (Shtilbans et al. 2000).

NARP/MILS (neuropathy, ataxia, retinitis pigmentosa/maternally inherited Leigh syndrome) readily explains how MILS is due to higher (90%) than lower (70%) NARP levels of the heteroplasmic mutation (m.8993T>G) in the *ATPase 6*

gene. This syndrome was first identified by the group of Anita Harding (Holt et al. 1990) and the importance of heteroplasmy was stressed by Tatuch and coworkers (1992). A different mutation at the same site (m8993T>C) caused a milder similar syndrome (Morava et al. 2006).

Maternally inherited Leigh syndrome (MILS) is a common result of mtDNA mutations, including many in the complex I submits (*ND1*, *ND3*, *ND5*) but, overall, LS is more often diagnosed as an autosomal recessive condition. This led to the discovery of innumerable mendelian inherited genes, some of which will be reported in the next section (NUCLEAR GENOME).

From a diagnostic standpoint, three techniques have contributed valuable clues to the diagnosis of mtDNA related diseases.

The first was the histochemical overlap of COX and SDH stains, introduced by my friend Eduardo Bonilla.

COX deficiency is the hallmark of mtDNA diseases, with four enzymatically active mtDNA-encoded subunits (COXI – COXIV) while SDH (complex II) is considered a marker of mitochondrial abundance as its four subunits are completely encoded by nDNA. In patients with mitochondrial diseases, cross sections of frozen muscle biopsies clearly show scattered COX-negative or COX-deficient fibers, which are often overstained with SDH due to abnormal compensatory mitochondrial proliferation (RRF). The brown stain of COX prevails over the blue stain of SDH in normal fibers while COX-negative fibers show a bright blue color ("ragged-blue fibers") and COX-deficient fibers will stain a milder bluish color (Tanji and Bonilla 2007) (Fig. 1.4). This COX/SDH combined staining has proven diagnostically useful generally in muscle biopsies, but has been equally valuable in brain sections from patients with mDNA related encephalomyopathies (Borthwick et al. 2006).

The second – even more sensitive – method was introduced by my valid collaborator, Carlos Moraes (Moraes et al. 1993), based as it is on PCR amplification of a novel mutation. In particular, this method measures the relative percentages of

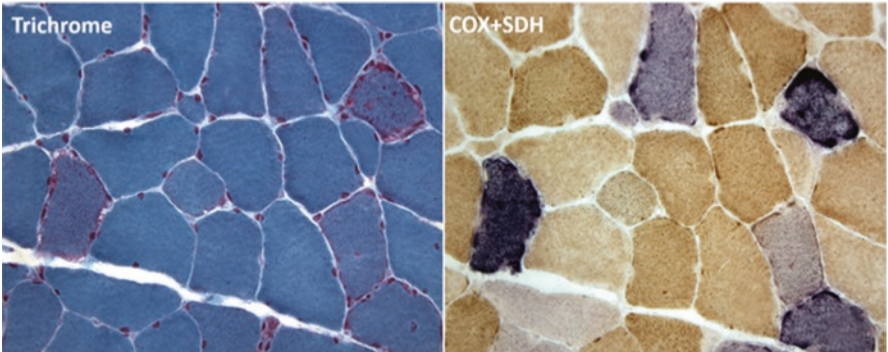


**Fig. 1.4 COX/SDH overlap staining in a muscle biopsy.** This patient with MERRF shows typical RRF by trichrome at left. However, COX/SDH overlap staining (at right) shows COX-negative, "ragged-blue" fibers, mixed with COX-deficient bluish fibers

wild-type and mutant mtDNA in COX-negative, COX-deficient, and normal-looking muscle fibers. These results give convincing clues to the pathogenicity of novel mtDNA variants. What was required meant plucking from thick histochemical muscle sections COX-negative, COX-deficient and normal-looking fibers and performing PCR on these tiny specimens (Fig. 1.5).

And the third useful method is to create a so-called “cybrid” (cytoplasmic hybrid) immortal fibroblast cell lines. It consists in transferring into rho-zero cells (deprived of their mtDNA) heteroplasmic mitochondria from cultured skin fibroblasts of patients carrying mtDNA mutations (King and Attardi 1989). These heteroplasmic or homoplasmic cybrids provided hallmark models to evaluate biochemical consequences and functional threshold effects in MELAS, MERRF, and KSS. And, to say the least, hybrid cell lines were employed in various therapeutic strategies to test different approaches to treat mtDNA related diseases.

And this concludes my historical perspective to the tale of the first genome.

**COX I gene mutation (G5920A)**

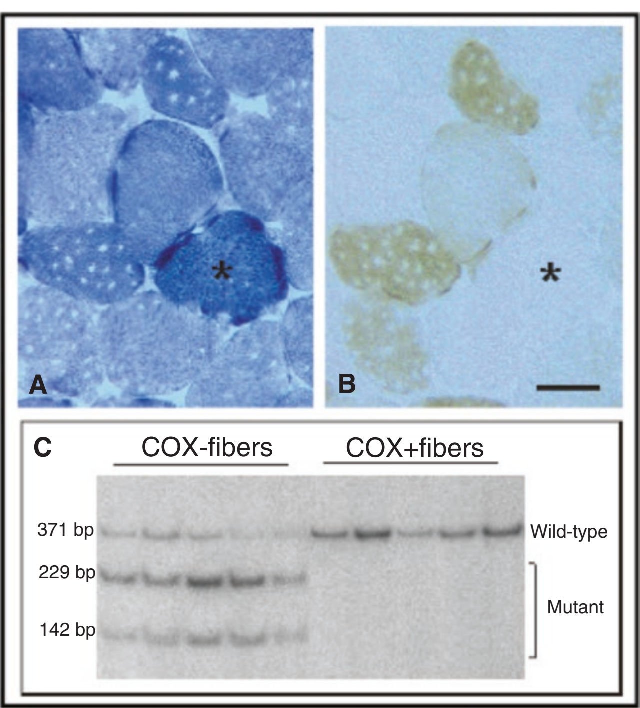


**Fig. 1.5 Evaluating the pathogenicity of a novel mtDNA mutation in single fibers by PCR.** In this young man with recurrent myoglobinuria, we identified a nonsense mutation in *COXI* gene of mtDNA (Karadimas et al. 2000). It is obvious to show abundant mutation in COX-negative fibers and lack of mutation in COX-positive fibers by PCR in single fibers

## 1.3 Nuclear Genome

1988 was the opening "mitochondrial genetics" but in 1989 Massimo Zeviani and Stefano DiDonato found several Italian families with autosomal dominant or recessive mitochondrial PEO and encephalomyopathy, in whose muscle biopsies they saw RRF and multiple deletions of mtDNA (Zeviani et al. 1989). They started a chase to the nuclear genes responsible for these syndromes. Their chase was concluded a few years later by the identification of mutations in the gene *SLC25A4* encoding ADP/ATP translocase 1 (Kaukonen et al. 2000), the identification of mutations in *POLG* (Van Goethem et al. 2001), and the identification of *TWNK*, the Twinkle gene (Deschauer et al. 2003). Although not surprisingly, these first three mutant nuclear genes belong to the third section of my presentation (INTERGENOMIC SIGNALING), I will now focus on some of the multiple nuclear genes that when mutant, affect the respiratory chain.

The respiratory chain consists of five complexes that are mostly encoded by nDNA: it is obvious that mutations in genes encoding proteins in each complex ("direct hits") are responsible for each complex dysfunction. According to the "all or none" effect of mendelian mutations and, in contrast to the variegated effect of heteroplasmic mtDNA mutations, disorders due to direct hits to the respiratory chain often manifest at or soon after birth with severe phenotype.

Direct hits. One of the first direct hits unsurprisingly involved a component of complex II (SDH A) and was associated with autosomal recessive Leigh syndrome (Bourgeron et al. 1995).

Many of the direct hits affecting 16 of the 45 structural subunits of complex I have been associated with Leigh syndrome (LS) or infantile cardiomyopathies (Distelmaier et al. 2009).

Primary Coenzyme $Q_{10}$ ($CoQ_{10}$) deficiencies. The story of $CoQ_{10}$ deficiency started early in 1989, when the group of Andrew G. Engel reported two sisters affected with a mitochondrial encephalomyopathy due to severe defect of $CoQ_{10}$ in their muscle mitochondria (Ogasahara et al. 1989). Both sisters had lipid storage myopathy, cerebellar ataxia, seizures, and recurrent myoglobinuria. In the following years, I observed several children with cerebellar atrophy and ataxia, muscle weakness, often associated with seizures, mental retardation, spastic paraparesis, or peripheral neuropathies (Musumeci et al. 2001; Lamperti et al. 2003). In a discussion, I predicted that the heterogeneous phenotypes of the patients with $CoQ_{10}$ deficiency were presumably attributed to mutations in genes encoding any of the 10 enzymes in the complex biosynthetic pathway of $CoQ_{10}$ (*COQ1* [*PDSS1* and *PDSS2*], – *COQ9*). The chase of these enzyme defects started in 2006 by the group of Michio Hirano, identifying two severe infantile encephalomyopaties (one similar to MELAS and the other to LS) due to mutations in the first two genes (*COQ2* and *PDSS2*) (Quinzii et al. 2006; Lopez et al. 2006). During the following 12 years, mutations were identified in genes encoding 8 more biosynthetic enzymes (Table 1.1), related to a variety of phenotypes, including infantile encephalomyopathies, juvenile cerebellar ataxia (*COQ8A*) and steroid-resistant glomerular nephropathy (Salviati et al. 1993).

**Table 1.1** Primary $CoQ_{10}$ deficiencie

| Gene | Phenotype | References |
|---|---|---|
| *PDSS1* | Infantile nystagmus, optic atrophy, neurosensory hearing loss, ataxia, dystonia, progressive nephrosis. | Mollet et al. (2007) |
| *PDSS2* | Infant with hypotonia, left seizures, severe nephrosis, cortical blindmess and Leigh syndrome | Lopez et al. (2006) |
| *COQ2* | Infantile proteinuria, hypotonia, optic atrophy, myoclonus, weakness, stroke-like lesions | Quinzii et al. (2006) |
| *COQ4* | Infantile-onset hypotonia, bradycardia, respiratory insufficiency, heart failure, epileptic encephalopatny | Brea-Calvo et al. (2015), Chung et al. (2015) |
| *COQ6* | Steroid-resistant nephrotic syndrome (SRNS), sensorineural haring loss | Heeringa et al. (2011), Park et al. (2017) |
| *COQ7* | A boy born small, with lung hypoplasia, renal insufficiency, left ventricular hypertrophy, polyneuropathy. 9-year-old with learning disability.<br>6-year-old girl with spasticity, bilateral sensorineural hearing loss. At 5 years she walks with a walker. | Freyer et al. (2015), Wang et al. (2017) |
| *COQ8* | Adult-onset cerebellar ataxia, seizures, dystonia, spasticity | Lagier-Tourenne et al. (2008), Mollet et al. (2008), Horvath et al. (2012) |
| *COQ9* | Neonate with lactic acidosis, intractable seizures, global developmental delay, renal tubular dysfunction | Duncan et al. (2009) |

Indirect hits. Nuclear encoded subunits of different complexes have to be imported into mitochondria, inserted in the IMM, assembled with their mtDNA encoded counterparts, acquiring prosthetic groups, and – if necessary- polymerizing into supercomplexes.

Mutations in genes affecting these pathways, ancillary "assembly" proteins. have been through linkage analysis, homozygosity mapping, monochromosomal transfer, gene complementation, and whole exome sequencing (WES) (Fig. 1.6).

Numerous indirect hits in assembly proteins of complex I caused a variety of clinical presentations in infancy, including LS but often leukodystrophy rather than gray matter involvement, or severe cardiomyopathy. Early onset and early death are common in these diseases but occasional cases have prolonged course with fluctuations (Distelmaier et al. 2009).

Secondary $CoQ_{10}$ deficiencies have been reported in patients without any direct hit to the biosynthetic pathway.

One dramatic example is the autosomal recessive ataxia, oculomotor apraxia syndrome (AOA1) in families with mutations in the *aprataxin* (*APTX*) gene (Quinzii et al. 2005). Uncertain remains the pathogenesis of $CoQ_{10}$ deficiency although *APTX* has a role in single-stranded DNA repair.

Another example of secondary $CoQ_{10}$ deficiency shows lipid storage myopathy in several families with mutations in the gene of terminal enzyme of beta-oxidation, electron-transferring flavoprotein dehydrogenase (*ETFDH*), known to be associated with glutaric aciduria type II (Gempel et al. 2007).

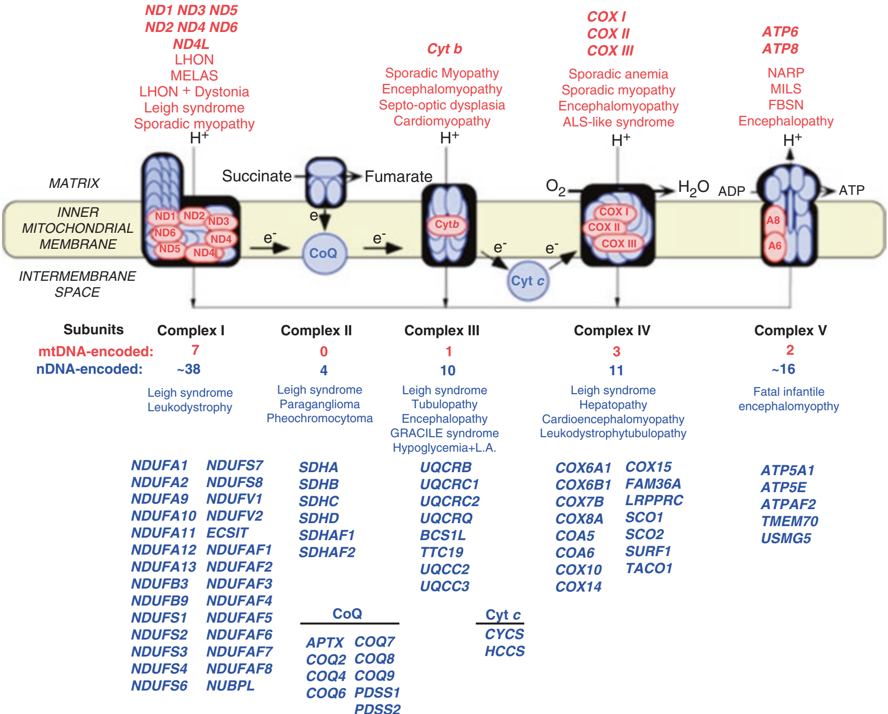


**Fig. 1.6 "Direct" and "indirect" hits affecting the respiratory chain complexes.** This schematic representation of the respiratory chain shows a good sample of "direct" and "indirect" hits. Acronyms are listed in the Appendix

The GRACILE syndrome, finely acronomyzed in Finland (growth retardation, aminoaciduria, cholestasis, iron overload, and early death) was due to mutations in *BCS1*, the gene that encodes a vital assembly protein needed to insert the Rieske iron-sulfur (FeS) into a subunit of complex III (Visapaa et al. 2002).

In 1998, a friendly competition of two groups, the one of Eric Shoubridge and the other of Massimo Zeviani, led to the discovery of mutations in *SURF1*, encoding an assembly protein for complex IV and responsible for COX-deficient LS. (Tiranti et al. 1998; Zhu et al. 1998).

Mutations in 14 genes encoding different COX assembly factors (*SCO1*, *SCO2*, *COX10*, *COX14*, *COX15*, *COA5*, *COA3*, *COA6*, *COX20*, *PET100*, *PET117*, *LRPPRC*, *TACO1*)(Ghezzi and Zeviani 2018; Signes and Fernandez-Vizarra 2018) have been associated with human diseases. Mutations in any of these genes cause encephalomyopathy but some show preferential tissue involvement, including the heart with cardiomyopathy (*SCO2*, *COX10*, *COX14*, *COX15*) and the liver (*SCO1*) (DiMauro et al. 2012).

Indirect hits affecting complex V were reported in infants with devastating disease, including a case of cerebro-oculofacioskeletal syndrome (COFS) due to

mutations in the *ATP12* gene (De Meirleir et al. 2004) and 23 cases from six pedigrees with microcephaly, psychomotor retardation, hypotonia, polyneuropathy, and hypertrophic cardiomyopathy due to mutations in *TMEM70*, the gene involved in the ATP synthase biogenesis (Cizkova et al. 2008). However, the newest hit was discovered jointly by two groups (Michio Hirano's and Marni Falk's) as an Ashkenazi Jewish founder homozygous mutation in *USMG5,* which encodes an essential protein for complex V dimerization and ATP production (Barca et al. 2018).

Other indirect hits include mutations in genes encoding mitochondrial transport proteins, such as *TIMM8A* causing the Mohr-Tranebjaerg deafness and dystonia syndrome(Roesch et al. 2002), and *HSP60* causing spastic paraplegia-13(Magen et al. 2008).

Defects of mtRNA translation. The mitochondrial genome is transcribed into nine monocistronic and two dicistronic mRNAs, which are translated into the 13 subunits of the respiratory chain through the mitoribosomes. The mitoribosome is composed by two subunits, a large one composed of 50 proteins and a smaller one composed of 30 proteins (Greber et al. 2015).

In the initiation phase of this translation process, the mRNA starting site is selected and the initiator tRNA (fMet tRNA) is base-paired with the mRNA. Two translation initiation factors ($_{mt}$IF2 and $_{mt}$IF3) are needed.

In the elongation phase, mRNA codons are sequentially read and the amino acids are incorporated into growing peptide chains by aminoacyl-tRNA synthetases. In addition, elongation factors (EF-Tu$_{mt}$, EF-Ts$_{mt}$, an EF-GI$_{mt}$) are required.

In the termination phase, complete polypeptides are released and ribosome complexes are recycled, requiring two release factors (eRF1 and ICT1).

Additional translation activators (TACO1, LRPPRC) and specific tRNA base modifiers (TRMU, MTO1, GTPBP3, NSUN3n, MTFMT and PUS1) are involved in the translation machinery (Boczonadi et al. 2018).

Envision mutations in genes encoding the multiplicity of translation factors, giving rise to a large group of novel mitochondrial diseases.

Mutations in genes encoding ribosomal proteins and assembly factors (*MRPL, MRPL44, MRPL12*, *MRPS16*, *MRPS22*, *MRSP34*, *ERAL1*, *RMND1*) cause severe infantile lactic acidosis, neurological problems (LS, leukoencephalopaty, ataxia), hepatocerebral syndrome, cardiomyopathy, Cornelia de Lange-like syndrome, and Perrault syndrome.

An interesting syndrome is the potentially reversible infantile cerebrohepatopathy due to mutations in tRNA-modifying gene *TRMU*, which modifies three tRNAs, including TNA$^{Glu}$. Mutations in *TRMU* explain the benign reversible COX deficiency myopathy (DiMauro et al. 1981), later recognized as a reversible infantile respiratory chain deficiency (RIRCD) myopathy associated with homoplasmic tRNA$^{Glu}$ mutation (Boczonadi et al. 2018).

Mutations in another RNA-modifying gene (*PUS1*) cause a peculiar syndrome defined as MLASA (myopathy, lactic acidosis, and sideroblastic anemia) (Bykhovskaya et al. 2004).

There are 19 genes encoding aminoacyl-tRNA synthetases and mutations in all of them have been associated with human diseases (Boczonadi et al. 2018). Of note,

mutations in *AARS2* encoding alanyl-tRNA synthetase cause either fatal infantile cardiomyopathy or childhood- and adult-onset leukoencephalopathy (Euro et al. 2015); mutations in *DARS2* encoding aspartyl-tRNA synthetase 2 cause leukoencephalopathy with brainstem, spinal cord, and high brain lactate (LBSL)(Isohanni et al. 2010); mutations in *EARS2* encoding glutamyl-tRNA synthetase cause leukoencephalopathy, thalamus, brainstem involvement and high lactate (LTBL) (Steenweg et al. 2012); mutations in *YARS2* encoding tyrosyl-tRNA synthetase cause MLASA syndrome (Sommerville et al. 2017); mutations in *RARS 2* encoding arginyl-tRNA synthetase cause infantile encephalopathy and pontocerebellar hypoplasia (Edvardson et al. 2007); mutations in *GARS* encoding glycyl-tRNA synthetase cause distal hereditary motor neuropathy, Charcot Marie Tooth disease type 2D (Lee et al. 2012); mutations in *HARS2* encoding histidyl-tRNA synthetase cause ovarian dysgenesis and sensorineural hearing loss (Perrault syndrome) (Pierce et al. 2011); mutations in *NARS2* encoding asparaginyl-tRNA synthetase cause LS, Alpers syndrome, and other infantile encephalomyopathies (Simon et al. 2015); mutations in *MARS2* encoding methionyl-RNA synthetase cause autosomal recessive ataxia and leukoencephalopathy (ARSAL) in French Canadian families (Bayat et al. 2012); mutations in *SARS2* encoding seryl-tRNA synthetase cause infantile hyperuricemia, pulmonary hypertension, renal failure and alkalosis (HUPRA) syndrome (Belostotsky et al. 2011); mutations in *FARS2* encoding phenylalanyl-tRNA synthetase cause a fatal infantile epileptic encephalopathy (Elo et al. 2012); mutations in *VARS* encoding valyl-tRNA symthetase and in *TARS* encoding threonyl-tRNA synthetase cause microcephaly, epilepsy, axial hypotonia, and psychomotor delay (Diodato et al. 2014); mutations in *LARS2* encoding leucyl-tRNA synthetase cause Perrault syndrome, hydrops, lactic acidosis, and sideroblastic anemia (Solda et al. 2016).

An additional notation refers to the interesting evidence that *overexpression* of the *LARS2* gene encoding leucyl-tRNA synthetase in cybrid lines from patients with MELAS, carrying the typical m.3243A>G mutation in the mtDNA $tRNA^{Leu(UUR)}$ gene improves the phenotype (Li and Guan 2010).

Defects of the lipid milieu. The phospholipid component of the inner mitochondrial membrane (IMM), where the respiratory chain resides, is much more than a cell scaffold and changes in the lipid milieu give rise to a series of mitochondrial encephalomyopathies.

To start with, changes in cardiolipin, the crucial dimeric molecule composed of two phosphatidylglycerol moieties joined by a glycerol group, have been found in X-linked Barth syndrome (Schlame et al. 2003). Mutations in the *TAZ* gene, encoding tafazzin, a phospholipid-lysophospholipid transacylase, are the basis for the common mitochondrial myopathy, cardiomyopathy, neutropenia, and stunted growth in boys (Barth syndrome) (Bione et al. 1996).

Next, autosomal recessive Sengers syndrome (Sengers et al. 1975) (mitochondrial myopathy, cardiomyopathy, and congenital cataracts) was attributed through WES to mutations in the *AGK* gene (Mayr et al. 2012), which encodes acylglycerol kinase, an enzyme that catalyzes the phosphorylation of diacylglycerol and monoacylglycerol to produce phosphatidic acid (PA) and lysopghosphatidic acid (LPA).

Considering that phosphatidic acid (PA) is a precursor of cardolipin mutations in *AGK* may easily explain the phenotypic similarities between Sengers and Barth syndromes.

To follow, new syndromes were due to novel defects of the IMM lipid milieu.

Megaconial encephalomyopathy was attributed – by analogy to the murine rostro-caudal muscular dystrophy – to mutations in *CHKB*, the gene encoding human choline kinase beta, resulting in a congenital encephalomyopathy with giant mitochondria displaced at the periphery of muscle biopsies (Gutierrez Rios et al. 2012).

The cause of MEGDEL syndrome (mehylglutaconic aciduria type IV, deafness and Leigh-like encephalopathy) was discovered by WES due to mutations in the gene *SERAC1* (Wortmann et al. 2009).

And the cause of recurrent myoglobinuria in children during febrile illnesses but rarely affected with inborn errors of metabolism was found in mutations of *LPIN1* that encodes the muscle-specific isoform of PA phosphatase (Zeharia et al. 2008).

My best friends, Eric Schon and Estela Area Gomez, called attention to a membranous structure they called MAM (mitochondria associated membrane), where mitochondria come in close contact with the endoplasmic reticulum, establishing important functional relationships, including lipid exchange, cholesterol synthesis, calcium control, and mitochondrial dynamics.

In addition to two above-mentioned disorders of lipid milieu – megaconial encephalomyopathy, and MEGDEL – due to defective phospholipids exchange, two more diseases were related to MAM dysfunction, a primary lateral sclerosis due to mutations in *ERLIN2*, and a juvenile ALS due to mutations in mutations in *SIGMAR*.

Schon and Area-Gomez have provided remarkable evidence of MAM dysfunction in Alzheimer disease (AD), supporting that mutations in presenilin-1 (*PSEN1*) and presenilin-2 (*PSEN2*) suppress the action of mitofusin-2, which crucially aids the connectivity of mitochondria to endoplasmic reticulum, thus impairing mitochondrial bioenergetics in AD (Schon and Area-Gomez 2013).

Defects of mitochondrial dynamics comprise a large and developing group of mitochondrial mendelian diseases, Mitochondria are highly dynamic organelles, they can fuse to form tubular structures, they can split to form punctate structures, they can travel long distances on microtubules within the cell (imagine going from the motoneuron soma to the neuromuscular junction) and short distances on actin cables, they can attach themselves to specific cellular structures (for example to the endoplasmic reticulum through mitofusin-2), and they can initiate mitophagy and apoptosis (Area-Gomez and Schon 2014).

The first mitochondrial dynamics disease, dominant optic atrophy (DOA) was the counterpart of mtDNA related LHON and was due to mutations in *OPA1*, the gene encoding a dynamic-related GTPase bound to the IMM, which together with another GTPase, mitofusin-1 bound the OMM participate to mitochondrial fusion (Alexander et al. 2000).

Mutation in two mitochondria fission genes encoding dynamic-related protein 1 (*DM1L*) and mitochondrial fission factor (*MFF*) cause, in addition to optic atrophy,

severe infantile encephalomyopathies (Waterham et al. 2007; Shamseldin et al. 2012).

Four types of Charcot-Marie-Tooth (CMT) disease are due to impaired mitochondrial trafficking: CMT 2E and CMT 4A/2K are related to defective mitochondrial motility, the first to mutations in *INF2* encoding neurofilament light polypeptide, and the latter to mutations in *GDPA1* encoding ganglioside-induced differentiation-associated protein 1. CMT 2A is related to defective communication between mitochondria and endoplasmic reticulum, due to mutations in *MFN2*, encoding the GTPase mitofusin-2 (), and variants of *DNM2* have been associated with CMT 2M (Zuchner et al. 2004).

More diseases belong to defects of mitochondrial quality control, including familial Parkinson disease (PD) type 2, with mutations in *PARK2* encoding E3 ubiquitin protein-ligase, and PD type 6, with mutations in *PINK1* encoding PTEN-induced putative kinase 1: both proteins operate in the same pathway removing dysfunctional mitochondria through mitophagy (Schon and Przedborski 2011).

Add to PD spastic paraplegia type 7 due to mutations in *SPG7* encoding the metalloprotease paraplegin; spinocerebellar ataxia 28 due to mutations in *AFLG3L2* encoding a metalloprotease paraplegia-like protein; ALS type 14 due to mutations in *VCP* encoding a valosin-containing protein, an ATPase.

## 1.4 Intergenomic Signaling

The partnership between the two genomes is not equal but overwhelmingly in favor of the nuclear genome, which controls mtDNA maintenance, its replication and integrity. Two groups of diseases in this category are usually characterized by the effects they have on mtDNA maintenance, leading to two subgroups: mtDNA depletion and multiple mtDNA deletions. Their mendelian genetic origin shares in common phenotypical variegated expression with mtDNA related diseases, due to the level of damage on mtDNA (concept somehow similar to what was described in mtDNA mutations).

The concept of mtDNA depletion was introduced in 1991 by my friend Carlos Moraes, showing complete or partial mtDNA reduction in different tissues from severely affected infants (Moraes et al. 1991). At the same time, my friend Eduardo Bonilla devised an immunochemical stain for mtDNA in contrast to bright nDNA to be applied in muscle biopsies (Tritschler et al. 1992).

Multiple genes are involved controlling nucleic acid metabolism and the intramitochondrial pool of deoxynucloside triphosphates (dTNPs), the building blocks of DNA.

Mutations in *DGUOK* encoding deoxyguanosine kinase were described in Israel, causing severe hepatocerebral syndrome with neonatal liver failure, nystagmus, and hypotonia (Mandel et al. 2001).

Mutations in *TK2* encoding thymidine kinase 2 became of great interest to us for the reasons I will briefly explain later in this chapter, and are among the most

common cause of mitochondrial depletion syndrome with about 200 patients that have been reported by two groups, Hirano' and Wong's (Garone et al. 2018; Wang et al. 2018). Most of the patients with *TK2* mutations have infantile onset form, presenting at or soon after birth with generalized weakness, respiratory insufficiency, and death at 1–3 years of age. Severe skeletal muscle mtDNA depletion is commonly observed.

Lately, a life-saving therapeutic approach for these infants has been developed by Michio Hirano and Caterina Garone. The therapy provides oral deoxynucleosides (the substrates of the TK2 enzyme) and showed efficacy in ameliorating mtDNA depletion and increased lifespan in mice (Lopez-Gomez et al. 2017; Hirano et al. 2018). Up to day, this treatment has been used in 16 patients worldwide under compassionate use, the exiting results obtained in this cohort will lead to a forthcoming clinical trial.

Mutations in *SUCLA2* encoding ADP-forming succinyl-CoA synthase usually cause severe infantile encephalomyopathies (Elpeleg et al. 2005) but it may present in childhood with psychomotor delay, deafness, myopathy, ataxia and chorea (Garone et al. 2017). Mutations in *SUCLG1* encoding succinyl-CoA ligase have been associated with infantile hepatocerebral LS-like syndrome (Van Hove et al. 2010).

Mutations in *RRM2B* encoding p53-inducible ribonucleoside reductase subunit 2B caused severe infantile disease, with hypotonia, tubulopathy, seizures, respiratory distress, diarrhea, and lactic acidosis (Bornstein et al. 2008) but application of WES has revealed unusual late-onset cases with PEO (Takata et al. 2011) or KSS (Pitceathly et al. 2011), and in a case of MNGIE syndrome (Shaibani et al. 2009).

Mutation in *TYMP* encoding thymidine phosphorylase (TP), identified by Michio Hirano (Nishino et al. 1999) were associated with the severe syndrome of MNGIE (mitochondrial neurogastrointestinal encephalomyopathy). The syndrome usually affects young adults with intestinal problems (increased borborigmi; intestinal pseudoobstruction; diarrhea), PEO, severe peripheral neuropathy (axonal or demyelinating), and leukoencephalopathy. Disease course is severe, evolving in pronounced cachexia and early death. The pathogenesis of MNGIE is attributed to excessive accumulation of two toxic compounds, thymidine and deoxyuridine, causing mtDNA alterations (depletion, multiple deletions, and site-specific point mutations, isolated or in combination) (Nishigaki et al. 2004).

A useful, if risky, therapy for patients with this devastating MNGIE syndrome was introduced by Michio Hirano. It consists in allogeneic hematopoietic stem cell transplant (AHSCT, i.e. bone marrow transplantation), which showed in an international clinical trial to be able to detoxify the body from thymidine and deoxyuridine, alleviating neuropathy, and increasing body weight (Halter et al. 2015).

Mutations in *MPV17* encoding a protein in the IMM of uncertain significance were discovered in 2006 by Massimo Zeviani's group (Spinazzola et al. 2006), causing a severe infantile hepatocerebral syndrome. To our recognition, we attributed *MPV17* mutations to the Navajo neurohepatopathy (NNH), an endemic disease in the Southwestern US, characterized by childhood neuropathy, acral mutilation, corneal scarring or ulceration, liver failure, and CNS demyelination (Karadimas

et al. 2006). The MPV17 protein has been involved for ribosome assembly in mitochondria (Dalla Rosa et al. 2014).

Impairing mtDNA replication may cause severe mtDNA depletion, exemplified by Alpers-Huttenlocher syndrome (AHS) due to mutations in *POLG* encoding polymerase gamma (Nguyen et al. 2005). AHS is characterized by a triad of refractory seizures, psychomotor regression, and liver disease in children. Patients with AHS do not long survive and are particularly vulnerable to administration of valproic acid (VPA) therapy.

However, innumerable mutations throughout the *POLG* gene – even a few heterozygous – cause a variety of phenotypes, including PEO-plus, sensory ataxic neuropathy, dysarthria, and ophthalmoparesis (SANDO), mitochondrial recessive ataxia syndrome (MIRAS), and PD, all reviewed by Dr. William Copeland (Wong et al. 2008).

Multiple mtDNA deletions were first attributed to mendelian genetics by my friends Massimo Zeviani and Stefano DiDonato (Zeviani et al. 1989) and, in short sequence, the gene *ANT1* (ADP-ATP translocase) was identified mutant in a Finnish family with autosomal dominant PEO and a woman was described by Suomalainen manifesting PEO, psychiatric disorder, and carrying multiple deletions of mtDNA in her muscle, brain, liver (Suomalainen et al. 1992).

Mutations in the gene *TWNK* (or *PEO*) encoding the helicase Twinkle have been widely reported in families with autosomal dominant PEO (Paradas et al. 2013a) plus cardiomyopathy (Hong et al. 2010). A frequent presentation of recessive *TWNK* mutations is infantile onset spinocerebellar ataxia (IOSCA) (Hakonen et al. 2008) (Table 1.2).

Multiple mtDNA deletions can be observed in diseases already discussed in this text, for example many PEO-plus syndromes haven associated with mutations in *OPA1* (Yu-Wai-Man et al. 2010).

Mutations in *TK2* show – beside infantile-onset myopathy – later-onset presentations with multiple mtDNA deletions in skeletal muscle (Paradas et al. 2013b)

**Table 1.2** Intergenomic defects. Acronyms are explained in the Appendix

| Gene | mtDNA depletion | mtDNA multiple deletions |
|---|---|---|
| *DGUOK* | Hepato-cerebral syndrome | Adult-onset PEO |
| *TK2* | Lethal infantile myopathy | Later-onset myopathies |
| *SUCLA2* | Infantile encephalomyopathy | Later-onset movement disorder |
| *SUCLG1* | Hepato-cerebral syndrome | |
| *RRM2B* | Infantile encephalomyopathy | Adult-onset PEO; KSS; MNGIE |
| *TYMP* | MNGIE | |
| *MPV17* | Hepato-cerebral syndrome; NNH | Adult-onset |
| *POLG* | Alpers-Huttenlocher syndrome | Adult-onset PEO; SANDO; MIRAS; PD |
| *POLG2* | Fatal infantile liver failure | Adult-onset PEO |
| *TWNK* | | AD PEO; IOSCA |
| *ANT1* | | Adult-onset PEO, dementia |
| *OPA1* | DOA | Adult PEO-plus |

(Garone et al. 2018). A case of my friend Darryl De Vivo was puzzling for typical SMA and mitochondrial myopathy, showing childhood mtDNA depletion and multiple deletions encephalomyopathy (Pons et al. 1996). There were several adults with PEO-plus and both childhood- and late-onset patients with *TK2* mutations respond to oral deoxynucleoside therapy (Hirano et al. 2018).

An unexpected adult with PEO-plus was affected with *MPV17* mutations (Garone et al. 2012) and adult-onset autosomal dominant or recessive PEO-plus was reported in patients with mutations in *RRM2B* (Shaibani et al. 2009; Takata et al. 2011; Pitceathly et al. 2011) and in patients with *DGUOK* mutations (Ronchi et al. 2012).

Finally, mutations in *POLG2*, encoding the accessory dimeric subunit POLG2, enhancing binding to DNA, cause adult-onset dominant PEO (Longley et al. 2006) although we have recently found a homozygous *POLG2* mutation causing fatal infantile hepatic failure with mtDNA depletion (Varma et al. 2016).

## 1.5 Conclusions

To conclude this autobiographical journey through too many mitochondrial genetic diseases crowded with acronyms (see Appendix), I must say what the future will further reveal.

New pathogenic mechanisms through impaired mitochondrial dynamics will be understood, revealing through WES unexpected mutant genes.

My optimistic forecast is on therapeutic approaches, starting with idebenone in LHON (Klopstock et al), metabolic bypass in TK2-deficient (encephalo)myopathy (Garone et al. 2018), bone marrow transplantation in MNGIE (Halter et al. 2015).

And my hope is to see in the next few years healthy IVF babies with nuclear genomes from mom (carrier of a mtDNA mutation) and dad, and normal mitochondria from a donor normal woman (mitochondria replacement therapy, MRT). This dream will be realized by Douglass Turnbull (Herbert and Turnbull 2017) and Michio Hirano (Engelstad et al. 2016) and many creatures will be free from devastating MELAS, MERRF, NARP/MILS.

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# Chapter 2
# Mutations in Assembly Factors Required for the Biogenesis of Mitochondrial Respiratory Chain

**Cristina Cerqua, Lisa Buson, and Eva Trevisson**

**Abstract** The mitochondrial respiratory chain, which provides to the cells most of their ATP requirement, is composed of five multisubunit complexes. Its biogenesis is a multi-step process characterized by the sequential formation of intermediate assemblies composed of subunits encoded by two distinct genomes, mitochondrial or nuclear DNA.

This process is assisted by a diverse set of ancillary proteins of nuclear origin called assembly factors that are not part of the final complexes and exert different functions.

Mutations in several genes encoding these proteins have been identified in patients affected by mitochondrial diseases, exceeding those found in genes encoding structural subunits for some complexes.

The hallmark of these disorders, which are often multisystemic and mainly affect high energy demanding organs, is the broad genetic and clinical heterogeneity, making their diagnosis problematic. The number of assembly factors associated with human diseases is rapidly increasing, owing to the employment of next generation sequencing methods in the diagnostic workflow.

Therapy for these conditions is mostly based on supportive care, emphasizing the need to elucidate their pathological mechanisms to find novel treatments.

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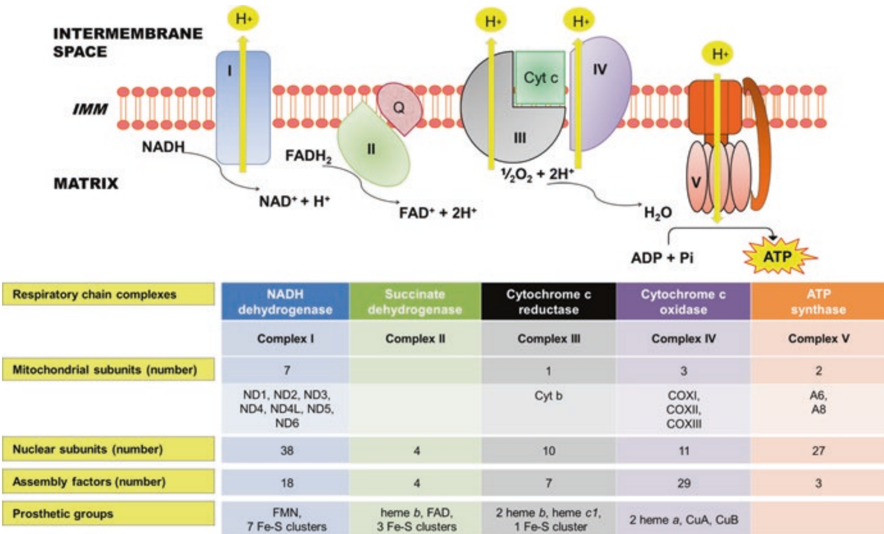
## 2.1 Introduction

The mitochondrial respiratory chain (MRC) is composed of five multiprotein complexes embedded in the inner mitochondrial membrane (IMM) and two mobile electron carriers [Coenzyme Q (CoQ) or ubiquinone and cytochrome *c* (cyt *c*)]. The first four complexes transport electrons to molecular oxygen through the oxidative phosphorylation, generating a proton gradient from the matrix to the intermembrane space (IMS) that sustains the production of ATP by Complex V (Fig. 2.1). MRC complexes contain a number of prosthetic groups and metal cofactors that constitute the redox centers required for the electron transport and ATP generation. This process provides to the cells most of the energy that they need, and its defects are the hallmark of mitochondrial diseases, among the most common inborn errors of metabolism (Frazier et al. 2019).

In higher eukaryotes, complexes I, III and IV associate with each other, restricting the electron carrier diffusion and forming supramolecular structures, called supercomplexes (Fig. 2.2). Based on *in vitro* experiments, it seems that they enhance MRC activity, stabilize the individual complexes and minimize electron leakage that could produce reactive oxygen species (ROS) (Lapuente-Brun et al. 2013; Letts and Sazanov 2017; Lobo-Jarne and Ugalde 2018).

The biogenesis of each respiratory complex and supercomplex is based on a tight regulation and coordination of both nuclear and mitochondrial genomes (Fontanesi 2013). Indeed, except for Complex II, which relies only on nuclear DNA for its biogenesis, proteins that compose the MRC are encoded by both the nuclear (nDNA)




| Respiratory chain complexes | NADH dehydrogenase | Succinate dehydrogenase | Cytochrome c reductase | Cytochrome c oxidase | ATP synthase |
|---|---|---|---|---|---|
| | Complex I | Complex II | Complex III | Complex IV | Complex V |
| Mitochondrial subunits (number) | 7 | | 1 | 3 | 2 |
| | ND1, ND2, ND3, ND4, ND4L, ND5, ND6 | | Cyt b | COXI, COXII, COXIII | A6, A8 |
| Nuclear subunits (number) | 38 | 4 | 10 | 11 | 27 |
| Assembly factors (number) | 18 | 4 | 7 | 29 | 3 |
| Prosthetic groups | FMN, 7 Fe-S clusters | heme *b*, FAD, 3 Fe-S clusters | 2 heme *b*, heme *c1*, 1 Fe-S cluster | 2 heme *a*, CuA, CuB | |

**Fig. 2.1** Composition of mitochondrial respiratory chain. The diagram reports the number of mitochondrial and nuclear subunits, assembly factors and prosthetic groups required for each complex

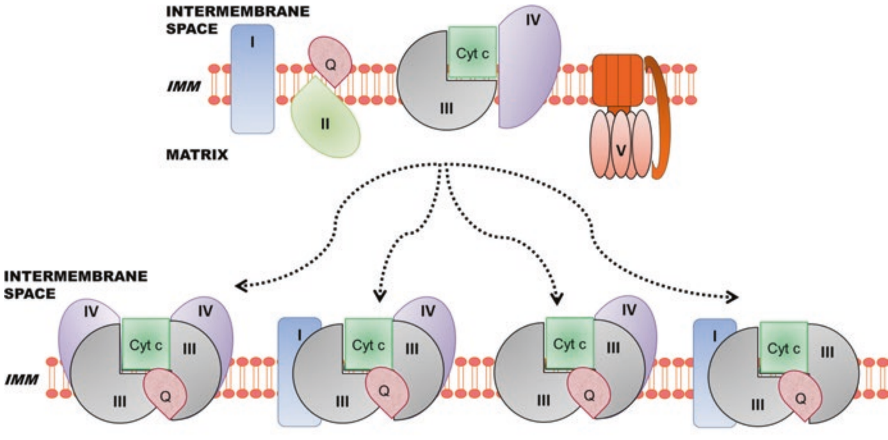



**Fig. 2.2** Representation of the current model of supercomplexes formation (Lobo-Jarne and Ugalde 2018). Within the inner mitochondrial membrane, single respiratory complexes coexist with supramolecular structures, called supercomplexes, which are formed by the association among complexes I, III and IV with different stoichiometry, and some of them are represented in the picture

and the mitochondrial (mtDNA) DNA (Fig. 2.1). Most of them are synthesized in the nucleus and subsequently transported into mitochondria (Wasilewski et al. 2017). The few mitochondrially encoded proteins (seven for Complex I, one for Complex III, three for Complex IV and two for Complex V) constitute the catalytic core of the complexes. The coordination and interplay between mitochondrial and nuclear genomes require specific proteins, called translational activators, which allow the translation of mitochondrial mRNAs (Rampelt and Pfanner 2016).

Most of the current knowledge on subunit composition and assembly of MRC complexes is based on studies performed in the yeast *Saccharomyces cerevisiae*, except for Complex I, which is not present in this unicellular eukaryote. The biogenesis of each respiratory complex is a multi-step process characterized by the sequential formation of different submodules that associate with each other to form the final functional holoenzyme. This pathway is supported by a plethora of ancillary accessory proteins called assembly factors that are not part of the final complexes, but are required for its formation and/or stability. Assembly factors exert different functions, including translation, co-factor synthesis and incorporation, subunit insertion into membranes, prevention of pro-oxidant partial assembled complexes and stabilization of intermediates (Barros and Mcstay 2020). Their importance is underlined by the variety and severity of phenotypes resulting from their loss of function mutations, and in fact, pathogenic variants in several distinct assembly factors have been identified in patients affected by mitochondrial disorders.

Mitochondrial diseases consist of a group of genetically and clinically heterogeneous conditions, which result in an impairment of ATP synthesis and/or chronic oxidative stress. They are typically multisystemic disorders and, although they

might affect any tissue, usually high energy-demanding organs, such as skeletal muscle, brain, heart or liver are the most frequently involved (Ghezzi and Zeviani 2018).

Their diagnosis can be challenging and requires a multidisciplinary approach; indeed, despite the advance in sequencing technologies, a subset of patients still lack a molecular diagnosis.

Treatment is mostly supportive and no etiologic cure for these disorders exists, mainly because of the poor knowledge of the pathogenetic mechanisms underlying them, and current interventions are only effective for a handful of these conditions (Pfeffer et al. 2013).

In this review, we will illustrate the current knowledge on assembly factors required for the proper functioning of MRC, on the syndromes associated with their defects and on the current available treatments. Proteins required for the biosynthesis of the CoQ, an electron carrier necessary for the proper functioning of the oxidative phosphorylation, will not be discussed in this issue, since a detailed description of its function and its involvement in human disorders is reported by Brea-Calvo and co-authors in a dedicated chapter in this book.

## 2.2 Assembly of MRC Complexes

### *2.2.1 Complex I*

The NADH:CoQ oxidoreductase, also called Complex I (CI), is the largest MRC complex and the major contributor of the proton motive force that generates cellular energy. In humans, it is composed of 45 structural subunits, including seven proteins (MTND1, 2, 3, 4, 4L, 5, 6) encoded by mtDNA.

CI is constituted by two arms: a hydrophilic peripheral one that extends into the matrix and a membrane arm (Agip et al. 2019). The peripheral arm consists of two modules: in the N module, Nicotinamide Dehydrogenase (NADH) is oxidized to $NAD^+$ by the primary electron acceptor flavin mononucleotide (FMN) and electrons are passed through a chain of iron-sulphur clusters to CoQ, that is then reduced in the Q module that connects the two arms. The P module of the membrane arm catalyzes proton transfer across the IMM. CI is also one of the most significant producer and regulator of mitochondrial ROS, and in humans, it is found exclusively bound to Complex III to form respiratory supercomplexes (Lenaz et al. 2016) (Fig. 2.2).

CI assembly requires the coordination of six assembly modules: ND1, ND2, ND4, ND5, embedded in the IMM, N and Q, that protrude to the matrix (Formosa et al. 2018). The entire process involves a series of assembly factors that are crucial also for preventing oxidative damage by ROS. The chaperones NDUFAF3, NDUFAF4 and NDUFAF6 bind to the Q module and are essential for the ND1 core subunit biogenesis. The hydroxylation of the NDUFS7 subunit mediated by NDUFAF5 is required for the incorporation of ND1 and ND2 and the formation of

the module containing NDUFS2, which is dimethylated by NDUFAF7. NUBPL binds the Fe–S clusters via a conserved CxxC motif and incorporates them in the subunits of N and Q modules. Also the assembly factor TIMMDC1A is involved in the ND1 module formation. NDUFAF1, Evolutionary Conserved Signaling Intermediate in Toll pathways (ECSIT), Acyl-CoA Dehydrogenase 9 (ACAD9) and TMEM126B bind to the ND2 module during its assembly and are necessary for its stabilization. They form the Mitochondrial Complex I Assembly (MCIA) complex. Other assembly factors that are linked to the ND2 module include TMEM186 and the chaperone COA1, which is also involved in Complex IV biogenesis. ND4 module formation is assisted by FOXRED1, DMAC2 and TMEM70, that participates also in Complex V assembly. DMAC1 is involved in ND5 formation and NDUFAF2 stabilizes the last intermediate that lacks only the N module (Mckenzie and Ryan 2010; Stroud et al. 2016).

### *2.2.2 Complex II*

The succinate:CoQ reductase, also named succinate dehydrogenase (SDH) or Complex II (CII), is the second and the smallest complex of the MRC. It is also part of the Krebs cycle (TCA cycle). After succinate oxidation, electrons are transferred to the cofactor flavin adenine dinucleotide (FAD) and through a chain of Fe–S clusters to ubiquinone, that is reduced to ubiquinol. SDH is a heterotetrameric complex composed by 4 subunits all encoded by nuclear genes: SDHA and SDHB are the catalytic subunits, which protrude to the matrix and are anchored to the IMM through the subunits SDHC and SDHD; the latter are integral membrane proteins and contain a heme *b* prosthetic group, which is crucial for their stability, and two CoQ binding sites (Sun et al. 2005).

CII assembly is a multistep process characterized by the maturation of three modules: SDHA, SDHB and that formed by SDHC and SDHD (Moosavi et al. 2019). Four assembly factors have been found to be required in this process: the chaperones SDHAF1, SDHAF2, SDHAF3, and SDHAF4. SDHAF2 flavinates the SDHA subunit and then binds to SDHAF4, which is located in the matrix and is involved in the binding between SDHA and SDHB. SDHAF1 and SDHAF3 contain a LYR motif characteristic of proteins involved in Fe–S delivery. They promote the maturation of the SDHB subunit, by cooperating for the Fe-S incorporation and protecting CII against ROS damage (Na et al. 2014).

### *2.2.3 Complex III*

Ubiquinol-cyt *c* oxidoreductase, also called bc1 complex or Complex III (CIII), catalyzes the electrons transfer from CoQ to cyt *c,* a soluble protein localized in the IMS. It is a homodimer, and each monomer contains three catalytic cores, Cyt *b*/

MT-CYB, Cyt $c_1$/CYC1, the Rieske Fe–S protein (Rip1/UQCRFS1) and other seven or eight supernumerary subunits, in yeast or in mammals, respectively (Xia et al. 2013). MT-CYB, the only mtDNA-encoded subunit and central core of the complex, contains two prosthetic groups, heme *b*562 ($b_H$) and heme *b*565 ($b_L$), and two CoQ binding sites. CYC1 contains a covalently attached c-type heme and Rip1 contains a 2Fe-2S cluster. After CoQ oxidation, one electron is transported to Rip1 and CYC1, which reduces cyt *c*. The second electron passes through heme $b_H$ and heme $b_L$, reducing ubiquinone to semiubiquinone. This electron transfer is coupled to the pumping of two protons across the IMM and this process is called Q-cycle (Crofts et al. 2008).

CIII biogenesis is a modular pathway with the sequential formation of five assembly intermediates (Ndi et al. 2018). The first step is the recruitment and stabilization of MT-CYB, mediated by a complex formed by assembly factors UQCC1 and UQCC2. The presence of one of these two proteins is required for the stability of the other. UQCC3 binds to cardiolipin and is involved in the stabilization of CIII-containing supercomplexes. The holocytochrome $c_1$ synthethase (HCC1S) is crucial for the haemylation of CYC1 and CCHL chaperones the heme to the apoprotein of cyt *c*. The assembly factors Bcs1/BCS1L and Mzm1/LYRM7 are involved in the formation of the fourth intermediate. They are required for the recruitment and stabilization of Rip1. The assembly factor TTC19 binds CIII, inducing the release of Rip1 N-terminal fragments to stabilize activity levels (Fernandez-Vizarra and Zeviani 2018).

### *2.2.4 Complex IV*

Cyt *c* oxidase (COX), also called Complex IV (CIV), is the terminal electron acceptor of the MRC, oxidizing cyt *c* to reduce molecular oxygen to water (Wikstrom et al. 2018). In humans, it is a homodimer composed of 14 subunits per monomer and four redox centers that enable electron transfer reactions. Three mtDNA-encoded core subunits, MT-CO1, MT-CO2, and MT-CO3 are integral proteins of the IMM and form the catalytic site. The other structural subunits and assembly factors are encoded by the nuclear DNA (Timon-Gomez et al. 2018). MT-CO1 contains two heme groups (*a* and $a_3$) and a $Cu_B$ center required for its stability and folding, as well as enzymatic activity. MT-CO2 contains a $Cu_A$ binuclear center that accepts electrons from ferrocytochrome *c* and passes them to the heme *a* group, from whom they are transferred to the heme $a_3$ paired with a $Cu_B$ site and finally to oxygen.

COX assembly has been extensively characterized by Blue-Native electrophoresis using *S. cerevisiae* and COX deficient patient-derived cell lines. It is a modular process that requires many COX assembly factors. In humans, the first step is constituted by the interaction of the structural subunits COX4I1 and COX5A that are stabilized by the assembly factor HIGD1A, one of the human homologues of yeast Rcf1, a protein involved in supercomplexes formation (Vukotic et al. 2012). This

complex binds to the first assembly intermediate, the MT-CO1 module, built up around MT-CO1. In yeast, Cox1 (the orthologue of MT-CO1), is inserted into the IMM by the insertase Oxa1, whereas human OXA1L is required also for CI and Complex V biogenesis (Stiburek et al. 2007). The MT-CO1 module is called MITRAC (mitochondrial translation regulation assembly intermediate of COX) and its gradual formation is assisted by many assembly factors: COX14 and COA3 (MITRAC12) are the first proteins to bind it. They are crucial also for MT-CO1 translation, which is regulated especially by the Leucine-rich pentatricopeptide repeat containing protein (LRPPRC), a post-transcriptional regulator of mitochondrial DNA expression, and by the translational activator TACO1. The twin $CX_9C$ protein CMC1 stabilizes the COX1-COX14-COA3 complex, while COX10, COX15, SURF1, CCHL and yeast Coa2 are involved in heme homeostasis (Kim et al. 2012). The metallochaperones COX17, COX11, CMC1, CMC2, COX19 and COX23 are required for $Cu_B$ biogenesis (Horn et al. 2010; Leary et al. 2013b): they contain twin $CX_9C$ motifs that are recognized by the MIA40–ERV1 import pathway within the IMS, which oxidizes them to form a pair of disulfide bonds. After the CMC1 release, the chaperones COA1 (MITRAC15), MITRAC7/SMIM20 and SURF1 bind to MITRAC. The MT-CO1 module joins to the second assembly intermediate, the MT-CO2 module, containing MT-CO2 and the structural subunits COX5B, COX6C, COX7C, COX8A and COX7B. COX18, the chaperone COX20 and TMEM177 are involved in the insertion and stabilization of MT-CO2 in the IMM. The chaperones COX16, COX17, SCO1, SCO2 and COA6 are responsible for copper metallation of $Cu_A$ site. COX17 receives copper from an anionic fluorescent molecule, the copper ligand (CuL), located in the matrix, and transfers it to the paralogs SCO2 and SCO1, which exert non-overlapping functions. They interact with COA6, that also contains a twin $CX_9C$ motif, then copper is finally transferred to the $Cu_A$ center (Jett and Leary 2018). COX16 is involved in the association between SCO1 and MT-CO2 and between MT-CO1 and MT-CO2 (Cerqua et al. 2018). Assembly factors involved in late stages of MT-CO2 assembly are the chaperones MR-1S, PET100 and PET117. The third intermediate, the MT-CO3 module containing MT-CO3 and the structural subunits COX6A1, COX6B1 and COX7A2 join to the preassembled CIV, whose maturation is achieved after the incorporation of the last subunit, NDUFA4. COX7A2L/COX7R/SCAFI, an orthologue of COX7A, and HIGD2A, a homologue of yeast Rcf1, are involved in the incorporation of CIV into supercomplexes (Chen et al. 2012; Perez-Perez et al. 2016).

### *2.2.5 Complex V*

The $F_1F_o$ATP synthase, or Complex V (CV) is the fifth enzyme of MRC, which utilizes the proton motive force created by the other complexes to produce ATP through ADP phosphorylation. Moreover, it plays a key role in shaping *cristae* structure. CV is a large multisubunit protein formed by a $F_o$ domain, located inside the IMM, which mediates the proton pumping, and a $F_1$ domain, which catalyzes the

ATP synthesis (Walker 2013). In humans, complex V is constituted by 29 subunits, which, except for MT-ATP6 and MT-ATP8 that are mtDNA-encoded, derive from nDNA.

The $F_1$ domain is formed by a globular hexamer and a central stalk, that connects it to the $F_o$ domain. The hexamer consists of three dimers of α/ATP5A1 and β/ATP5B subunits. Each dimer contains a nucleotide binding pocket. The γ/ATP5C1 subunit, which binds to δ/ATP5D and ε/ATP5E subunits, forming the central stalk, is situated at the center of the hexamer. The $F_o$ domain is formed by subunits *a*/MT-ATP6, A6L/MT-ATP8, *b*/ATP5F1 and *c*/Atp9p. The c8-ring is constituted by eight subunits *c* that bind to MT-ATP6. The peripheral stalk is formed by subunits *b*/ATP5F1, *d*/ATPH, F6/ATP5J, OSCP/ATP5O, *e*/ATP5I, *g*/ATP5L and *f*/ATPJ2. MT-ATP8 forms a domain with subunits *e*/ATP5I and *g*/ATP5L and its C terminus protrudes to the matrix as part of the peripheral stalk (Srivastava et al. 2018).

CV assembly is characterized by the sequential formation of three modules: the $F_1$, the $c_8$-ring and the peripheral stalk (He et al. 2018). $F_1$ biogenesis is assisted by the chaperones ATPAF1/ATP11 and ATPAF2/ATP12, which bind ATP5B and ATP5A1, respectively. Then $F_1$ binds to the $c_8$-ring and to the peripheral stalk. Subunits *e*/ATP5I, *f*/ATPJ2, *g*/ATP5L, *k*/DAPIT and *i*/*j*/6.8PL are involved in the stability of CV and TMEM70 is required for the incorporation of subunit *c* into the rotor structure of the enzyme (Kovalcikova et al. 2019).

## 2.3 Pathogenesis

Despite the pathophysiology of many mitochondrial disorders is not completely understood, and distinct defects may display tissue-specific effects and/or peculiar phenotypic features, failure to correctly assemble the MRC complexes results in some common pathogenetic mechanisms, which can be summarized in bioenergetic defect and oxidative damage. In fact, since mutations of assembly factors impact on the correct formation of the functional respiratory complexes and supercomplexes, the consequent mitochondrial dysfunction causes a decreased oxygen consumption and ATP synthesis, and induces an excess of ROS generation (Distelmaier et al. 2009). Organs with high metabolic requirements are the most affected by the impairment of energy supply. Indeed, tissues and cells of patients with mitochondrial diseases caused by mutations in various assembly factors display reduced MRC activities. The reduced ATP production can also affect cellular calcium dynamics, inducing an abnormal calcium uptake (Granatiero et al. 2017).

The increase in ROS production can result from a strong decrease of respiratory complex activity and/or from disruption of supercomplexes (Lobo-Jarne and Ugalde 2018). The consequent oxidative stress within mitochondria can lead to apoptosis, which is the main feature of the neurodegenerative phenotypes that characterize many mitochondrial disorders. Other consequences are mitochondrial fragmentation, resulting from the decreased MRC activity and ROS dysregulation, and a decrease of membrane potential, that is deleterious for energy production and

import of mitochondrial proteins (Koopman et al. 2016). There is evidence that also *cristae* organization may be affected, with consequent dysfunctional mitochondria, as in the case of mutations of CV assembly factors (Cameron et al. 2011).

The clinical variability observed in some patients with mitochondrial diseases due to defects in assembly factors may rely on different issues, including: (i) the type of genetic variants (hypomorphic versus null alleles); (ii) the specific function(s) of the assembly factor (some inactivating mutations are associated with relatively high residual MRC activity, possibly due to redundant functions, as in the case of COX16 (Cerqua et al. 2018); (iii) the presence of different isoforms for some nuclear genes encoding assembly (and structural) factors that often display specific spatial-temporal patterns of expression and are transcribed under specific conditions. This is the case for example of SCO2 and SURF1, two assembly factors required for the assembly of CIV, which exhibit tissue-specific functional differences required for COX biogenesis (Stiburek et al. 2005); also BCS1L, a factor required for CIII assembly, whose mutations have been associated with a wide clinical spectrum, displays a tissue-specific expression profile that may influence phenotypic variability (Ramos-Arroyo et al. 2009).

The phenotype associated with defects in assembly (or structural) factors of CII differs from those associated with the other MRC complexes. It should keep in mind that CII, which is the only complex that does not require mtDNA for its biogenesis, participates in electron transport of MRC and it is also required for the conversion of succinate to fumarate in the TCA cycle.

While biallelic pathogenic variants in some structural (SDHA, SDHB and SDHD) or ancillary (SDHAF1) proteins have been reported in patients with CII deficiency (Alston et al. 2012, 2015; Ghezzi et al. 2009a), manifesting the typical symptoms of other MRC defects, including Leigh syndrome (Burgeois et al. 1992), heterozygous mutations in some genes required for CII functioning (*SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*) have been found to confer a predisposition to paraganglioma, tumors arising from the neuroendocrine tissue (Baysal et al. 2000; Burnichon et al. 2010; Hao et al. 2009). Their development has been associated with the accumulation of succinate, which, inhibiting the prolyl hydroxylase (PHD), leads to the accumulation of hypoxia-inducible factor (HIF) and inflammation. Consequently, cells become more resistant to apoptosis and activate a pseudohypoxic program that favors glycolysis (Moosavi et al. 2020). The succinate accumulation has also an epigenetic effect, increasing histone methylation (Tretter et al. 2016).

## 2.4 Diagnosis of Assembly Factors Defects

Mitochondrial disorders due to defects in oxidative phosphorylation may be associated with a broad range of clinical manifestations, making the diagnosis challenging. Despite organs with high energetic requirements are typically affected, virtually any tissue may be involved.

Important clues for the diagnosis may derive from brain magnetic resonance imaging (MRI) (see below), as in the case of Leigh syndrome. Lactic acidosis, which is often but non invariably present in patients with mitochondrial disorders, results from an accumulation of NADH that inhibits the TCA cycle and leads to an increase of pyruvate produced through glycolysis, which in turn is converted in lactate. High levels of serum lactate in the presence of encephalomyopathy with or without other symptoms is highly suggestive of a mitochondrial disorder; however, the sensitivity of this laboratory parameter is low, since a normal value does not exclude this diagnosis, but it may be increased by measuring cerebrospinal fluid lactate.

The classical approach for the diagnosis of mitochondrial disorders includes the biochemical assessment of MRC complexes activities by spectrophotometric assay in biopsied skeletal muscle (Spinazzi et al. 2012). This analysis allows to confirm and define a defect in MRC activity, which can be isolated (CI and CIV deficiencies are the most frequent) or combined (I, III and IV). The specific combination of defects might in turn provide clues on which genome is involved (for instance, a combined defect of all complexes except for CII is observed in the presence of mutations in mtRNA genes) or suggests an electron carrier defect (an impairment of combined activities of Complex I+III and II+III with normal individual complex activity indicates a CoQ deficiency) (Alcazar-Fabra et al. 2018).

Pathogenic mutations in assembly factors are usually characterized by isolated biochemical defects, affecting a single complex. Exceptions are provided by few ancillary proteins that participate in the assembly of more complexes, such as OXA1L, which is required for the cotranslational membrane insertion of a subset of mtDNA-encoded subunits and for the insertion of nDNA-encoded IMM proteins: its deficiency in humans causes a combined oxidative phosphorylation defect, with impairment of Complex I, IV and V (Thompson et al. 2018).

Biochemical defects may also be measured on patients' skin fibroblasts (Montero et al. 2008; Rustin et al. 1994; Pereira et al. 2018); however, partial or tissue-specific deficiencies may be difficult to detect, as in the case of *SCO2* mutations, where patient's muscle displays severe COX deficiency, while skin fibroblasts exhibit only a mild defect (Salviati et al. 2002), further complicating the diagnostic assessment.

The spectrophotometric measurement of MRC complex activities is considered a third-level analysis, which is usually not routinely performed in most laboratories, since it requires high specialization. In the last decade, this limitation has been mostly overcome by the development of cost-effective next generation technologies, which allows high throughput gene analyses, including all the known genes encoding mitochondrial proteins or even the whole exome (WES) or genome (WGS), at limited costs and in a relatively short time (Thompson et al. 2020; Wong 2013). This innovation has led to an overturning of the diagnostic workflow, making biochemical and functional assessments secondary to genetic variant identification.

It should be noted that, considering the complexity of the biogenesis of MRC and the incomplete knowledge of its formation and regulation, many patients still lack a molecular diagnosis.

## 2.5 Clinical Manifestations of Assembly Factors Defects

The clinical manifestations associated with mutations of nuclear assembly factors rely on the biochemical defect(s) and on the resulting pathophysiological mechanisms, which consist mainly (but not exclusively) of a bioenergetic defect and a ROS overload. Therefore, apart from few exceptions, the phenotype is not specific for a given complex and many of these conditions manifest with a similar symptomatological constellation.

The main clinical presentations comprise encephalopathy (a broad collection of neurological manifestations that include psychomotor retardation/regression, seizures, hypotonia, ataxia) and Leigh syndrome (LS, MIM #256000). LS is a genetically and biochemically heterogeneous neurodegenerative disorder, with onset in infancy (usually before 2 years of age) and a subsequent rapid progression (Aulbert et al. 2014). Clinical features include a severe brainstem and/or basal ganglia dysfunction with neurodevelopmental delay, hypotonia, ataxia, epilepsy, lactic acidosis and respiratory distress. Death usually is caused by respiratory failure consequent to brainstem dysfunction. The typical neuroradiological features observed at the MRI direct the diagnosis and consist of symmetrical hyperintensity in T2-weighted images in basal ganglia and/or brainstem; spectroscopy may show a lactate peak in affected areas; additionally, other sites that may be involved comprise cerebellum, thalamus, cerebral white matter and spinal cord.

LS is extremely heterogeneous from a genetic viewpoint: the number of genes that have been associated with this condition is over 75 so far and is still increasing, including genes encoding structural and assembly factors, beyond those required for the interplay between mtDNA and nDNA. Despite the genetic heterogeneity, among MRC assembly defects, LS is associated more frequently with CI or CIV deficiency (Schubert Baldo and Vilarinho 2020).

Mutations in several genes encoding MRC assembly factors have been identified in patients manifesting various mitochondrial disorders (Table 2.1). Except for few exceptions, most of these conditions are inherited in an autosomal recessive manner. In the following section, we will describe for every MRC complex the different phenotypes related to these mutations.

### *2.5.1 Mutations of CI Assembly Factors*

CI deficiency (MIM# 252010), the most common biochemical manifestation of MRC defects in infancy and childhood, is a severe multisystem disorder, that frequently presents with neuromuscular and neurodegeneration features (Rodenburg 2016). A signature of a defect in CI is leukoencephalopathy (Baertling et al. 2014). Onset of the disease is typically early, with rapid progression and a poor prognosis.

CI deficiency can be either found in isolation isolated or combined with defects of other complexes. The main pathogenetic mechanisms follow those of other MRC

**Table 2.1** Human MRC assembly factors with their corresponding yeast orthologues. For those that have been identified mutated in patients, the associated phenotype is reported

| Yeast | | Human | | |
|---|---|---|---|---|
| Gene | Protein | Gene | Protein | Associated phenotype |
| **COMPLEX I** | | | | |
| – | – | *ACAD9* | ACAD9 | Hypertrophic cardiomyopathy, severe lactic acidosis, Leigh syndrome, leukodystrophy, exercise intolerance (Haack et al. 2010; Nouws et al. 2014; Lagoutte-Renosi et al. 2015; Collet et al. 2016; Leslie et al. 2016; Dewulf et al. 2016; Schrank et al. 2017; Fragaki et al. 2017; Finsterer and Zarrouk-Mahjoub 2017; Repp et al. 2018; Kadoya et al. 2019) |
| | | [a, b]*AIFM1* | AIFM1 | Encephalomyopathy, axonal sensorimotor neuropathy, deafness and cognitive impairment (Ghezzi et al. 2010; Diodato et al. 2016) |
| – | – | [a]*COA1* | COA1 | |
| – | – | *ECSIT* | ECSIT | |
| – | – | *FOXRED1* | FOXRED1 | Leigh syndrome, microcephaly, cortical blindness, hypertrophic cardiomyopathy, leukodystrophy (Calvo et al. 2010; Fassone et al. 2010; Zurita Rendon et al. 2016; Apatean et al. 2019; Barbosa-Gouveia et al. 2019) |
| – | – | *NDUFAF1* | CIA30 | Cardioencephalomyopathy, Leigh syndrome, leukodystrophy, retinopathy (Dunning et al. 2007; Fassone et al. 2011; Wu et al. 2016) |
| – | – | *NDUFAF2* | B17.2 L | Encephalopathy, Leigh syndrome, leukodystrophy, hyperactivity disorder (Ogilvie et al. 2005; Barghuti et al. 2008; Calvo et al. 2012; Herzer et al. 2010) |
| – | – | *NDUFAF3* | NDUFAF3 | Macrocephaly, axial hypotonia, brain leukomalacia, fatal infantile lactic acidosis, encephalopathy (Saada et al. 2009; Baertling et al. 2017a; Ishiyama et al. 2018) |
| – | – | *NDUFAF4* | NDUFAF4 | Cardiomyopathy, Leigh syndrome, leukodystrophy, encephalopathy (Baertling et al. 2017b) |
| – | – | *NDUFAF5* | NDUFAF5 | Leigh syndrome, extrapyramidal choreodystonic movement disorder, fatal infantile lactic acidosis (Sugiana et al. 2008; Gerards et al. 2010; Saada et al. 2012; Simon et al. 2019) |
| – | – | *NDUFAF7* | NDUFAF7 | |
| – | – | *NUBPL* | NUBPL | Leukoencephalopathy, spasticity, Leigh syndrome (Calvo et al. 2010; Kevelam et al. 2013; Protasoni et al. 2020; Friederich et al. 2020) |

(continued)

**Table 2.1** (continued)

| Yeast | | Human | | |
|---|---|---|---|---|
| Gene | Protein | Gene | Protein | Associated phenotype |
| – | – | *TIMMDC1* | TIMMDC1 | Peripheral neuropathy, Leigh syndrome (Kremer et al. 2017) |
| – | – | *TMEM126B* | TMEM126B | Exercise intolerance, hypertrophic cardiomyopathy, renal tubular acidosis, myalgia (Sanchez-Caballero et al. 2016; Alston et al. 2016) |
| – | – | *TMEM186* | TMEM186 | |
| | | *DMAC1* | DMAC1 | |
| – | – | *DMAC2* | DMAC2 | |
| – | – | [a]*TMEM70* | TMEM70 | Lactic acidosis, cardiomyopathy, 3-methylglutaconic aciduria (Cizkova et al. 2008; Honzik et al. 2010; Spiegel et al. 2011; Torraco et al. 2012; Catteruccia et al. 2014; Diodato et al. 2015; Magner et al. 2015; Braczynski et al. 2015; Sarajlija et al. 2017; Hirono et al. 2019; Staretz-Chacham et al. 2019) |
| **COMPLEX II** | | | | |
| *SDH6* | Sdh6 | *SDHAF1* | SDHAF1 | Spastic quadriplegia, infantile leukoencephalopathy (Ghezzi et al. 2009a; Ohlenbusch et al. 2012; Zhu et al. 2015; Maio et al. 2016) |
| *SDH5* | Sdh5 | *SDHAF2* | SDHAF2 | Pheochromocytoma, paraganglioma (Bayley et al. 2010; Rattenberry et al. 2013; Casey et al. 2014; Zhu et al. 2015; Wolf et al. 2019; Roose et al. 2020) |
| *SDH7* | Sdh7 | *SDHAF3* | SDHAF3 | Paraganglioma (Dwight et al. 2017) |
| *SDH8* | Sdh8 | *SDHAF4* | SDHAF4 | |
| **COMPLEX III** | | | | |
| *CBP3* | Cbp3 | *UQCC1* | UQCC1 | |
| *CBP6* | Cbp6 | *UQCC2* | UQCC2 | Delayed psychomotor development, hearing impairment, hypotonia, seizures, metabolic acidosis, renal tubular dysfunction (Tucker et al. 2013; Feichtinger et al. 2017) |
| *CBP4* | Cbp4 | *UQCC3* | UQCC3 | Lactic acidosis, hypoglycemia, hypotonia, delayed psychomotor development, intellectual disability (Wanschers et al. 2014) |

(continued)

**Table 2.1** (continued)

| Yeast | | Human | | |
|---|---|---|---|---|
| Gene | Protein | Gene | Protein | Associated phenotype |
| *BCS1* | Bcs1 | *BCS1L* | BCS1L | Tubulopathy, GRACILE syndrome, Bjornstad syndrome, Leigh syndrome, bilateral cataracts, psychomotor retardation, liver disease, encephalopathy (de Lonlay et al. 2001; Fernandez-Vizarra et al. 2007; Hinson et al. 2007; Ramos-Arroyo et al. 2009; Tuppen et al. 2010; Falco et al. 2017; Shigematsu et al. 2017; Olahova et al. 2019) |
| *MZM1* | Mzm1 | *LYRM7* | LYRM7 | Hypotonia, leukoencephalopathy, neurologic dysfunctions, intellectual disability, metabolic acidosis (Invernizzi et al. 2013; Dallabona et al. 2016; Kremer et al. 2016; Hempel et al. 2017) |
| – | – | *TTC19* | TTC19 | Developmental disability, atrophy, Leigh syndrome, psychiatric manifestations (Ghezzi et al. 2011; Nogueira et al. 2013; Atwal 2014; Morino et al. 2014; Ardissone et al. 2015; Koch et al. 2015; Conboy et al. 2018; Habibzadeh et al. 2019) |
| *CYT2* | Cyt2 | [b]*HCCS* | CCHL | Microphtalmia with linear skin defects (Wimplinger et al. 2006) |
| **COMPLEX IV** | | | | |
| *OXA1* | Oxa1 | *OXA1L* | OXA1L | |
| SHY1 | Shy1 | *SURF1* | SURF1 | Leigh syndrome, intellectual disability, Charcot-Marie-Tooth Disease Type 4 K (Tiranti et al. 1998; Piekutowska-Abramczuk et al. 2009; Echaniz-Laguna et al. 2013; Aulbert et al. 2014; Ribeiro et al. 2016; Maalej et al. 2018; Danis et al. 2018; Li et al. 2018) |
| – | – | [a]*COA1* | COA1 | |
| *COA3* | Coa3 | *COA3* | COA3 | Peripheral neuropathy (Ostergaard et al. 2015) |
| *PET191* | PET191 | *COA5* | COA5 | Cardioencephalomyopathy (Huigsloot et al. 2011) |
| *COA6* | Coa6 | *COA6* | COA6 | Hypertrophic cardiomyopathy (Baertling et al. 2015) |

(continued)

**Table 2.1** (continued)

| Yeast | | Human | | |
|---|---|---|---|---|
| Gene | Protein | Gene | Protein | Associated phenotype |
| – | – | *COA7* | COA7 | Spinocerebellar ataxia with axonal neuropathy-3, leukoencephalopathy (Martinez Lyons et al. 2016; Higuchi et al. 2018) |
| – | – | *COA8* | COA8 | Leukodystrophy (Melchionda et al. 2014; Sharma et al. 2018) |
| *COX10* | Cox10 | *COX10* | COX10 | Pyramidal syndrome, hypertrophic cardiomyopathy, sensorineural hearing loss, Leigh syndrome, leukodystrophy, tubulopathy (Valnot et al. 2000b; Antonicka et al. 2003a) |
| *COX11* | Cox11 | *COX11* | COX11 | |
| *COX14* | Cox14 | *COX14* | COX14 | Brain hypertrophy, hepatomegaly, hypertrophic cardiomyopathy, renal hypoplasia, adrenal hyperplasia, fatal neonatal lactic acidosis (Weraarpachai et al. 2012) |
| *COX15* | Cox15 | *COX15* | COX15 | Leigh syndrome, cardioencephalomyopathy (Antonicka et al. 2003b; Bugiani et al. 2005; Alfadhel et al. 2011; Miryounesi et al. 2016; Halperin et al. 2020) |
| *COX16* | Cox16 | *COX16* | COX16 | |
| *COX17* | Cox17 | *COX17* | COX17 | |
| *OXA1* | Oxa1 | *COX18* | COX18 | |
| *COX19* | Cox19 | *COX19* | COX19 | |
| *COX20* | Cox20 | *COX20* | COX20 | Dystonia, ataxia, cerebellar atrophy, CoQ deficiency (Szklarczyk et al. 2013; Doss et al. 2014; Otero et al. 2019; Xu et al. 2019) |
| *COX23* | Cox23 | *CHCHD7* | CHCHD7 | |
| *CMC1* | Cmc1 | *CMC1* | CMC1 | |
| *CMC2* | Cmc2 | *CMC2* | CMC2 | |
| *PET100* | Pet100 | *PET100* | PET100 | Leigh syndrome, severe acidosis (Lim et al. 2014; Olahova et al. 2015; Mansour et al. 2019) |
| *PET117* | Pet117 | *PET117* | PET117 | Neurodevelopmental regression (Renkema et al. 2017) |
| *SCO1* | Sco1 | *SCO1* | SCO1 | Neurologic dysfunctions, hypertrophic cardiomyopathy, encephalopathy, hepathopathy (Valnot et al. 2000a; Stiburek et al. 2009; Leary et al. 2013a; Brix et al. 2019) |

(continued)

**Table 2.1** (continued)

| Yeast | | Human | | |
|---|---|---|---|---|
| Gene | Protein | Gene | Protein | Associated phenotype |
| *SCO2* | Sco2 | *SCO2* | SCO2 | Cardioencephalomyopathy (Papadopoulou et al. 1999; Jaksch et al. 2001; Pronicki et al. 2010; Rebelo et al. 2018; Barcia et al. 2019) |
| – | – | *LRPPRC* | LRP 130 | French Canadian type of Leigh syndrome (Mootha et al. 2003; Gohil et al. 2010; Debray et al. 2011) |
| – | – | *TACO1* | TACO1 | Leigh syndrome (Weraarpachai et al. 2009; Makrythanasis et al. 2014) |
| – | – | *SMIM20* | SMIM20 | |
| – | – | *PNKD* | MR-1S | [c]Paroxysmal nonkinesigenic dyskinesia-1 (Ghezzi et al. 2009b) |
| – | – | *TMEM177* | TMEM177 | |
| **COMPLEX V** | | | | |
| *ATP11* | Atp11 | *ATPAF1* | ATPAF1 | |
| *ATP12* | Atp12 | *ATPAF2* | ATPAF2 | Encephalopathy (de Meirleir et al. 2004) |
| – | – | [a]*TMEM70* | TMEM70 | Lactic acidosis, cardiomyopathy, 3-methylglutaconic aciduria (Cizkova et al. 2008; Honzik et al. 2010; Spiegel et al. 2011; Torraco et al. 2012; Catteruccia et al. 2014; Diodato et al. 2015; Magner et al. 2015; Braczynski et al. 2015; Sarajlija et al. 2017; Hirono et al. 2019; Staretz-Chacham et al. 2019) |

[a]Assembly factors involved in more than one complex biogenesis
[b]The condition is transmitted in an X-linked manner
[c]This condition is autosomal dominant and it is due to a deleterious action of the mitochondrial targeting sequence

defects, resulting in defective ATP production paralleled by increased ROS, mitochondrial fragmentation and a decrease of membrane potential (Distelmaier et al. 2009).

Isolated CI deficiency has mostly been documented in patients with mutations in CI structural subunits, but pathogenic variants in its assembly factors represent an increasing cause of this biochemical defect (Alston et al. 2017).

Mutations in the assembly factors *NDUFAF1*, *NDUFAF2*, *NDUFAF3*, *NDUFAF4, NDUFAF5* and *NDUFAF6* cause cardiomyopathy, infantile encephalopathy, fatal infantile lactic acidosis or Leigh syndrome, the most common mitochondrial disease (Kohda et al. 2016; Bianciardi et al. 2016; Wu et al. 2016; Baertling et al. 2017a, b; Tong et al. 2018; Simon et al. 2019). Specific phenotypes associated with mutations in CI assembly factors were described in the case of an ultrarare intronic variant in *NDUFAF6* identified in nine patients with the Acadian variant of Fanconi syndrome, that affects kidney with tubulopathy and lung (Hartmannova et al. 2016),

and a missense heterozygous variant in *NDUFAF7* segregating in a family with pathologic myopia, although this finding needs further confirmation.

ACAD9, a FADH2-dependent acyl-CoA dehydrogenase required for fatty acid oxidation, is found frequently mutated in patients with a severe multisystemic disorder (Schrank et al. 2017; Fragaki et al. 2017; Finsterer and Zarrouk-Mahjoub 2017; Repp et al. 2018; Kadoya et al. 2019). FOXRED1 mutations have been described in five patients with different clinical pictures, including neurodevelopmental disorders, epilepsy, ataxia and pulmonary hypertension (Zurita Rendon et al. 2016; Apatean et al. 2019; Barbosa-Gouveia et al. 2019). A *TIMMDC1* mutation was identified in patients with Leigh syndrome or a neurodegenerative condition with severe epilepsy (Kremer et al. 2017).

Cardiomyopathy, skeletal muscle involvement and kidney disease with tubulopathy have been documented in patients harboring mutations of *TMEM126B* (Sanchez-Caballero et al. 2016; Alston et al. 2016), whereas pathogenic variants of *NUBPL*, a member of the Mrp/NBP35 ATP-binding proteins required for Fe–S centers incorporation into CI, have been identified in patients with leukoencephalopathy and a complex multisystemic syndrome (Protasoni et al. 2020; Friederich et al. 2020).

### *2.5.2 Mutations of CII Assembly Factors*

Mitochondrial disorders caused by defects in assembly factors required for CII are rare (Hoekstra and Bayley 2013) and include mutations in *SDHAF1*, *SDHAF2* and *SDHAF3*.

Mutations of the assembly factor *SDHAF1* cause an infantile leukoencephalopathy (Ghezzi et al. 2009a; Hao et al. 2009; Ohlenbusch et al. 2012; Rattenberry et al. 2013) associated with CII deficiency. Indeed, mutant SDHAF1 results in the degradation of the structural subunit SDHB by the mitochondrial protease LONP1 (Maio et al. 2016).

Notably, heterozygous loss-of-function mutations of *SDHAF2* and a hypomorphic variant of *SDHAF3* were identified in patients with hereditary paraganglioma (OMIM:601650), a neuroendocrine tumor that occurs in cells of the neural crest and is associated also with mutations in CII structural subunits (Hao et al. 2009; Bayley et al. 2010; Kunst et al. 2011; Piccini et al. 2012; Casey et al. 2014; Zhu et al. 2015; Hoekstra et al. 2017; Dwight et al. 2017; Wolf et al. 2019; Roose et al. 2020).

### *2.5.3 Mutations of CIII Assembly Factors*

Mitochondrial CIII deficiency (MIM: 124000) is characterized by a wide range of clinical symptoms, with a poor prognosis, since most patients die in early childhood (Meunier et al. 2013). Although pathogenic variants in some CIII subunits have been identified, many patients with this biochemical phenotype lack an etiologic diagnosis.

Mitochondrial diseases associated with mutations in CIII assembly factors are not common and are caused by inherited recessive mutations. Patients often display also CI and CIV deficiencies. Mutations of assembly factors *BCS1L*, *TTC19*, *LYRM7*, *UQCC2* and *UQCC3* have been identified in patients with different clinical symptoms (Ghezzi and Zeviani 2018). Most pathogenic variants were associated with *BCS1L* and impacted on Rip1 incorporation into CIII. The most severe *BCS1L* mutations have been associated with a lethal disorder called GRACILE (Growth Retardation, Aminoaciduria, Cholestasis, Iron overload, Lactic acidosis and Early death, MIM 603358), that was first identified in patients of Finnish descent and is characterized by encephalopathy, tubulopathy and liver disease. *BCS1L* missense pathogenic variants resulted also in a less-severe disease, the Bjornstad syndrome (MIM 262000), an autosomal recessive disorder characterized by neurosensory hearing loss and brittle hair (Hinson et al. 2007).

### *2.5.4 Mutations of CIV Assembly Factors*

Impairment of COX activity impacts on ATP synthesis and induce ROS production, causing COX deficiency (MIM #220110), a disorder characterized by early onset encephalocardiomyopathy or multisystem disorders (Ghezzi and Zeviani 2018). COX deficiency can be either isolated or combined with defects of other MRC complexes. Indeed, COX assembly intermediates that accumulate during CIV deficiency are involved in supercomplexes formation (Moreno-Lastres et al. 2012).

Most mutations causing COX deficiency were found in nuclear genes encoding COX assembly factors and mutations in proteins involved in the same pathway exhibit similar clinical phenotypes. Pathogenic variants were identified in *COA7*, *COA8*, *COX14*, *COX20* and *PET117* (Weraarpachai et al. 2012; Szklarczyk et al. 2013; Doss et al. 2014; Melchionda et al. 2014; Martinez Lyons et al. 2016; Renkema et al. 2017). Mutations of *SCO1*, *SCO2*, *COX15*, *COA5*, and *COA6* resulted mostly in an early onset form of fatal infantile encephalocardiomyopathy (Valnot et al. 2000a; Jaksch et al. 2000, 2001; Stiburek et al. 2009; Pronicki et al. 2010; Calvo et al. 2012; Huigsloot et al. 2011; Leary et al. 2013a; Baertling et al. 2015), while Leigh syndrome was documented in patients with dysfunctional SURF1 (the primary cause of CIV defects), COX10, COX15, TACO1, PET100 and COX8 (Valnot et al. 2000b; Antonicka et al. 2003a, b; Aulbert et al. 2014; Olahova et al. 2015; Ribeiro et al. 2016; Hallmann et al. 2016). *SURF1* and *SCO2* pathogenic variants were also associated with Charcot–Marie–Tooth disease type 4, an axonal neuropathy characterized by muscle weakness and atrophy (Rebelo et al. 2018). Heterozygous *SCO2* mutations were proposed to be associated with severe myopia, but this finding was not confirmed in a large cohort of carriers of the common pathogenic mutation E140K in *SCO2* (Piekutowska-Abramczuk et al. 2009). Compound heterozygous mutations of *COA3* were identified in a patient with a mild phenotype characterized by neuropathy, exercise intolerance, obesity, and short stature (Ostergaard et al. 2015). Mutations of *LRPPRC,* that caused a reduced amount of

COX transcription, were associated with the French-Canadian type Leigh Syndrome (LSFC, MIM 220111), an early-onset progressive neurodegenerative disorder (Mootha et al. 2003; Gohil et al. 2010; Debray et al. 2011). In LSFC patients cells, COX activity levels were different, depending on the tissue (Sasarman et al. 2015), and in a *Lrpprc* heart knockout mice, the MRC dysfunction was associated with an ATP synthase assembly defect (Mourier et al. 2014).

### 2.5.5 Mutations of CV Assembly Factors

Isolated CV deficiency usually affects pediatric population and presents fatal outcome (Houstek et al. 2006). ATP synthase defects are mostly caused by mutations of the mtDNA-encoded subunits or nuclear assembly factors and result in a hypertrophic cardiomyopathy characterized by different symptoms. Most pathogenic variants were found in *TMEM70* and patients presented also other respiratory complexes deficiencies (Torraco et al. 2012; Catteruccia et al. 2014; Diodato et al. 2015; Braczynski et al. 2015; Magner et al. 2015). A mutation of *ATPAF2* was found in a patient who manifested encephalopathy (de Meirleir et al. 2004).

## 2.6 Treatments

Treatments of mitochondrial disorders, including those related to assembly factors defects, are mainly supportive. Here we summarize the various strategies and different molecules that have been proposed for the treatment of these conditions.

### 2.6.1 Antioxidants and Cofactors

A potential strategy for CI deficiency has been the use of antioxidants and cofactors, such as vitamin E and its analogues, that reduce ROS levels and reverse the membrane potential depolarization, increasing ATP production in patients fibroblasts (Roestenberg et al. 2012; Koopman et al. 2016). Some antioxidants, such as Omaveloxone, have been tested in clinical trials for mitochondrial diseases, but the results are not available yet.

The antioxidant idebenone, structurally related to CoQ, has been approved for some disorders that are caused by CI deficiency, since it can transfer electrons directly to CIII (Viscomi and Zeviani 2020).

Patients with *ACAD9* mutations improved after a high dosage of riboflavin, the vitamin precursor of the FAD moiety, that synthesizing FMN and FAD, increased CI activity (Gerards et al. 2011).

Expression of the alternative oxidase (AOX) enzyme, which in lower eukaryotes can transfer electrons from CoQ to molecular oxygen, rescued some biochemical and clinical defects in a mouse model of CIII deficiency (Rajendran et al. 2019).

Some patients affected by CI deficiency showed some clinical improvement after following a high-fat and ketogenic diet. The rationale is that the increased fatty acids would stimulate the beta-oxidation and the consequent production of ketone bodies, which can function as energy substrates for the brain (Scholl-Burgi et al. 2015; Theunissen et al. 2017).

In the case of specific defects of assembly factors required for metallation of structural subunits, a bypass therapy with copper has been proposed. Cells from patients harboring mutations in *SCO2*, a COX-assembly factor required for copper metallation of the COX2-associatad $Cu_{A,}$ showed a partial complementation of the bioenergetic defects after copper supplementation (Salviati et al. 2002; Casarin et al. 2012). Remarkably, treatment with copper increased COX activity and restored normal levels of COX subunits also in cells lacking COX16, a COX assembly factor involved in the maturation of COX2 (Cerqua et al. 2018).

### *2.6.2 Mitochondrial Biogenesis*

A master gene crucial for the treatment of mitochondrial disorders is the nuclear *PPARγ coactivator-1 α* (PGC-1α or PPARGC1A), whose protein product up-regulates mitochondrial biogenesis, inducing the recruitment of a broad range of transcription factors that induce mitochondrial respiration and fatty acids oxidation (Wenz 2013; Hofer et al. 2014).

Some cell and animal models of MRC deficiency improved after PGC-1α overexpression (Srivastava et al. 2009; Wenz et al. 2009). PGC-1α can be activated by AMP or its analogues, that induce the overexpression of AMPK or histone deacetylation by the NAD dependent deacetylase sirtuin-1 (Sirt1), which is activated by $NAD^+$(Gerhart-Hines et al. 2007; Wenz et al. 2008).

Interestingly, the biochemical defect of some patients cells or mice with CI or CIV deficiency was restored by the treatment with the PGC-1α inducers Bezafibrate and AICAR (5-aminoimidazole-4-carboxamide ribonucleotide) (Bastin et al. 2008; Golubitzky et al. 2011; Casarin et al. 2012; Viscomi et al. 2011; Yatsuga and Suomalainen 2012; Cerutti et al. 2014), with nicotinamide riboside (NR), a vitamin B3 and NAD(+) precursor (Khan et al. 2014), or with resveratrol, that stimulates the transcription of many MRC genes through the activation of estrogen receptors (Jardim et al. 2018).

Clinical trials are ongoing using some of the substances that induce mitochondrial biogenesis and elamipretide, a compound that binds the IMM phospholipid cardiolipin and regulates MRC complexes and *cristae* remodeling (Viscomi and Zeviani 2020; Szeto and Birk 2014).

Many cellular and animal models of mitochondrial diseases improved after the treatment with rapamycin or its analogues, that can induce autophagy, inhibiting a

mTOR-containing complex, mTORC1, that regulates cellular translation (Civiletto et al. 2018). This would stimulate mitochondrial biogenesis and increase mitophagy, with the consequent elimination of dysfunctional mitochondria. A rapamycin analogue, Nab-sirolimus, is tested in patients affected by Leigh syndrome. with various responses (Sage-Schwaede et al. 2019).

Finally, the overexpression of Opa1, an IMM protein essential for mitochondrial dynamics, increased the MRC efficiency, improving the myopathic phenotype of a muscle-specific Cox15 conditional knockout mouse (Civiletto et al. 2015).

### *2.6.3 Gene Therapy*

The expression of the wild-type form of some "therapeutic genes", obtained using adeno-associated viral vectors (AAVs), could ameliorate many phenotypes of mouse models of mitochondrial diseases, such as the *Ndufs4*$^{-/-}$ mice manifesting Leigh syndrome (di Meo et al. 2017). Despite the promising results, the use of these vectors for gene therapy has still some problems related to the limited cloning capacity and the difficult achievement of an effective gene expression in some tissues, particularly the brain, that is protected by the blood-brain barrier. Nevertheless, the presence of several AAV serotypes with specific tissue tropism and transfection efficiency is promising and many trials are ongoing (Viscomi and Zeviani 2020).

## 2.7 Concluding Remarks

The assembly of MRC is essential for the correct functioning of mitochondria and its importance is underlined by the variety of disorders caused by mutations of several genes encoding different MRC assembly factors. These diseases mainly affect high energy demanding organs and are characterized by an isolated or combined deficiency of specific complexes activity.

The number of pathogenetic variants associated with new or already known MRC assembly factors has conspicuously grown in the last years thanks to the advent of next generation sequencing technologies.

Many patients still lack a molecular diagnosis and no effective treatment is available for these disorders. Nevertheless, the combination between deep sequencing analyses for adequate patient genotyping and functional characterization of novel variants found in old and novel genes will pave the way to a better comprehension of MRC biogenesis and functioning, fostering our understanding on the pathogenetic mechanisms underlying these conditions and increasing the chance to develop new therapeutic strategies to fight them.

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# Chapter 3
# Mitochondrial DNA: Defects, Maintenance Genes and Depletion

**Miguel A. Fernández-Moreno, Luis Vázquez-Fonseca, Sara Palacios Zambrano, and Rafael Garesse**

**Abstract** Mitochondria constitute a graticule dynamic cellular compartment present in the vast majority of eukaryotic cells. They produce most of the cellular energy by burning metabolic fuels. In addition, these organelles are involved in other essential processes such as nucleotide and iron-sulfur cluster biosynthesis, amino acid metabolism, fatty acid oxidation, calcium homeostasis, apoptosis, etc. Mitochondria contain their own genome, mtDNA, reminiscent of their bacterial origin. The mtDNA encodes 13 out of the 1500 estimated mitochondrial proteins and part of the machinery to translate them: 2 ribosomal RNAs (mt-rRNAs) and 22 tRNAs (mt-tRNAs). The remaining mitochondrial proteins are encoded in the nucleus, synthesized in the cytosol and imported into the mitochondria. The 13 mtDNA-encoded proteins are structural subunits of the energy producing system (the OXPHOS system), whose alterations provoke so-called mitochondrial OXPHOS diseases, a genetic and clinical heterogeneous group of disorders. The cellular mtDNA content, different according to the cell's metabolic and energy requirements, is based on its replication machinery which is formed by a number of nuclear-encoded proteins, including DNA polymerase γ subunits PolG1 and PolG2, mtDNA helicase (Twinkle), single stranded binding protein (mtSSB), mtRNA polymerase (POLRMT/mtRNAP) and others. Functional alterations in any of these proteins or in those involved in the supply of deoxyribonucleotide triphosphate (dNTP) or in those controlling mitochondrial dynamics may provoke disorders characterized by the instability of the mtDNA, causing deletions and depletion. This chapter focuses on the molecular basis of these disorders.

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## 3.1 Introduction

Approximately 3 billion years ago, photosynthetic bacteria massively proliferated increasing the levels of oxygen, a toxic gas, in the earth atmosphere (Hohmann-Marriott and Blankenship 2011). In this context, according to the endosymbiotic theory, the anaerobic ancestor of the eukaryotic cells engulfed a α-proteobacterium able to use oxygen in its metabolism but, instead of digesting, kept it inside and they divided together. Thus, the bacteria received a safer environment and easy nutrients and the host overcame the toxicity of oxygen (Sagan 1967; Archibald 2015). Several billion years of later co-evolution have led both biological systems to a deep mutual integration at structural and functional levels becoming a single living entity. So, at present, almost every eukaryotic cell includes mitochondria within their cytosol (Archibald 2015).

The knowledge of mitochondrial structure and function underwent two main hits in the middle of the twentieth century with the first high-resolution electron micrographs in 1952 (Palade 1952) and the proposal of the chemiosmotic theory as a general mechanism of oxidative phosphorylation in 1966 (Mitchell 1961). The former showed a round shape organelle with two membranes where the internal membrane was projected into the matrix to form the so-called cristae, where the energy production machinery is located. This structure produces four compartments: outer membrane, intermembrane space, inner membrane and matrix. The development of new technologies and tools in the last decades has gone further, drawing a different and modern view of the cellular organization and ultrastructure of mitochondria as an elongated and branched 3D network of tubular structures, usually denser around the nucleus and with different cristae shape according to their function (Frey et al. 2002; Collins et al. 2002; Cogliati et al. 2013). Furthermore, the current mitochondria undergo a complex biogenesis involving strictly regulated processes of proliferation (forming new mitochondrial mass) and differentiation (cristae formation) in order to yield the suitable network to satisfy the energy demand of the tissue where they belong. Thus, mitochondria from different tissues, and in some cases from different cells in the same tissue, show different morphology, density, fuel preferences and proteome components (Johnson et al. 2007; Pagliarini et al. 2008; Vafai and Mootha 2012). In addition, the mitochondrial network is a dynamic structure in a continuous highly regulated process of remodeling through fission and fusion events (Pernas and Scorrano 2016; Tilokani et al. 2018). This dynamic is closely related to mitochondrial homeostasis and therefore to their function (Rampelt et al. 2017; Farmer et al. 2018). In a general sense**,** fusion of mitochondria generates elongated fibers which are associated to higher ATP synthesis and respiration rates (Wai and Langer 2016; Sprenger and Langer 2019). On the contrary, mitochondria fission is related to less active mitochondria that usually goes either to mitophagy (Hamacher-Brady and Brady 2016) or to daughter cells during cell division (Horbay and Bilyy 2016).

Concurrently to the evolutionary adaptive changes in structure, mitochondrial function was also fitting to the new symbiotic situation. Hence, most of the ancient

bacterial functions were assumed by the host, a few were kept and new ones appeared (Roger et al. 2017). At present, mitochondria is deeply integrated with the cell physiology, being involved in many functions essential for the cell/tissue/organism such as synthesis of iron-sulfur clusters (Stehling and Lill 2013), calcium homeostasis (Bagur and Hajnoczky 2017), ageing (Michikawa et al. 1999; Shpilka and Haynes 2018), development (Tuzlak et al. 2016), apoptosis (Wang and Youle 2009; Tuzlak et al. 2016), thermogenesis (Chouchani et al. 2019), heme biosynthesis (Piel et al. 2019), fatty acid metabolism (Nowinski et al. 2018) and ROS signaling (Hou et al. 2014) among others. In fact, mitochondria have gone even further and have become a signaling center for the cell (Chandel 2015). Although all those roles are critical, the most recognized function of mitochondria is to produce the majority of the energy required for respiring cells in form of ATP, which is executed by the OXPHOS system. This system is composed by five multiprotein complexes embedded into the mitochondrial inner membrane and two electron carriers (ubiquinone, or CoQ, and cytochrome C) (Vafai and Mootha 2012). Complexes I to IV and the electron carriers constitute the Electron Transport Chain (CTE), responsible of transferring electrons from reduced metabolic substrates to the final acceptor, the molecular oxygen, coupling this transfer to the pump of protons into the mitochondrial intermembrane space by Complexes I, III and IV. This produces a proton gradient whose dissipation through Complex V, or ATP Synthase, is in its turn coupled to the ADP phosphorylation generating ATP. In fact, there is a supramolecular organization of OXPHOS Complexes in which they interact in several ways to form the so-called supercomplexes to probably increase the efficiency of the OXPHOS system (Enriquez 2016).

OXPHOS complexes are formed by approximately 90 structural subunits from which 13 are encoded by the mtDNA: seven out of 45 subunits in complex I (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6); one out of 11 subunits in complex III (cytochrome b); three out of 13 subunits in complex IV (COI, COII, and COIII) and two out of 18 subunits in complex V (ATP6 and ATP8). Complex II is exclusively formed by nuclear encoded subunits (Alston et al. 2017). Thus, biogenesis of OXPHOS system is supported by two different genomes located on two different cellular compartments, requiring, therefore, a tightly coordinated expression and a complex machinery to assemble the individual subunits with the correct stoichiometry to yield functional respiratory complexes (Formosa and Ryan 2018; Tang et al. 2020; Enriquez 2016).

Since the mitochondrial OXPHOS function lies in a dual genetic expression, mutations either in nuclear (nDNA) or in mitochondrial genomes are able to provoke mitochondrial OXHOS diseases. One group of these disorders is formed by the intergenomic communication defects, which are defined by functional alterations of proteins involved in the maintenance and expression of mtDNA, all of which are encoded in the nucleus. Intergenomic communication defects provoke mtDNA loss or instability affecting its synthesis, integrity or stability and leading to the occurrence of multiple deletions or depletion syndromes (Spinazzola and Zeviani 2009).

## 3.2 mtDNA: General Characteristics and Defects

The presence of DNA molecules inside mitochondria was firstly described by Nass in 1960s (Nass and Nass 1963). In the 1980s it was completely sequenced (Anderson et al. 1981) and the two first human diseases associated to mtDNA mutations were described (Holt et al. 1988; Wallace et al. 1988). Since then, hundreds of pathogenic mtDNA mutations have been described (http://www.mitomap.org/MITOMAP (Lott et al. 2013)) and the knowledge about mitochondrial genome nature and metabolism has increased enormously. mtDNA is a covalently closed circular DNA molecule of 16,569 nucleotides in size, with an extraordinarily compact organization that does not strictly follow the universal genetic code. In addition, there are several important aspects that characterize mitochondrial genetics and differentiate it from nuclear genetics: maternal inheritance, mitotic segregation, polyplasmy and the threshold effect (Gorman et al. 2016; Craven et al. 2017). Mitotic segregation means the random distribution of mitochondria during cell division. Polyplasmy refers to the presence of numerous molecules of mtDNA per cell, from ~100 copies in sperm cells to hundreds of thousands in oocytes. Polyplasmy allows for the coexistence of mtDNA molecules harboring mutations with molecules that are mutation-free. Mutations can be in all of the mtDNA molecules of a cell ("homoplasmy") or only in a population ("heteroplasmy"), being the degree of the latter different between cells from different tissues or organs, and even between cells from same tissue, due to the mitotic segregation (Stewart and Chinnery 2015). Heteroplasmy gives rise to a threshold effect, a point where the proportion of mutated mtDNA molecules produce a clinical manifestation. In addition to heteroplasmy, the threshold also depends on the type of the mutated molecules (a mt-tRNA dysfunction provokes more severe consequences than that of an individual mt-protein), the grade of the dysfunction of the mutated molecule and the energy demand of the tissue (Stewart and Chinnery 2015). In some cases, a detectable phenotype requires until 90% of heteroplasmy (Holt et al. 1990). Deleterious mutations are not usually homoplasmic because it would be incompatible with survival, however, some of them are mild enough to be tolerated in homoplasmy such as those causing aminoglycoside-induced deafness or Leber's hereditary optic neuropathy (LHON) (Carelli and La Morgia 2018).

Although historically the mtDNA has been considered a naked and unprotected molecule, it has recently been described in detail that the mtDNA, in fact tightly interacts with proteins to form discrete structures called nucleoids, with a single mtDNA molecule per nucleoid. An impressive variety of technical approaches demonstrates that TFAM is the main protein component of nucleoid. TFAM, initially described as a transcription factor and later as a histone-like for mtDNA is more than that since, in addition to its binding to the mtDNA molecule at high ratios, 1000:1 in mammals, TFAM bends the mtDNA chain and leads to different degrees of nucleoid compaction, what is essential for mtDNA metabolism (Bonekamp and Larsson 2018). In addition to TFAM, many other mitochondrial proteins are co-purified with nucleoids, highlighting key elements of the

mitochondrial replication, transcription and mtRNAs processing machineries as well as quality control proteases, RNA helicases and mitoribosome proteins, building a large nucleoprotein complex that interacts with the mitochondrial inner membrane and play a central role in mtDNA replication, gene decoding, segregation to daughter cells and distribution on the mitochondrial net of the cell (Bonekamp and Larsson 2018; Lee and Han 2017).

In spite of the mtDNA being a small molecule with no introns, with barely any intergenic regions and including only a small non-coding control region of ~1 kb in length (Fig. 3.1), its copy number and expression are highly regulated according to the metabolic demands of the cell, although the control mechanisms underlying are

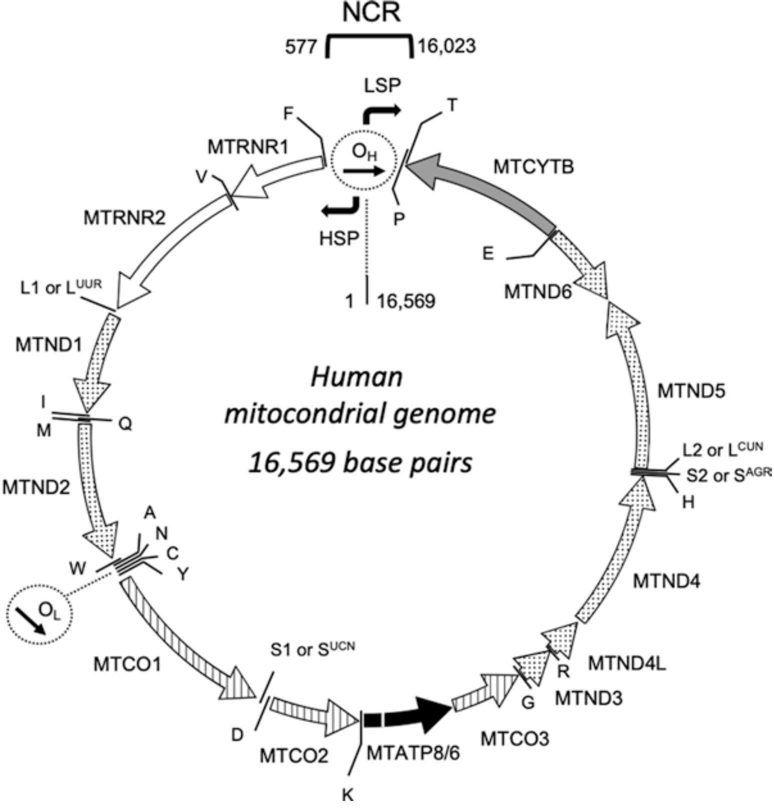



**Fig. 3.1 Mitochondrial DNA coding genes and control region.** The mtDNA molecule is a covalent closed circular 16,569 bp in length. White arrows mean ribosomal RNA (rRNA); dotted arrows show Complex I (NADH dehydrogenase); lined arrows represent Complex IV (cytochrome C oxidase); black arrows show Complex V (ATP Synthase); Grey arrow represents complex III (ubiquinol-cytochrome C oxidoreductase). The 22 tRNA genes are interspersed between the peptide-encoding or rRNA genes. $O_H$ and $O_L$ (origins of replication of Heavy and light strand, respectively, are inside a dotted circle). LSP: Light Strand Promoter; HSP: Heavy Strand Promoter. NCR: Non-Coding Region, where interact control elements for replication and transcription (limits positions are shown)

poorly understood (Pearce et al. 2017; Nicholls and Gustafsson 2018; Nissanka et al. 2019). Mitochondrial genome expression involves a complex machinery supporting a series of linked processes including mtDNA replication, mtDNA maintenance, mtDNA transcription, mtRNAs maturation and mt-mRNAs translation to finally synthesize the 13 proteins that interact in a specific order with a myriad of nuclear-encoded assembly factors and OXPHOS subunits to assemble the mitochondrial respiratory complexes (Gustafsson et al. 2016). From those processes, only defects in the mtDNA replication and maintenance and some elements of the transcription machineries are involved in mtDNA stability defects causing deletion and depletion syndromes.

Mitochondrial genome shows a mutation rate much higher than that of the nuclear genome, probably due to its proximity to the source of reactive oxygen species produced during oxidative phosphorylation (Craven et al. 2017). Genetically, mtDNA primary mutations (those located into the mtDNA molecule) can be classified into three categories according to the affected gene: (i) genes encoding proteins, (ii) genes encoding rRNA and tRNA and (iii) mtDNA rearrangements. The first two are usually maternally inherited, and the latter typically arises *de novo* during embryonic development.

Although mitochondrial diseases are individually considered rare diseases, taken together form the largest group of inborn errors of metabolism (Craven et al. 2017). They usually do not affect human embryonic development due to its non-aerobic metabolism, being the prevalence of the diseases caused by primary mutations in DNA estimated at 1:5000 in newborns (Distelmaier et al. 2009). Debut can occur at any age although are more typical in adults (Haas and Zolkipli 2014), progressively worsening symptoms (Gorman et al. 2015). mtDNA mutations may provoke well characterized clinical syndromes, such as MELAS (Mitochondrial Encephalomyopathy with Lactic Acidosis and Stroke-like episodes), MERRF (Myoclonic Epilepsy with Ragged-Red Fibers), NARP (Neurogenic weakness with Ataxia and Retinitis Pigmentosa), LHON, CPEO (Chronic Progressive External Ophthalmoplegia), KSS (the Kearns–Sayre syndrome) or PS (Person syndrome) (Carelli and La Morgia 2018). However, one of the hallmarks of diseases associated with mitochondrial genome mutations is the high heterogeneity of their clinical phenotypes, encompassing from muscle weakness to devastating syndromes, and typically affecting tissues or organs with a high energy demand such as nervous system, skeletal muscle and cardiac muscle (Gorman et al. 2016). This heterogeneity is well illustrated when point mutations on different genes can provoke the same pathology and a specific point mutation may cause different phenotypes, making it difficult to understand the molecular pathway from genotype to phenotype and the develop of therapeutic approaches (Suomalainen 2011; Gorman et al. 2016).

Although knowledge about mtDNA has enormously progressed in the last decades, we are still learning about new aspects of its metabolism, sensitivity, regulation of expression and role in cell physiology. Thus, it has been described the influence of environmental factors as disease triggers (Giordano et al. 2015; Aw et al. 2019) or the presence of epigenetic marks (methylation and hydromethylation)

into mitochondrial genome correlating to clinical phenotypes and aging (Bacalini et al. 2017; D'Aquila et al. 2017).

## 3.3 mtDNA Replication and Maintenance Genes

Mitochondrial functionality depends on the active and continuous control of mtDNA maintenance and decoding and on a suitable capacity of the cell to keep a healthy mitochondrial network, removing damaged material and allowing for the exchange of molecules and the correct transmission to daughter cells. These requirements rest on the proteins that replicate the mtDNA molecule, on the regular supply of deoxyribonucleotide triphosphates (dNTPs) and on appropriate mitochondrial dynamics. mtDNA point mutations and rearrangements represent primary mtDNA defects, and mutations in any of the components affecting mtDNA metabolism and maintenance constitute secondary mtDNA disorders. The latter, known as Mitochondrial DNA maintenance defects (MDMDs), produce mtDNA instability and can cause quantitative defects such as mtDNA depletion syndromes or qualitative alterations such as multiple DNA deletions, showing a Mendelian or sporadic (*de novo*) inheritance behaves.

### *3.3.1 mtDNA Replication*

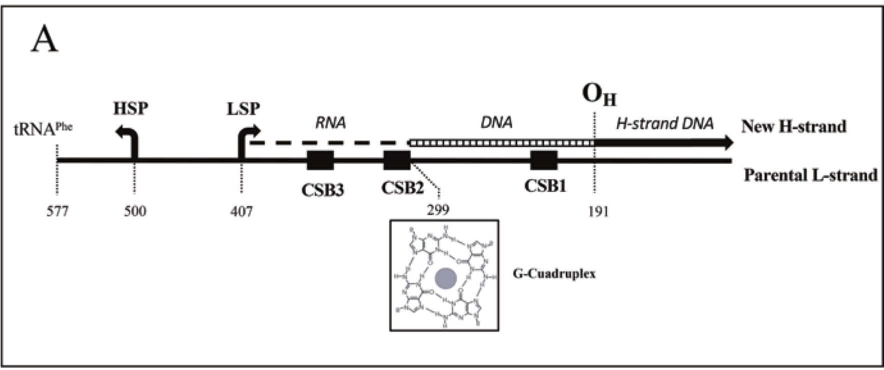
Mitochondrial genome replication occurs independently to that of nuclear DNA, through a different mechanism and utilizing specific machinery (Nicholls and Gustafsson 2018; Gustafsson et al. 2016). Thus, mtDNA replicates, unlike nuclear DNA, in differentiated cells such as neuronal and cardiac cells (Yasukawa and Kang 2018). It is accepted than mtDNA nucleotide variations, pathogenic or not, are mostly the result of errors in the replication process, becoming a relevant source of mtDNA-associated mitochondrial diseases and aging (Zheng et al. 2006; Gustafsson et al. 2016). The two strands of mtDNA contain different base composition and can be separated in denaturing density gradients, being described as heavy (H-strand) and light strand (L-strand) (Chinnery and Hudson 2013).

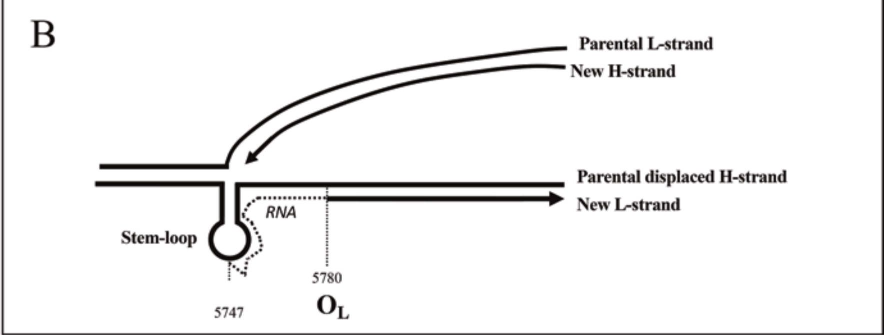
There are three proposed models for the replication of mtDNA strands, with evidences supporting and contradicting all of them, suggesting that we still lack some information to completely characterize this crucial process (Zinovkina 2019). The controversial models are: the Strand-Displacement Model (SDM) (Robberson and Clayton 1972), the Ribonucleotide Incorporation Throughout the Lagging Strand model (RITOLS) (Yasukawa et al. 2006) and the strand-coupled model (SCD) (Holt et al. 2000). The components of the mtDNA replication machinery are mostly the same in the three models although different to those found in the nucleus. However, some of them (DNA polymerase (PolG1), mt-helicase (Twinkle) and mt-DNA-dependent RNA polymerase (POLRMT)) show a strong resemblance to

the corresponding enzymes from T-odd bacteriophages (Nicholls and Gustafsson 2018) and the transcription factor mtTFB2, also involved in mtDNA replication, derives from a rRNA methyltransferase (activity still conserved (Cotney et al. 2009)) of α-proteobacteria, reflecting the heterogenous origin of the mitochondrial genome machinery (Shutt and Gray 2006).

#### 3.3.1.1 The Strand-Displacement Model of mtDNA Replication

The SDM is the most accepted model. Accordingly, the mtDNA replication process has two unidirectional and independent origins of replication, one for the H-strand ($O_H$) located into the non-coding region (position 191) and one for the light strand ($O_L$) located 2/3 of the mtDNA molecule away from $O_H$ in the direction of H-strand chain synthesis (position 5747) (Fig. 3.1). Replication from $O_H$ and from $O_L$ are asynchronous processes that occur in a continuous way from both strands, without Okazaki fragments involvement. Replication from $O_H$ starts with the transcription of a short RNA molecule from the Light Strand Promoter that is located approximately 200 nts upstream of $O_H$ (LSP; Fig. 3.2a). This action is executed by the key components of the mitochondrial transcription machinery: the mitochondrial transcription factor A (TFAM), that bends the mtDNA strands recruiting POLRMT, and the final incorporation of the mitochondrial transcription factor mtTF2 to enhance POLRMT activity (Fig. 3.2c). When the RNA transcribed from LSP is approximately 100 nt in length, the transcription machinery reaches a G-rich region named CBS2 (Conserved Sequence Block 2) where the transcription stops due to a stable G-quadruplex structure in DNA, giving rise an RNA primer for replication (Fig. 3.2a) (Wanrooij et al. 2010; Gustafsson et al. 2016). Once the RNA primer is formed, the synthesis of DNA begins by DNA polymerase-γ (Polγ). Human Polγ is a heterotrimeric enzyme involved in mtDNA replication and repair. It is composed by three subunits, POLγA or POLG1 and two copies of POLγB or POLG2 (Yakubovskaya et al. 2006). POLG1 is a 140 kDa protein that contains the DNA-dependent DNA polymerase activity, a 3′ to 5′ exonuclease activity required for proofreading and a 5′-deoxyribose lyase activity involved in DNA repair. It is one of the most accurate DNA polymerases in nature. POLγB or POLG2, 55 kd in size, enhances DNA binding and increases the processivity of PolG1 (Lim et al. 1999). However, Polγ cannot use dsDNA as a template, requiring additional elements to start the mtDNA heavy chain replication from the RNA primer. Thus, mtDNA helicase, TWINKLE, helps Polγ at replication fork unwinding the ds-mtDNA from 5′ to 3′, although to a limit of approximately 2 kb. The help of a third component of replication machinery overcomes this limitation. Thus, the mitochondrial ssDNA-binding protein (SSB) enhances the helicase activity of TWINKLE and Polγ processivity, allowing the complex to completely replicate the entire mtDNA molecule (Korhonen et al. 2003, 2004). In addition, mtSSB tightly binds as tetramer and coats the single stranded heavy-strand displaced during Polγ advance to stabilize, to protect from degradation and to prevent its renaturation (Fig. 3.2c) (Korhonen et al. 2004; Almannai et al. 2018). Polγ, Twinkle and mtSSB forms the



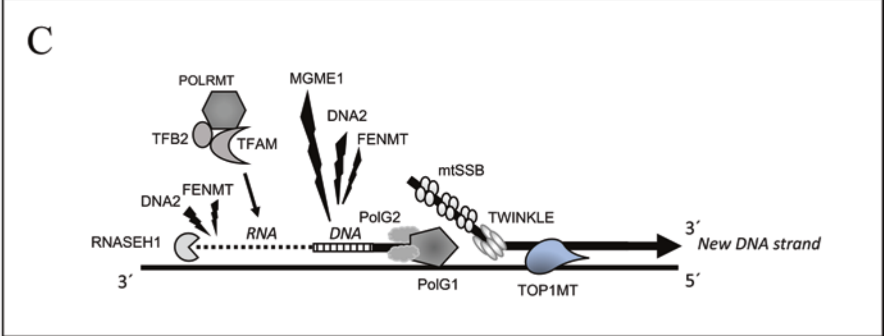




**Fig. 3.2 Replication of human mitochondrial genome according to the Strand-Displacement Model.** (**a**) Origin of replication of human mtDNA heavy strand. HSP: Heavy Strand Promoter; LSP: Light Strand Promoter; CSB: Conserved Sequence Block; stripe line: RNA primer transcribed from LSP; lined bar: DNA next to the primer, it will be removed; full arrowed bar: newly synthesized H-strand, arrow show the direction of synthesis; $O_H$: origin of replication of the H-strand. G-cuadruplex structure at CSB2 that stops transcription to give rise the replication primer is shown. (**b**) Origin of replication of human mtDNA light strand. When the H-strand replication fork reaches $O_L$ region (origin of replication of L-strand) POLRMT recognizes the DNA stem-loop structure, transcribes an RNA primer and Polγ starts polymerization of the new L-strand. (**c**) Diagram of mitochondrial replication machinery showing components involved in the RNA primer synthesis, the replication fork and those to leave the 5′ end of the H-strand, what involves RNA primer and a short stretch of DNA removing. Since the 3′ end of the newly synthesized H-strand is not shown mtLigIII (the ligase activity which seal the nick finishing the replication process) is absent

so-called minimal mtDNA replisome (Korhonen et al. 2004). Finally, the unwinding of DNA in front of a replication fork generates torsional tension, particularly in circular genomes, that must be relieve by topoisomerase activity. In mtDNA replication this activity is executed by TOP1MT, probably the Top3α isoform, (Fig. 3.2c) (Sobek and Boege 2014; Zinovkina 2019).

Once H-strand mtDNA synthesis has progressed, the RNA primer must be removed. In fact, the primer synthesized from LSP starts 191 nts before the 5′ end of the heavy chain but RNA primer is only 100 nts in length. That means that approximately 100 nts DNA next to the RNA primer must be also removed (Fig. 3.2a). The mitochondrial DNA polymerase Polγ possesses limited strand displacement activity, suggesting that primer flap intermediates are likely to be involved in primer removal (Uhler and Falkenberg 2015). The enzymes involved in leaving the suitable 5′ end of mtDNA H-strand are RNASEH1, that digest the RNA primer (Holmes et al. 2015), MGME1 (Mitochondrial Genome Maintenance Exonuclease-1) that remove approximately 100 DNA nucleotides from the 5′ end exposed by RNASEH1 action (Zinovkina 2019; Nicholls et al. 2014), and DNA2 and FENMT, that plays several roles y replication and repairing, either in nucleus and in mitochondria, that it is believed they facilitate RNA primer displacement and DNA flap degradation (Uhler and Falkenberg 2015).

Replication of the light strand according the SDM starts at position 5747, on a tRNA-rich coding region and, therefore, with high possibilities of forming secondary structures. During replication, H-strand replication fork reaches $O_L$ position and the maternal H-strand, coated by mtSSB, acquires a stem-loop structure that cannot be recognized by mtSSB, being exposed to POLRMT that binds and transcribes a 25 nt in length RNA primer (Fig. 3.2b) (Miralles Fuste et al. 2014). Then, POLRMT is replaced by Polγ and the L-strand synthesis starts unidirectionally in the opposite direction to that of the heavy-strand DNA. It is interesting to note that the L-strand synthesis depends on H-strand synthesis and not on TWINKLE, because of the template for L-strand replication is ssDNA, that does not require the helicase activity for unwinding (Gustafsson et al. 2016).

The replication finishes when the 3′ end reaches the 5′ end of the same strand. At this point, DNA Ligase III may seals the nick or previously Polγ may initiates successive cycles of polymerization-degrading, creating a 5′-flap and removing with its 3′ to 5′ exonuclease activity to finally rendering a ends substrates for DNA Ligase III (Macao et al. 2015).

#### 3.3.1.2 Alternative Models for the mtDNA Replication

A similar but alternative model to SDM for the mtDNA replication is the ribonucleotide incorporation throughout the lagging strand model (RITOLS), also called bootlace model. Basically, it consists on the observation of tracts of RNA associated with replicating molecules of mtDNA. In RITOLS model, supported by *in vivo* and *in vitro* approaches, the parental H-strand is coated by processed mtRNA molecules instead of by mtSSB tetramers (Yasukawa et al. 2006). Those RNAs are threaded

onto the lagging-strand template as the replication fork advances and will be removed during DNA lagging-strand synthesis (Reyes et al. 2013). However, as arguments against, the mechanisms and enzyme machinery required for the RNA hybridization to DNA and further RNA displacement have not been described and the pattern of *in vivo* occupancy of mtSSB on mtDNA correlates with the replication expected for the strand displacement model of mtDNA synthesis, not with RITOLS' (Miralles Fuste et al. 2014; Zinovkina 2019).

A third model for the mtDNA replication is the strand-coupled mtDNA replication model (SCD) (Holt et al. 2000). This model shows many properties of conventional, coupled leading- and lagging-strand DNA synthesis, resembling to the replication of circular bacterial genomes. Thus, the main characteristics of this model includes (1) Several origins of replication, (2) bidirectional replication from these origins, (3) each fork has the leading and lagging strands, being the latter synthesized as Okazaki fragments, (4) The fork moving towards the NCR direction stops in the $O_H$ region, after which DNA synthesis becomes unidirectional (Bowmaker et al. 2003; Holt and Jacobs 2014). However, neither mechanism of lagging-strand synthesis has been described yet nor the mitochondrial primase responsible for the generation of these Okazaki fragments has been identified.

Since there are evidences supporting and open questions in the three mtDNA replication models, it may well be possible that they are not completely selective, but perhaps in particular processes or specific physiological situations two models, or more, may coexist in a cell or in a tissue.

### *3.3.2 Deoxyribonucleotide Triphosphates (dNTPs) Supply*

Deoxyribonucleotide triphosphates are essential substrates of the replication enzymatic machinery to synthesize new DNA molecules. Nuclear DNA replicates once per cell cycle but mtDNA replicates continuously and independently of cell division. The synthesis of dNTPs to satisfy the nuclear genome duplication comes from a *de novo* pathway, which is cell cycle-regulated and works mostly at S-phase cells. However, mitochondria lack this capacity and, in addition, the mitochondrial inner membrane is impermeable to charged molecules, including nucleotides. Thus, mitochondria need to keep a constant dNTPs supply, and do it by import cytosolic dNTPs, via specific transporters, and through the mitochondrial nucleotide salvage (recycling) pathway, where preexisting deoxynucleosides are converted to dNTPs within the mitochondrial matrix (Suomalainen and Isohanni 2010; El-Hattab and Scaglia 2013). Figure 3.3 shows a simple diagram of the mitochondrial nucleotide salvage pathway and cytosolic dNTPs import.

The synthesis of dNTPs by the mitochondrial nucleotide salvage pathway starts with the conversion of pyrimidine deoxynucleosides deoxycytidine (dC) and thymidine (T) to deoxycytidine monophosphate (dCMP) and thymidine monophosphate (TMP), respectively, by action of the mitochondrial thymidine kinase 2 (TK2) (Johansson and Karlsson 1997). In the same manner, the

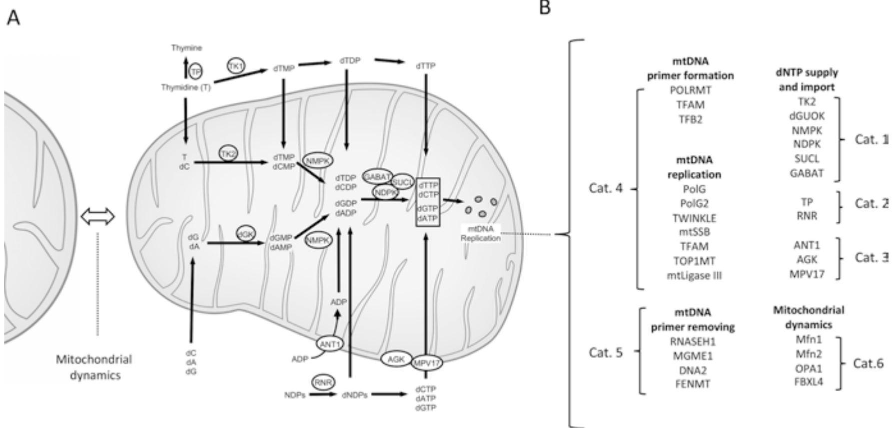



**Fig. 3.3 mtDNA replication and maintenance: proteins involved and dNTP supply to mitochondria.** (**a**) Diagram of the mitochondrial nucleotide salvage pathway and cytosolic nucleotides import. (**b**) Proteins involved in mtDNA replication and maintenance. The proteins are grouped in six categories: *1.- Enzymes of mitochondrial nucleotide salvage pathway*: Thymidine kinase 2 (**TK2**; encoded by TK2) and deoxyguanosine kinase (**DGK**; encoded by DGUOK) convert the deoxyribonucleosides (T/dC and dA/dG, respectively) to deoxyribonucleotide monophosphates (dNMPs). Nucleotide monophosphate kinase (**NMPK**; encoded by DTYMK) converts dNMPs to deoxyribonucleotide diphosphates (dNDPs). Nucleotide diphosphate kinase (**NDPK; encoded by NME4**), in a complex with succinyl-CoA ligase (**SUCL** composed of an alpha subunit encoded by SUCLG1 and a beta subunit encoded by either SUCLA2 or SUCLG2) and gamma-aminobutyrate transaminase (**GABAT**; encoded by ABAT) converts dNMPs to deoxyribonucleotide triphosphates (dNTPs). *2.- Enzymes of cytosolic nucleotide metabolism*: Ribonucleotide reductase (**RNR**; composed of 2 catalytic subunits and two small subunits converts ribonucleotide diphosphates (NDPs) to dNDPs. Thymidine phosphorylase (**TP**; encoded by TYMP) converts thymidine to thymine. *3.- Proteins involved in mitochondrial nucleotide transport*: adenine nucleotide translocator 1 (**ANT1**; encoded by SLC25A4), acyl-glycerol kinase (**AGK**; encoded by AGK), and **MPV17** protein (encoded by MPV17). *4.- Enzymes involved in mtDNA synthesis*: **Twinkle** (encoded by TWNK or *c10orf2*) which is a DNA helicase that separates the DNA stands, **DNA polymerase γ** (pol γ; consisting of one catalytic subunit encoded by POLG and two accessory subunits encoded by POLG2) which needs a RNA primer (synthesized by **POLRMT**, **TFAM** and **TFB2**) to initiate DNA synthesis, **mtSSB** (single stranded binding protein; encoded by mtssb) to protect ssDNA exposed on replication process, **mitochondrial DNA Topoisomerase** (**TOP1MT; encoded by TOP1MT**) to relax the tensional torsion of unwinding in front of the replication fork) and a DNA ligase (**mtLigIII; encoded by DNA Ligase 3**) to seal the nick and finish replication. *5.- Nucleases removing RNA primers and flap intermediate*: **RNase H1** (encoded by RNASEH1), DNA helicase/nuclease 2 (**DNA2**; encoded by DNA2), RNA and DNA 5′ flap endonuclease **FENMT** and mitochondrial genome maintenance exonuclease 1 (**MGME1**; encoded by MGME1). *6.- Proteins involved in mitochondrial fusion*: mitofusin 1 (**MFN1**; encoded by MFN1), mitofusin 2 (**MFN2**; encoded by MFN2), dynamin-related GTPase **OPA1** (encoded by OPA1) and F-box and leucine-rich repeat 4 (**FBXL4**; encoded by FBXL4). nde

mitochondrial deoxyguanosine kinase (DGK; encoded by DGUOK) phosphorylates the purine deoxyribonucleosides deoxyguanosine (dG) and deoxyadenosine (dA) to deoxyguanosine monophosphate (dGMP) and deoxyadenosine monophosphate (dAMP), respectively (Johansson and Karlsson 1996). Then, the deoxyribonucleotide

monophosphates (dNMPs) are phosphorylated by nucleotide monophosphate kinase (NMPK) yielding deoxyribonucleotide diphosphates (dNDPs) that, in their turn, are finally phosphorylated to dNTPs by the nucleotide diphosphate kinase (NDPK) that forms a complex with both succinyl CoA ligase (SUCL, formed by subunits a and b) and the gamma-aminobutyrate transaminase (GABAT) that act at this point in addition to their "main" activities in Krebs cycle and synthesizing the inhibitory neurotransmitter GABA (Besse et al. 2015; Kowluru et al. 2002).

In addition to the mitochondrial nucleotide salvage pathway, the import from the cytosol is the second important source for dNTPs obtaining by mitochondria. There are two main cytosolic enzymes involved in an adequate supply of dNTPs precursors to mitochondria, the Ribonucleotide reductase (RNR) and Thymidine phosphorylase (TP) (El-Hattab et al. 2017). RNR reduces ribonucleotides (NDPs) to dNDPs for both genomes, nuclear and mitochondrial. RNR is a heterotetramer (R1-R1-R2-R2 subunits) that actively works during the S-phase of the cell cycle. R2 is degraded in late mitosis but then a p53R2 subunit is synthesized. Thus, RNR harboring R1-R1-p53R2- p53R2 subunits is the form of the enzyme essential for dNTPs supply to mitochondria (Pontarin et al. 2008). Thymidine phosphorylase (TP; encoded by TYMP) reversibly catalyzes the conversion of the ribonucleoside thymidine to thymine being essential for the cytosolic nucleotide salvage pathway (Lopez et al. 2009).

Obviously, if the import of dNTPs or precursors from cytosol to mitochondria is one of the pillars where relies a balanced mitochondrial dNTPs pool, the mitochondrial nucleotide transporters also play an essential role. There are three proteins involved, directly or indirectly, in nucleotides transport through the mitochondrial membranes: ANT, AGK and MPV17. ANT (Adenine nucleotide Translocator) is a homodimer located into the mitochondrial inner membrane which form a channel that import ADP and export ATP respect to mitochondria, supplying energy to the cell and regulating the ADP/ATP ratio, essential for OXPHOS function. There are three isoforms of ANT: ANT1 (encoded bySLC25A4) is expressed in post-mitotic cell types, ANT2 is expressed in proliferating cells and ANT3 is ubiquitously expressed (El-Hattab et al. 2017). ANT is bound to the inner membrane lipids phosphatidic acid and cardiolipin. Interestingly, mitochondrial acyl-glycerol kinase (AGK; encoded by AGK), has an essential function in phosphatidic acid synthesis and therefore in the synthesis of phospholipids including cardiolipin, becoming indirectly important for ANT function by maintaining the integrity of the mitochondrial inner membrane (Mayr et al. 2012). Finally, MPV17 protein (encoded by MPV17) is also located into the mitochondrial inner membrane and is essential in maintaining a balance dNTPs pool inside mitochondria. It has been suggested that MPV17 forms a channel in the mitochondrial inner membrane that functions in importing dNTPs into the mitochondria (Lollgen and Weiher 2015).

### *3.3.3 Mitochondrial Dynamics*

In addition to the components of the mtDNA replication machinery and to the proteins involved in a constant supply of deoxyribonucleotide triphosphates (dNTPs), functional mitochondria relies on the capacity of the cell to keep a healthy mitochondrial network.

Mitochondria network is undergoing continuous fusion and fission events and, although both are essential for the homeostasis and mitochondrial function, each process plays different roles. Mitochondrial fusion generates the elongation of mitochondrial tubular network, increases ATP production and enhances transfer of mitochondrial material among the newly fused mitochondria, homogenizing and balancing their metabolites and protein content including the enzymes needed for mtDNA synthesis (Farmer et al. 2018). This may be particularly important under compromising conditions for the cell such as stress or starvation in which fusion events can help to overcome the situation (Silva Ramos et al. 2016). On the contrary, mitochondrial fission leads to the fragmentation of the mitochondrial network in small pieces with two putative opposite functions, making easier the mitochondrial distribution on daughter cells during cell division or generating a substrate easier to degrade by mitophagy, an essential process for mitochondrial turnover (Twig et al. 2008).

Mitochondrial fusion requires the coordinated fusion of both the outer and inner membranes. Among proteins promoting mitochondrial fusion highlights the three GTPases Mitofusin-1 (MFN1; encoded by MFN1) and Mitofusin-2 mitofusin 2 (MFN2; encoded by MFN2) controlling the Outer Mitochondrial Membrane (OMM) fusion, and OPA1 that involved in fusion events of the Inner Mitochondrial Membrane (IMM) (Chen et al. 2010). OPA1-exon4b, an alternatively spliced OPA1 isoform, is associated with the mitochondrial inner membrane and directly interacts with the nucleoids regulating their even distribution within the mitochondrial network. In addition, OPA1-exon4b also promotes mtDNA replication potentially by interacting with the replisome and hence participating in mtDNA copy number and expression (Elachouri et al. 2011). Finally, FBXL4 (F-box and leucine-rich repeat4; encoded by FBXL4) is located in the mitochondrial intermembrane space. FBXL4 has a protein-protein interaction domain that makes it to form a quaternary protein complex and has been found essential for the formation of a normal mitochondrial network probably through interaction with other mitochondrial fusion proteins (Bonnen et al. 2013).

The interaction between nucleoids and IMM and their relationship with mitochondrial dynamics is object of interest due to the putative role in control the mtDNA replication, mtDNA distribution and its association with depletion syndromes. Thus, due to the large nucleoids size, probably they cannot move within mitochondria graticule in spite of fusion events. It is likely that nucleoid distribution depends on continuous events of fusion and fission of the mitochondrial network as a distribution tool of mitochondrial content (Bonekamp and Larsson 2018). Furthermore, recent studies indicate a close relationship between mitochondrial

fission and nucleoid maintenance and distribution since loss of the GTPase Drp1, necessary for mitochondrial division, results in nucleoid clustering and dysfunction (Ishihara et al. 2015).

## 3.4 mtDNA Instability: Deletions and Depletion Syndromes

Mutations on nuclear genes involved in nuclear-mitochondrial intergenomic communication cause Mendelian inherited disorders characterized by producing mtDNA instability that leads to mtDNA large-scale rearrangements (multiple deletions) or by severe reduction of the mtDNA copy number (mtDNA depletion). These genes encode proteins belonging to at least the three pathways seen above: mtDNA replication and maintenance, nucleotide supply, and mitochondrial dynamics. Their function has been shown in previous points of this chapter and here we will see some aspects of the defects associated to their dysfunction. As discussed above, one of the hallmarks of mtDNA disorders is their clinical and genetic heterogeneity. Particularly, mtDNA depletion may affect either a specific tissue (most commonly muscle or liver and brain) or multiple organs, including heart, brain, and kidney.

When defects in mtDNA maintenance were described, they were arranged in two groups according to clinical criteria: (1) Mitochondrial DNA depletion syndromes typically infancy-onset and characterized by severe clinical phenotypes and shortened life expectancy; and (2) Multiple mtDNA deletion syndromes that typically debut in adulthood and characterized by milder phenotypes including progressive external ophthalmoplegia (CPEO) and myopathy (El-Hattab et al. 2018). At date, we can consider as the most frequent clinical presentations of defects in mtDNA maintenance (modified from Viscomi and Zeviani (2017) and El-Hattab et al. (2018)):

1. autosomal dominant or recessive adult-onset encephalomyopathy, with CPEO and multiple deletions of mtDNA.
2. an autosomal recessive multisystem disorder known as mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), with combined accumulation of multiple deletions and incomplete depletion of mtDNA.
3. a spectrum of recessive neurologic syndromes ranging from typical infantile hepatopathic poliodystrophy (Alpers-Huttenlocher syndrome) to juvenile-onset sensory-ataxia neuropathy, dysarthria, and ophthalmoplegia (SANDO) to a combination of spinocerebellar ataxia and epilepsy (SCAE) with or without external ophthalmoplegia.
4. early-onset, organ-specific autosomal recessive syndromes associated with profound mtDNA depletion that can be present as a Myopathic form, Encephalomyopathy forms or Hepatocerebral form.

Currently, there are pathogenic variants in more than 20 nuclear genes associated with mtDNA maintenance defects that can be classified according the affected process: mtDNA synthesis, mitochondrial nucleotide salvage pathway, cytosolic

**Table 3.1** Mitochondrial DNA Maintenance Defects: Genes and relevant characteristics

| Gene (protein) | Activity | Main function | mtDNA defect | Main clinical phenotype | Inherit. |
|---|---|---|---|---|---|
| *Genes involved in mitochondrial DNA synthesis* | | | | | |
| *POLG* (POLG) | Mitochondrial DNA polymerase (catalytic subunit) | mtDNA synthesis | Multiple deletions, Depletion | arCPEO, Alpers-Huntenlocher, SANDO/SCAE | AD, AR AR |
| *POLG2* (POLG2) | Pol γ accessory subunit | Increases POLG processivity | Multiple deletions, | adCPEO | AD |
| *TWNK* (TWINKLE) | Mitochondrial DNA helicase | Separates DNA strands | Multiple deletions, Depletion | adCPEO, IOSCA, Alpers-like | AD, AR AR |
| *TFAM* (TFAM) | Replication and transcription | Primer formation; mtDNA stability | Depletion | Hepatocerebral syndrome | AR |
| RNASEH1 (*RNase H1*) | Exobinuclease | Primer removal | Multiple deletions, | arCPEO | AR |
| *MGME1* (MGME1) | Mitochondrial genome maintenance exonuclease 1 | Primer removal | Multiple deletions, | arCPEO, | AR |
| *DNA2* (DNA2) | DNA helicase/ nuclease 2 | Primer removal | Multiple deletions, | adCPEO | AD |
| *Genes involved in mitochondrial nucleotide salvage pathway* | | | | | |
| *TK2* (TK2) | Thymidine kinase | Pyr deoxynucleosides > Pyr dNMPs | Multiple deletions, Depletion | Myopathy, arCPEO | AR AR |
| *DGUOK* (DGK) | Deoxyguanosine kinase | Pur deoxynucleosides > Pur dNMPs | Multiple deletions, Depletion | Hepatocerebral syndrome, Myopathy with or w/o CPEO, lower motor neuron syndrome | AR AR |
| *DTYMK* (NMPK) | Nucleotide monophosphate Kinase | dNMPs > dNDPs | Depletion | Hepatocerebral syndrome | AR |
| *NME4* (NDPK)[a] | Nucleotide diphosphate Kinase | dNDPs > dNTPs | Depletion | Hepatocerebral syndrome | AR |
| *SUCLAG2 or SUCLA2* (SUCL β-subunit) | ATP-dependent Succinate-CoA ligase. Synthesis dNTPs | Complex with NDPK | Depletion | Hepatocerebral syndrome | AR |
| *SUCLG1* (SUCL α-subunit) | GTP-dependent Succinate CoA ligase. Synthesis dNTPs | Complex with NDPK | Depletion | Hepatocerebral syndrome | AR |
| *ABAT* (GABAT*)* | Gamma-aminobutyrate transaminase | Complex with NDPK | Multiple deletions | Encephalomyopathy | AR |

(continued)

**Table 3.1** (continued)

| Gene (protein) | Activity | Main function | mtDNA defect | Main clinical phenotype | Inherit. |
|---|---|---|---|---|---|
| *Genes involved in cytosolic nucleotide metabolism* | | | | | |
| *TYMP* (TP) | Thymidine phosphorylase | Thymidine > thymine | Multiple deletions, Depletion | MNGIE | AR |
| *RRM2B* (p53R2) | p53-Ribonucleotide reductase small subunit | NDPs > dNDPs | Multiple deletions, Depletion | adCPEO, myopathy and tubulopathy | AD AR |
| *Genes involved in mitochondrial nucleotide import* | | | | | |
| *SLC25A4* (ANT1) | Adenine nucleotide translocator | Nucleotide transport | Multiple deletions, Depletion | ad/arCPEO, myopathy and cardiomyopathy | AD, AR AD |
| *AGK* (AGK) | Acyl-glycerol kinase | Nucleotide transport | Depletion | Congenital cataract, hypertrophic cardiomyopathy, skeletal myopathy and lactic acidosis | AR |
| *MPV17* (MPV17) | Mt inner membrane protein. dNTPs transport | Nucleotide transport | Multiple deletions, Depletion | Hepatocerebral syndrome, arCPEO, leukoencephalopathy and, parkinsonism | AR AR |
| *Genes involved in mitochondrial dynamics* | | | | | |
| *OPA1* (OPA1) | Dynamin-related GTPase. IMM fusion factor | IMM fusion. | Multiple deletions, | DOA | AD |
| *MFN2* (MFN2) | GTPase. OMM fusion factor | OMM fusion | Multiple deletions, | DOA plus | AD |
| *FBXL4* (FBXL4) | Mitochondrial LRR F-Box protein. IMM integrity | Mitochondrial fusion | Depletion | Encephalomyopathy | AR |

By alphabetical order. *AD* autosomal dominant, *AR* autosomal recessive, *CPEO* chronic progressive external ophthalmoplegia, *DOA* Dominant optic atrophy, *dNMPs* deoxyribonucleotide monophosphates, *dNDPs* deoxyribonucleotide diphosphates, *dNTPs* deoxyribonucleotide triphosphates, *IMM* Inner mitochondrial Membrane, *IOSCA* Infantile onset spinocerebellar ataxia, *OMM* outer mitochondrial Membrane, *Pur* Purine, *Pyr* Pyrimidine, *SANDO* sensory-ataxia neuropathy, dysarthria, and ophthalmoplegia

[a]NDPK forms a complex with both SUCL (succinyl-CoA ligase) and GABAT (gamma-aminobutyrate transaminase). SUCL is formed by an alpha subunit (encoded by *SUCLG1*) and a beta subunit (encoded by either *SUCLA2* or *SUCLG2*)

nucleotide metabolism, mitochondrial nucleotide import, and mitochondrial dynamics. In Fig. 3.3 it is shown a simple diagram of these process, in Fig. 3.3 legend is shown the protein and their coding gene and function and in Table 3.1 are shown the most relevant characteristics of each defect.

## 3.5 Therapy for mtDNA Defec

For the vast majority of mitochondrial diseases only symptomatic therapies, usually with very poor benefits, are available. In fact, at date, only those diseases provoked by defects on the synthesis of Coenzyme $Q_{10}$ really respond to treatment using supplements of coenzyme $Q_{10}$ (Hernandez-Camacho et al. 2018; Lopez-Lluch et al. 2019). The main problems to develop treatments for mitochondrial disorders include the rarity, heterogeneous clinical phenotypes, pathogenic complexity and etiological heterogeneity (Hirano et al. 2018). This is even more complex in the case of mtDNA-based diseases since at date we barely have access to it. However, there has been important progress in our understanding of the molecular genetic causes, pathomechanisms, and the clinical presentations of mitochondrial diseases (Gorman et al. 2016; Pfeffer 2013 #143). Furthermore, in the last years there have been important, a sometimes amazing, advances in Molecular and Cellular Biology that allow us to maintain hope for a future improvement in treatment, limiting transmission and a possible cure for this group of devastating diseases.

The current approaches and promising research for treatment and therapeutic options against mtDNA-based mitochondrial diseases can be grouped, in a simple manner, in three categories: pharmacological, genetic and cellular approaches.

### *3.5.1 Pharmacological Approaches*

Mutations in almost every mtDNA genes have been implicated in encourage leakage of electrons from the electron transport chain and increased reactive oxygen species (ROS) production that may lead to the pathogenic cellular damage and disease. Thus, antioxidants are frequently used in the treatment of mitochondrial patients independently of the origin of their mitochondrial defect, highlighting idebenone, EPI-743 and vitamin C. However, efficacy of antioxidants in patients with mitochondrial diseases remains controversial, with some promising but not conclusive results and uncertain efficiency (Yu-Wai-Man 2014 #144; Hirano et al. 2018). In addition, a recent review of mitochondrial therapies has found little evidence supporting the use of any vitamin or cofactor for treatment (Pfeffer et al. 2012).

A particularly promising pharmacological therapy for some mtDNA depletion syndromes is the supplement with of deoxyribonucleotides and deoxyribonucleosides. Thus, mutations in components of the dNTP salvage pathway such as TK2 (thymidine kinase 2), dGK (deoxyguanosine kinase) or TP (thymidine phosphorylase) impairs the availability of one or more dNTPs. Addition to cellular and animal models of deoxyribonucleosides or derivatives have shown as an effective pharmacological approach for treating mitochondrial depletion syndromes due to defects in dNTP homeostasis (Camara et al. 2014).

### *3.5.2 Genetic Approaches*

The genetic approach for secondary mtDNA disorders should rely on well-known, although still improving, gene replacement/rescue techniques, some of which have shown successful results (Young et al. 2006; Wirth et al. 2013). However, the location and nature of mtDNA supposes a much more complex problem for gene therapy related to primary mtDNA mutations. Our access to mtDNA is extremely limited being, at date, only possible to send proteins to mitochondria, but not nucleic acids. However, this opens and indirect way of mtDNA manipulation. Thus, Moraes and collaborators targeted the bacterial restriction enzyme *PstI* to mitochondria, where it recognized and cut mtDNA PstI sites. When the transfecting cultured cells harbored mtDNA molecules included PstI recognition sites the treatment led to a complete loss of mtDNA. However, when mtDNA molecules included PstI recognition sites were in heteroplasmy, its percentage was modified, being a proof of concept for mtDNA manipulation inside mitochondria (Srivastava and Moraes 2001). This approach can, however, be used only when a restriction site is introduced by the mutation and it is absent in the wild type molecules. An improved version of Moraes' experiment was the use of TALE and zinc finger nucleases (TALEN and ZFN) that use an common restriction enzyme (FokI) fused to appropriate ZFN or TALE modules to recognize specific sites in the mtDNA (Gammage et al. 2014; Bacman et al. 2013). The main limitation of this approaches is that they both require quite large constructs not easily fitted into AAV vectors (Viscomi 2016).

One of the most exciting gene therapy approach to primary mtDNA mutations is the gene replacement/rescue. Thus, an mtDNA-coding gene is cloned preceded in frame by a mitochondrial targeting sequence and allotopically expressed in the nucleus. This approach has been tried in an animal model of LHON (Ellouze et al. 2008) and is currently in clinical trials of gene replacement therapy in a patient harboring mutation in the mitochondrially-encoded MT-ND4 gene (Slone and Huang 2020).

The possibility of using the CRISPR/Cas9 genomic edition system is a really attractive and challenging possibility and it is being tried in many laboratories around the world. The biggest drawback for it is the strictly requirement of import the guide RNA into mitochondria. The only two published attempts of using CRISPR/Cas9 to edit mtDNA show many ambiguous evidences (Slone and Huang 2020). However, the speed of new advances appearance in relation to the CRISPR/Cas9 system makes us to speculate that we will soon have this powerful genomic editing tool applicable to mtDNA and revolutionize the field of mitochondrial diseases.

### 3.5.3 Cellular Approaches

The use of cells as a tool to improve some aspects of mtDNA-associated mitochondrial deficiencies is focused mainly on two points: curing or ameliorating and avoiding transmission from oocyte to offspring.

The therapeutic use of cells in mitochondrial diseases is illustrated by cell replacement approaches and the promising and potential use of induced pluripotent stem cells (iPSCs). Thus, in a patient defective in Thymidine phosphorylase activity, and therefore with a severe impaired supply of dNTPs, the use of erythrocyte-encapsulated thymidine phosphorylase made improved clinical and biochemical symptoms (Bax et al. 2013). The implementation of induced pluripotent stem cells (iPSCs) in biomedical research more than a decade ago, resulted in a huge leap forward in the potential treatment of primary mtDNA disorders. Thus, reprogramming mtDNA-harboring mutations fibroblasts from a patient to iPSCs, removing their mtDNA with available techniques, repopulation those mtDNA-free iPSCs with mtDNA from a healthy donor (for example by fusion with his/her platelets) would give rise iPSCs containing mtDNA mutation-free. Of course, there are numerous variants of these techniques waiting to be found. Finally, cells manipulations let Mitalipov and collaborators to obtain zygotes free of mtDNA mutations starting from oocytes containing mutated mtDNA molecules by nuclear transfer. Basically, there are two ways to do it. On one hand, an oocyte from a woman with a mitochondrial genetic disease is fertilized and the pronucleus is removed and inserted into an egg from a healthy woman previously fertilized and enucleated. On the other hand, the pronucleus of the patient's oocyte is removed and inoculated into the donor's enucleated egg, then it is then fertilized (Amato et al. 2014). In both cases, the new fertilized oocyte contains genomic information from three parents.

Finally, there are other promising therapeutic approaches for mitochondrial diseases that could ameliorates patients, or in that case, animal model phenotypes. Among them, are remarkable those associated to exercise since muscle is an exocrine organ that produces signaling molecules which stimulates mitochondrial biogenesis (Tarnopolsky 2014) or the use of alternative enzymes to bypass mitochondrial respiratory chain defects such as the use of Alternative Oxidase from *Ciona intestinalis* to bypass complex III–IV deficiencies in human cells and Drosophila (Dassa et al. 2009).

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# Chapter 4
# Mitochondrial Translation Deficiencies

**Veronika Boczonadi, Juliane S. Müller, and Rita Horvath**

**Abstract** Most mitochondrial diseases are disabling and progressive, usually affecting multiple organs with high energy demand such as the brain, liver, skeletal muscle and heart. Although defective oxidative phosphorylation is the common final pathway, it is unknown why different mutations result in largely heterogeneous clinical presentations. Currently there are very few effective therapies and treatment is usually symptomatic.

Mitochondrial respiratory chain deficiencies are caused by mutations in the mtDNA and in over 300 nuclear genes. The translation of the 13 mtDNA-encoded proteins require a unique apparatus of over 150 genes, which are distinct from the genes involved in the translation of the nuclear-encoded mitochondrial proteins. In addition to the mtDNA encoded tRNA and rRNA genes, several nuclear genes are involved in the various steps of mitochondrial protein synthesis. The mitochondrial respiratory chain enzymes are composed of both mtDNA and nuclear encoded proteins, therefore a controlled interaction between nuclear and mitochondrial protein synthesis is essential for the optimal function of mitochondria. In this chapter we discuss the molecular mechanism of mitochondrial translation and provide an update on diseases caused by a defect of mitochondrial protein synthesis.

## 4.1 Introduction

Mitochondrial diseases affect at least 1 in 4300 of the population and produce diverse clinical phenotypes often presented as multi-systemic disorders (DiMauro et al. 2013; Gorman et al. 2016; Vafai and Mootha 2012; Ylikallio and Suomalainen

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2012). In addition to the nucleus, human cells also harbour DNA in the mitochondria (mtDNA), which is essential for cell viability (Tuppen et al. 2010). This small (16.5 kb) genome is found in multiple copies in mitochondria, the subcellular organelles that are the main sources of energy production within the cells. OXPHOS (oxidative phosphorylation) is responsible for the production of ATP by generating a proton gradient across the inner membrane of the mitochondria which is used by the mammalian cells (Greaves et al. 2012). The mitochondrial OXPHOS system comprises hundreds of different proteins out of which only 13 polypeptide subunits are encoded by the mtDNA. In addition, the mtDNA encodes the small and large rRNAs, and 22 distinct mitochondrial tRNAs that are necessary for the translation of only the mitochondrial-encoded proteins (Boczonadi and Horvath 2014; Chrzanowska-Lightowlers et al. 2011; Rotig 2011; Smits et al. 2010). The nuclear-encoded subunits of the respiratory chain (RC) complexes as well as proteins that are inevitable for normal mitochondrial protein synthesis (such as OXPHOS assembly, mtDNA metabolism and maintenance, mitochondrial cofactor biosynthesis, mitoribosomal subunits and assembly factors, regulators of mitochondrial expression and translation, etc.) are encoded by the nuclear genome (nDNA) and synthetized in the cytosol before transported into the organelle (Vafai and Mootha 2012). The mitochondrial ribosomal proteins assemble with mitochondrial ribosomes 12S rRNA and 16S rRNA to form the mitochondrial ribosome (Pietromonaco et al. 1991).

## 4.2 The Machinery of Mitochondrial Translation

The proteins responsible for mitochondrial translation are different from their cytosolic counterparts and they are more related to those of bacteria, however the mechanisms of the translation follow the same major steps: initiation, elongation, termination and recycling of the ribosome (Christian and Spremulli 2012).

The process of mitochondrial translation starts with the formation of the initiation complex. In the first step the two mitochondrial ribosomal subunits (28S and 39S) are getting separated (Kuzmenko et al. 2014) and form a complex which consists of the 28S subunit, mRNA, the formylmethionine-tRNA and the $IF2/3_{mt}$ (Christian and Spremulli 2009; Koc and Spremulli 2002). This is followed by the entry of the mRNA into the $IF3_{mt}$:28S subunit complex. $IF3_{mt}$ is thought to support the correct position of mRNA to bind the small subunit at the peptidyl (P) site of the mitoribosome. The association of the mitoribosome stimulates the release of initiation factors and the elongation on the 55S ribosome. When the appropriate start codon is present, the formylmethionin-tRNA can bind to the first codon with the help of $IF2_{mt.}$ The importance of this step in mitochondrial translation is illustrated by the fact, that mutations in MTFMT, which is responsible for the Met-tRNA(Met) formylation is

critical for efficient human mitochondrial translation (Neeve et al. 2013; Tucker et al. 2011).

The mitochondrial elongation factor (EF-Tu$_{mt}$-GTP) and an amino-acylated tRNA arrives to the A-site of the mitoribosome and upon correct codon-anticodon pairing the EF-Tu$_{mt}$-GDP leave the mitoribosome and the aminoacyl-tRNA moves into the P site where peptide bond formation is catalysed extending the growing polypeptide chain. Another translation elongation factor, EF-Ts$_{mt}$ plays a role as a nucleotide exchange factor and converts EF-Tu$_{mt}$ to an active form (EF-Tu$_{mt}$-GTP). The GTP bound EFG1$_{mt}$ catalyses the translocation of the ribosome during the A and P site and tRNAs move to the P and exit (E) sites of the mitoribosome. This elongation step repeats itself until the stop codon (UAA, UAG, AGA or AGG) is encountered in the A-site. Mutations of the mitochondrial elongation factors typically associated with encephalopathy and other organ involvement (liver, heart) in early infancy and the affected children die early (Coenen et al. 2004; Smeitink et al. 2006; Smits et al. 2011; Valente et al. 2007).

The stop codon is recognized by the mitochondrial release factor (mtRF1a), which binds to the mitoribosome and induces hydrolysis of the peptidyl-tRNA bond in the A-site releasing the mature protein from the site. Other termination release factors such as mtRF1, C12orf65 and ICT1 are also thought to play an essential role in the termination (Richter et al. 2010). As a last step mitochondrial recycling factors (mtRRF1 and mtRRF2) translocate to the A-site to induce the release of the mRNA (Chrzanowska-Lightowlers et al. 2011). Up to date from the factors involved in translation termination only the *C12orf65* has been identified as a disease causing gene (Antonicka et al. 2010).

## 4.3 Diseases Caused by Abnormal Mitochondrial Protein Synthesis

As it was predicted, mutations in the nuclear genes coding for various components of the mitochondrial translation machinery could give rise to a wide spectrum of diseases and phenotypes (Boczonadi and Horvath 2014; Chrzanowska-Lightowlers et al. 2011) (Table 4.1). As we illustrated above, mitochondrial protein synthesis requires several nuclear-encoded factors, such as ribosomal proteins, ribosome assembly proteins, aminoacyl-tRNA synthetases, tRNA modifying enzymes and initiation, elongation and termination factors (Rotig 2011). Autosomal recessive mutations have been reported in several of these factors in association with variable clinical presentations (Chrzanowska-Lightowlers et al. 2011). Here we note that disorders of mitochondrial protein synthesis usually result in combined RC deficiencies and associated with abnormal mitochondria (ragged red fibres, COX negative fibres) on histology. However, a defect of translation activation factors or post-transcriptional regulators of mammalian mtDNA expression may cause impairment in the stability of certain mitochondrial transcripts leading to isolated enzyme

**Table 4.1** Summary of the clinical presentations of the known human disease genes involved in mitochondrial protein synthesis

| Gene | Protein | Clinical presentation | Age of onset | OMIM | References |
|---|---|---|---|---|---|
| ***Nuclear genes involved in impaired mitochondrial translation – tRNA-modifying enzymes*** | | | | | |
| *PUS1* | Pseudouridine synthase | Myopathy, lactic acidosis, and sideroblastic anemia (MLASA1) | Early childhood | 608,109 | Bykhovskaya et al. (2004) |
| *TRMU* | tRNA 5-methylaminomethyl-2-thiouridylate methyl-transferase | Reversible infantile liver failure | Infantile | 613,070 | Gaignard et al. (2013), Schara et al. (2011), Zeharia et al. (2009) |
| *MTO1* | Mitoch. translation optimization 1 homolog | Hypertrophic cardiomyopathy and lactic acidosis | Infantile | 614,702 | Ghezzi et al. (2012) |
| *MTFMT* | Mitoch. methionyl-tRNA formyltransferase | Leigh syndrome | Early childhood | 611,766 | Tucker et al. (2011), Neeve et al. (2013) |
| *TRMT5* | tRNA methyltransferase 5 | Failure to thrive and hypertrophic cardiomyopathy or exercise intolerance | Infantile, adulthood | 616539 | Powell et al. (2015) |
| *TRMT10C* | tRNA methyltransferase 10 | Lactic acidosis, hypotonia, feeding difficulties, deafness | Childhood | 616974 | Metodiev et al. (2016) |
| *ELAC2* | ElaC ribonuclease Z 2 | Hypertrophic cardiomyopathy, poor growth, hypotonia, delayed psychomotor development and lactic acidosis | Infantile | 615440 | Haack et al. (2013), Shinwari et al. (2017) |
| *TRIT1* | tRNA isopentenyltransferase | Epileptic encephalopathy, myoclonic jerks and developmental delay | Childhood | N/A | Yarham et al. (2014) |
| ***Nuclear genes involved in impaired mitochondrial translation – Ribosomal proteins*** | | | | | |
| *MRPL3* | Mitochondrial ribosomal protein L3 | Hypertrophic cardiomyopathy and psychomotor retardation | Infantile | 614,582 | Galmiche et al. (2011) |
| *MRPS16* | Mitochondrial ribosomal protein S16 | Corpus callosum agenesia, hypothonia and fatal neonatal lactic acidosis | Neonatal | 610,498 | Miller et al. (2004) |
| *MRPS22* | Mitochondrial ribosomal protein S22 | Cornelia de Lange-like syndrome Oedema, cardiomyopathy, tubulopathy | Neonatal | 611,719 | Saada et al. (2007), Smits et al. (2011) |

(continued)

**Table 4.1** (continued)

| Gene | Protein | Clinical presentation | Age of onset | OMIM | References |
|---|---|---|---|---|---|
| *MRPL12* | Mitochondrial ribosomal protein L12 | Growth retardation, failure to thrive, muscle weakness | Neonatal | 602,375 | Serre et al. (2013) |
| *MRPL44* | Mitochondrial ribosomal protein L44 | Hypertrophic cardiomyopathy | Neonatal | 611,849 | Carroll et al. (2013) |
| ***Nuclear genes involved in impaired mitochondrial translation – Aminoacyl -tRNA synthetases*** | | | | | |
| *DARS2* | Aspartyl-tRNA sythetase 2 | Leukoencephalopathy with brainstem and spinal cord involvement (LBSL) | Childhood or adulthood | 610,956 | Scheper et al. (2007) |
| *RARS2* | Arginyl-tRNA Synthetase 2 | Pontocerebellar hypoplasia type 6 (PCH6) | Neonatal | 611,523 | Edvardson et al. (2007) |
| *EARS2* | Glutamyl-tRNA synthetase 2 | Leukoencephalopathy with thalamus and brainstem involvement and high lactate (LTBL) | Infantile | 612,799 | Steenweg et al. (2012) |
| *MARS2* | Methionyl-tRNA synthetase 2 | Autosomal recessive spastic ataxia with leukoencephalopathy | Juvenile or adulthood | 609,728 | Bayat et al. (2012) |
| *FARS2* | Phenylalanyl-tRNA synthetase 2 | Alpers syndrome, encephalopathy, epilepsy, lactic acidosis | Neonatal and infantile | 611,592 | Elo et al. (2012), Shamseldin et al. (2012) |
| *AARS2* | Alanyl-tRNA synthetase 2 | Hypertrophic cardiomyopathy | Infantile | 614,096 | Gotz et al. (2011) |
| *YARS2* | Tyrosyl-tRNA synthetase 2 | Myopathy, lactic acidosis, and sideroblastic anemia (MLASA2) | Infantile | 613,561 | Riley et al. (2010) |
| *SARS2* | Seryl-tRNA synthetase 2 | HUPRA (hyperuricemia, pulmonary hypertension, renal failure, alkalosis) | Infantile | 613,845 | Belostotsky et al. (2011) |
| *HARS2* | Histidyl-tRNA synthetase 2 | Perrault syndrome (sensorineural deafness, ovarian dysgenesis) | Juvenile or adulthood | 600,783 | Pierce et al. (2011) |
| *LARS2* | Leucyl-tRNA synthetase 2 | Perrault syndrome (sensorineural deafness, ovarian dysgenesis) | Juvenile | 604,544 | Pierce et al. (2013) |

(continued)

**Table 4.1** (continued)

| Gene | Protein | Clinical presentation | Age of onset | OMIM | References |
|---|---|---|---|---|---|
| *TARS2* | Threonyl-tRNA synthetase | Mitochondrial encephalomyopathy, axial hypotonia, psychomotor delay, and high levels of blood lactate. | Infantile | 615918 | Diodato et al. (2014) |
| *NARS2* | Asparginyl-tRNA synthetase | Non-syndromic deafness, Leigh syndrome, Alpers syndrome, infantile onset neurodegenerative disorder | Infantile | 616239 612,803 | Mizuguchi et al. (2017), Sofou et al. (2015), Vanlander et al. (2015) |
| *CARS2* | Cysteinyl-tRNA synthetase | Severe epileptic encephalopathy and complex movement disorders | Juvenile | 616672 | Coughlin 2nd et al. (2015) |
| *IARS2* | Ileucyl-tRNA synthetase | Cataracts, growth hormone deficiency, sensory neuropathy, sensorineural hearing loss, and skeletal dysplasia | Infantile | 616,007 612,801 | Moosa et al. (2017), Schwartzentruber et al. (2014) |
| *VARS2* | Valyl-tRNa synthetase | Mitochondrial encephalomyopathy Psychomotor delay | Juvenile | 615917 | Baertling et al. (2017), Diodato et al. (2014) |
| *WARS2* | Tryptophanyl-tRNA synthetase | Autosomal recessive intellectual disability | Juvenile | N/A | Musante et al. (2017) |
| *PARS2* | Prolyl-tRNa synthetase | Non-syndromic hearing loss, Leigh syndrome, intellectual disability with epilepsy and severe myopathy | | 616239 | Mizuguchi et al. (2017), Sofou et al. (2015) |
| ***Nuclear genes involved in impaired mitochondrial translation – Initiation, elongation and termination factors*** | | | | | |
| *RMND1* | RMND | Deafness, hypotonia, developmental delay, epilepsy, myopathy, renal involvement | Neonatal | 614,917 | Garcia-Diaz et al. (2012), Janer et al. (2012) |
| *TUFM* | Elongation factor Tu, mitochondrial (EF-$TU_{mt}$) | Lactic acidosis, leukoencephalopathy and polymicrogyria | Neonatal | 610,678 | Valente et al. (2007) |
| *TSFM* | Elongation factor Ts, mitochondrial (EF-$Ts_{mt}$) | Encephalomyopathy, hypertrophic cardiomyopathy | Neonatal | 610,505 | Smeitink et al. (2006) |

(continued)

**Table 4.1** (continued)

| Gene | Protein | Clinical presentation | Age of onset | OMIM | References |
|---|---|---|---|---|---|
| *GFM1* | Elongation factor G 1, mitochondrial ($EFG1_{mt}$) | Encephalopathy with or without liver involvement | Neonatal | 609,060 | Coenen et al. (2004), Smits et al. (2011), Valente et al. (2007) |
| *C12orf65* | Chromosome 12 open reading frame 65 | Leigh syndrome with optic atrophy or Behr syndrome (optic atrophy, motor neuropathy, spastic paraparesis, ataxia) | Infantile | 613,559 | Antonicka et al. (2010), Pyle et al. (2014) |
| ***Nuclear genes involved in impaired mitochondrial transaltion – Translation activators and mRNA stability factors*** | | | | | |
| *LRPPRC* | Leucine-rich PPR-motif containing protein | Leigh syndrome French–Canadian variant (LSFC) | Infantile | 220,111 | Mootha et al. (2003) |
| *TACO1* | Translational activator of cytochrome c oxidase 1 | Late-onset Leigh syndrome with optic atrophy | Juvenile | 612,958 | Weraarpachai et al. (2009) |
| *MTPAP* | Mitochondrial poly-A polymerase | Progressive spastic ataxia with optic atrophy | Juvenile (early childhood) | 613,672 | Crosby et al. (2010) |

defect, as reported in patients with *TACO1* and *LRPPRC* mutations (Harmel et al. 2013; Weraarpachai et al. 2009).

## 4.4 Defective Mitochondrial Translation Due to mtDNA Mutations

Frequent causes of impaired mitochondrial translation are mtDNA rearrangements (e.g. Kearns-Sayre syndrome) that affect mitochondrial tRNA and/or rRNA genes or single mt-tRNA point mutations. About half of the mtDNA mutations causing diseases in humans occur in tRNA genes (MELAS, MERRF, etc) and the heterogeneous clinical manifestations usually reflect variable heteroplasmy (Gorman et al. 2016; Greaves et al. 2012; Tuppen et al. 2010). However, the phenotypic diversity of the m.3243A > G mutation cannot be fully explained by the different heteroplasmy rate and most likely further genetic, epigenetic and/or environmental factors may contribute to the disease phenotype (Mancuso et al. 2014). The expression of mitochondrially encoded genes requires the efficient processing of long precursor RNAs at the 5′ and 3′ ends of tRNAs and the m.3243A > G and also the m.3302A > G mutations in the mt-tRNALeu(UUR) have been shown to cause RNA processing

defects and altered RNA processing may add to the variability of clinical manifestations (Siira et al. 2017).

Homoplasmic tRNA mutations with variable penetrance and clinical presentations also occur and further indicate that so far unknown genetic or epigenetic modifiers or environmental factors are important modifiers of mitochondrial translation (Taylor et al. 2003). Although most of these conditions are progressive and fatal, reversible infantile cytochrome c oxidase deficiency myopathy (or reversible infantile respiratory chain deficiency), due to a homoplasmic mt-tRNA$^{Glu}$ mutation m.14674 T > C stands out by showing spontaneous recovery (Horvath et al. 2009). The "reverse" disease course may be explained by some very unique molecular mechanism underlying mitochondrial translation, and these mechanisms are targets of current investigations (Boczonadi et al. 2015).

MtDNA deletions and depletion are diseases of mtDNA maintenance, but the molecular mechanism of these diseases also involves a defect of mitochondrial translation, however these diseases will not be discussed here.

## 4.5 Defective Mitochondrial Translation Due to Nuclear Gene Defects

The currently defined disorders caused by nuclear defects of mitochondrial protein synthesis are usually early-onset, severe, often fatal diseases (Table 4.1) with extremely variable clinical presentations. Approximately 150 nuclear encoded proteins are part of the mitochondrial translation apparatus and their mutations result in human disease with tissue specific clinical presentations, suggesting that in different organs these proteins may have different molecular function (Boczonadi and Horvath 2014).

## 4.6 Translation Initiation, Elongation and Termination Factor Mutations

Patients with translation elongation factor or mitochondrial ribosomal protein defects had an early age of onset and a severe multisystem disease with symptoms already present at birth or even prenatal in a few cases. For example, patients with mutations in genes *TUFM* (Tu translation elongation factor), *TSFM* (Ts translation elongation factor) and *GFM1* (G elongation factor) illustrate a severe phenotype, typically with lethal outcome, associated with combined respiratory chain deficiency (Smeitink et al. 2006; Valente et al. 2007). Regarding the mitochondrial ribosomal proteins, mutations in *MRPS16*, *MRPS22*, *MRPL3, MRPL44* and *MRPL12* have been associated with hypertrophic cardiomyopathy and neonatal lactic acidosis and neurological deterioration (Miller et al. 2004; Saada et al. 2007). Mutations

in these genes lead to impaired assembly of the small or large mitoribosomal subunit and eventually decreased rRNA levels. *C12orf65* is a protein of so far unknown function in mitochondrial translation, which belongs to the ribosomal release factors and mutation in this gene have been identified as a disease causing gene (Antonicka et al. 2010). Affected patients develop a very characteristic complex clinical phenotype (Behr syndrome) presenting with optic atrophy, ophthalmoplegia, spasticity, motor neuropathy and occasionally Leigh-like lesions (Pyle et al. 2014).

*RMND1* acts to anchor or stabilize the mitochondrial ribosome near the sites where the mRNAs are matured, coupling post-transcriptional handling of mRNAs with their translation (Janer et al. 2012), and mutations in *RMND1* are associated with a unique constellation of clinical phenotypes including congenital sensorineural deafness, hypotonia, developmental delay, renal involvement and lactic acidosis, that vary with the severity of the mitochondrial translation defect (Ng et al. 2016).

## 4.7 Mutations in tRNA Modifying Factors

Functional tRNA molecules provide the direct link between the DNA and protein, enabling the co-ordinated recruitment of amino acids based on the DNA transcript that determines the protein. All tRNA molecules require a wide variety of post-transcriptionally modified nucleosides which stabilize tRNA structure, enable efficient interaction with the ribosome, and fine-tune the translation machinery (Machnicka et al. 2014). Several enzymes have been shown to post-transcriptionally modify cytosolic (Machnicka et al. 2013) and mitochondrial (Suzuki and Suzuki 2014) tRNAs. The location and type of modification vary greatly between different tRNA molecules, organisms and tissues. Physiological environment and growth conditions influence the RNA modification pattern, raising the possibility that tRNA modification may be important in synchronizing translation to the needs of the organism at that particular time (Machnicka et al. 2013). However, the exact role of these modifications is largely unknown. It is also intriguing that cytoplasmic and mitochondrial translation share several key components. For example, aminoacyl-tRNA synthetases are important for either cytosolic or mitochondrial translation, however two aminoacyl tRNA synthetases (GARS, KARS) are present in both the cytoplasm and mitochondria encoded by the same gene, and the addition of the mitochondrial targeting signal differentiates the mitochondrial form (Oprescu et al. 2017). Myopathy, lactic acidosis and sideroblastic anaemia (MLASA) have been associated with mutations in pseudouridylate synthase 1 (*PUS1*), an enzyme located in both nucleus and mitochondria. PUS1 converts uridine into pseudouridine in several cytosolic and mitochondrial tRNA positions, thereby increasing the efficiency of protein synthesis in both compartments (Fernandez-Vizarra et al. 2009). Mutations in the methionyl-tRNA formyltransferase (*MTFMT)* gene, responsible for the N-formylation of the initiator $tRNA^{Met}$ in mitochondria, result in Leigh syndrome. Unlike all other translation systems, mitochondria use a single $tRNA^{Met}$ for

both initiation and elongation. A portion of Met-tRNA$^{Met}$ is formylated for initiation, whereas the remainder is used for elongation (Boczonadi and Horvath 2014). Autosomal recessive mutations in the tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase (*TRMU)* enzyme have been reported in reversible infantile liver disease. TRMU is responsible for the thiouridylation of the uridine at the first anticodon position (U34, wobble position), only present in the anticodon of 3 mt-tRNAs (Glu, Lys and Gln). The 2-thio group is required for the efficient codon recognition and interestingly, a modifying role of dietary cysteine intake has been suggested in the manifestation of the disease (Boczonadi and Horvath 2014). Deficiency of the mitochondrial translation optimization factor 1 (*MTO1*) enzyme that catalyzes the 5-carboxymethylamino-methylation of the same U34 at the wobble position in mt-tRNA$^{Glu}$, mt-tRNA$^{Gln}$ and mt-tRNA$^{Lys}$ were reported in patients with infantile hypertrophic cardiomyopathy (Boczonadi and Horvath 2014). Furthermore, a tissue-specific regulatory role of MTO1 has been recently suggested in fine tuning mitochondrial translation and balancing mitochondrial and cellular secondary stress responses (Tischner et al. 2015).

Mutations in *TRIT1* encoding a human tRNA isopentenyltransferase, which is responsible for i6A37 modification of the anticodon loops of a small subset of cytosolic and mitochondrial tRNAs, were identified in a patient with severe epileptic encephalopathy, diabetes mellitus. Here the abnormal mitochondrial protein synthesis led to a combined defect of mitochondrial respiratory chain complexes which were responsible for the human disease (Yarham et al. 2014). These data demonstrate that deficiencies of i6A37 tRNA modification should be considered a potential mechanism of human disease caused by both nuclear gene and mitochondrial DNA mutations. Altered tRNA modification was also documented to contribute to the pathomechanism of the two common pathogenic mt-tRNA mutations (m.3243A > G which causes mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes [MELAS]; and m.8344A > G, which causes myoclonic epilepsy with ragged-red fibres [MERRF]) (Suzuki and Suzuki 2014), and in m.343A > G cybrids, the expression of mt-tRNA modifying enzymes was modulated by microRNA-9/9*.

RNA in mitochondria is transcribed as one long polycistronic transcript; protein and rRNA encoding genes are interspersed by tRNA genes. Processing of the RNA molecules consist in the excision of mitochondrial the tRNAs from these polycistronic transcripts (Sanchez et al. 2011). Cleavage at the 5′ and 3′ termini of the tRNAs relies on the RNase P complex and the RNase Z activity of ELAC2 (ElaC Ribonuclease Z 2), respectively (Sanchez et al. 2011). The mitochondrial RNase P in animals is composed of three proteins, MRPP1, MRPP2, and MRPP36 (encoded by *TRMT10C, HSD17B10*, and *KIAA0391*, respectively) (Holzmann et al. 2008). *ELAC2* mutations were shown to cause severe infantile hypertrophic cardiomyopathy and complex I deficiency (Haack et al. 2013; Shinwari et al. 2017). Unprocessed mitochondrial RNA precursors were found to accumulate in muscle tissue and fibroblast cell lines from *ELAC2* patients, leading to blocking of mitochondrial translation and a reduction in OXPHOS capacity of the cells (Haack et al. 2013).

The *TRMT10C* gene (TRNA Methyltransferase 10C, Mitochondrial RNase P Subunit) encodes the MRPP1 subunit of RNAse P. Patients with *TRMT10C* mutations presented with neonatal mitochondrial disease and died at the age of 5 months (Metodiev et al. 2016). In addition to the RNAseP function of MRPP1, a stable complex of MRPP1 and MRPP2 has been described to have m1R9 methyltransferase activity. It is unclear which of the roles of MRPP1 is impaired at a molecular level in the patients.

Another mitochondrial tRNA processing and modifying enzyme, TRMT5 (tRNA Methyltransferase 5), has been identified to cause a mitochondrial disorder when mutated. TRMT5 methylates the guanosine at position 37 in the tRNA anticodon loop (next to the anticodon) in selected tRNAs using S-adenosyl methionine (Brule et al. 2004). This modification has been shown to significantly enhance translational efficiency. Powell and colleagues reported two affected individuals with *TRMT5* mutations presenting with lactic acidosis and evidence of multiple mitochondrial respiratory-chain-complex deficiencies (Powell et al. 2015). Severity of clinical symptoms varied considerably between the two patients. The identification of disease-casing *TRMT5* mutations further corroborates the importance of mitochondrial-tRNA-modifying enzymes for mitochondrial function.

## 4.8 Mitochondrial tRNA Synthetases

Human cells contain 17 cytoplasmic ARS polypeptides, including the bifunctional glutamyl-prolyl-tRNA sythetase (EPRS), and 18 mitochondrial ARS2 enzymes. Two ARS genes encode proteins with dual localisation, present both in the cytoplasm and mitochondria (GARS, KARS), and the transport of the mitochondrial isoforms is ensured by a mitochondrial targeting signal. Beside disorders due to impaired mitochondrial translation, several human disorders such as some forms of Charcot-Marie-Tooth disease (CMT), distal hereditary motor neuropathies (dHMN) or leukoencephalopathy with vanishing white matter (VWM) (Oprescu et al. 2017) are caused by altered cytosolic translation (Yao and Fox 2013). Interestingly mt-ARS diseases also lead to tissue specific clinical presentations mainly affecting brain, spinal cord and peripheral neurons,. Further implications of altered mitochondrial translation are highlighted by variable and complex clinical presentations, including diseases of eye, cartilage, skin, hair and even cancer.

Mutations in all mitochondrial ARSs have been reported in human disease with strong tissue specificity. Some gene defects affect most specifically the central nervous system, as exemplified by mutations in the mitochondrial aspartyl-tRNA synthetase 2 (*DARS2)* in leukoencephalopathy with brainstem involvement and high lactate, *EARS2* mutations cause leukoencephalopathy with thalamus and brainstem involvement (LTBL) (Steenweg et al. 2012), while mutations in the argynyl tRNA synthase 2 (*RARS2)* result in pontocerebellar hypoplasia and mitochondrial encephalomyopathy (PCH6) (Edvardson et al. 2007). Further neurological presentations often in association with epilepsy were reported in association with mutations in

*CARS2, FARS2, MARS2, PARS2, NARS2, WARS2* (Musante et al. 2017; Oprescu et al. 2017). Mitochondrial tyrosyl-tRNA synthase 2 (*YARS2*) mutations have been identified in families with mitochondrial myopathy, lactic acidosis and sideroblastic anemia (MLASA), very similar, but earlier onset compared to the phenotype caused by *PUS1* deficiency (Riley et al. 2010). Another very characteristic tissue specific disease is HyperUricemia, Pulmonary hypertension, Renal failure and Alkalosis (HUPRA) syndrome, which is caused by mutations in the mitochondrial seryl-tRNA synthetase 2 (*SARS2*) (Belostotsky et al. 2011). Mutations in the mitochondrial histidyl-tRNA synthetase 2 (*HARS2*) and *LARS2* were found in Perrault syndrome, characterized by ovarian dysgenesis and sensorineural hearing loss (Demain et al. 2017). While patents with fatal infantile hypertrophic cardiomyopathy had mutations in the catalytic domain of the mitochondrial alanyl-tRNA synthetase 2 (*AAR2*) gene (common mutation p.Arg592Trp) (Euro et al. 2015), mutations in other domains of *AARS2* result in leukoencephalopathy and ovarian failure, suggesting further clinical heterogeneity of tRNA synthetase mutations based on the localisation of the mutation (Euro et al. 2015). The frequent occurrence and the extreme variability and relative strict tissue specificity of the diseases caused by mutations in mitochondrial tRNA synthetase genes further illustrate the importance of understanding the factors influencing mitochondrial translation in different tissues.

It is often not easy to define the pathogenic role of novel ARS mutations. Beside the genetic prediction, population frequency of the variants and segregation analysis within the family, there are additional model systems to study the pathogenicity of mutations by functional studies in vitro in human patient cells, in yeast and worm (Oprescu et al. 2017).

As a novel therapeutic approach it has been recently shown that leucyl tRNA synthetase is able to partially rescue defects caused by mutations in cognate, but also some non-cognate mt-tRNAs (Hornig-Do et al. 2014; Perli et al. 2014). Furthermore, a C terminal peptide alone can enter mitochondria and interact with the same spectrum of mt-tRNAs as the entire synthetase in intact cells. These data support the possibility that a small peptide may correct the biochemical defect associated with many mt-tRNA mutations, inferring a novel therapy for these disorders causing abnormal mitochondrial translation.

## 4.9 Translation Activators of Single mtDNA Encoded Proteins

The expression of mitochondrial proteins is regulated by their own translational activators that bind mitochondrial mRNAs usually to their 5`-untranslated regions, and each mitochondrial mRNA has its own translational activator, which has been first shown in yeast (Herrmann et al. 2013). These translational activators can be part of feedback control loops which only permit translation if the downstream assembly of nascent translation products can occur (Herrmann et al. 2013). There are a few known translational activators of single mtDNA encoded proteins,

including *LRPPRC, TACO1* and *MSS51*. Mutations in nuclear-encoded translational activators of mitochondrial proteins such as *LRPPRC* and *TACO1* were also implicated in human disease (Mootha et al. 2003; Weraarpachai et al. 2009). The French-Canadian variant of COX-deficient Leigh syndrome has been shown to be caused by a founder mutation in the *LRPPRC* gene, however mutations in other populations were also described with intermittent severe lactic acidosis and early-onset neurodevelopmental problems with episodes of deterioration. In addition, some patients have had neonatal cardiomyopathy or congenital malformations, most commonly affecting the heart and the brain. All patients who were tested had isolated COX deficiency in skeletal muscle (Olahova et al. 2015). *TACO1* mutations were reported in a single family to date with optic atrophy and late-onset Leigh syndrome and an isolated defect of COX (Seeger et al. 2010; Weraarpachai et al. 2009). Other factors, such as MTERF3, have been also implicated to coordinate crosstalk between transcription and translation for the regulation of mammalian mtDNA (Wredenberg et al. 2013). Furthermore, a regulatory role of aminoacyl-tRNA synthetases has been suggested in both cytosolic and mitochondrial translation (Yao and Fox 2013).

## 4.10 Other Defects of Mitochondrial Protein Synthesis

PNPT1 is a subunit of the mitochondrial degradosome complex, which is involved in 3` to 5` exoribonuclease activity for RNA processing and degradation (Portnoy et al. 2008). The enzyme is predominantly located in the mitochondrial intermembrane space and is involved in import of RNA to mitochondria (Vedrenne et al. 2012). Biochemical studies showed that the mutation impaired normal import of several RNA species into mitochondria and caused a defect in mitochondrial translation, resulting in deafness or severe encephalomyopathy, choreoathetotic movements, and combined mitochondrial respiratory chain deficiency (Vedrenne et al. 2012; von Ameln et al. 2012).

GTPBP3 (GTP Binding Protein 3, mitochondrial) is a protein localised to the mitochondria and may play a role in mitochondrial tRNA modification. GTPBP3 and MTO1 have been proposed to generate the 5-taurinomethyluridine ($\tau m^5U34$) modification in mammalian mitochondrial tRNAs, but this has not yet been confirmed (Villarroya et al. 2008). Patients with mutations in *GTPBP3* presented with lactic acidosis, hypertrophic cardiomyopathy, and combined respiratory chain complex deficiencies in skeletal muscle. On a molecular level, mutations in *GTPBP3* cause a mitochondrial translation defect; the exact biochemical mechanism causing the translational defect, however, has not yet been determined (Kopajtich et al. 2014).

SFXN4 (Sideroflexin protein 4) is a member of a gene family encoding predicted mitochondrial transmembrane proteins. SFXN4 is assumed to be a transporter transmembrane protein of the inner mitochondrial membrane, and is required for mitochondrial respiratory homeostasis and erythropoiesis. Two patients with recessive *SFXN4* mutations have been reported to date the mutations are associated with

mitochondriopathy and macrocytic anaemia (Hildick-Smith et al. 2013). The function of SFXN4 is not yet well characterised and the molecular mechanism causing the diseases remains to be elucidated.

AIFM1 (Apoptosis Inducing Factor, Mitochondria Associated 1) encodes a flavoprotein essential for nuclear disassembly in apoptotic cells. It is found in the mitochondrial intermembrane space, and functions both as NADH oxidoreductase and as regulator of apoptosis (Susin et al. 1999; Wang et al. 2002). Mutations in *AIFM1* were reported to cause severe X-linked mitochondrial encephalomyopathy (Ghezzi et al. 2010) and also X-chromosome linked Cowchock syndrome (CMTX4), a slowly progressive disorder with axonal motor and sensory neuropathy, deafness, and cognitive impairment (Rinaldi et al. 2012). Patient cells displayed multiple respiratory chain deficiencies and an increased susceptibility to AIF-mediated programmed cell death (Ghezzi et al. 2010).

The majority of mitochondrial proteins are encoded by the nuclear genome and have to be imported into the mitochondria post-translationally. The mitochondrial protein import presequences are cleaved by specialised proteases, the mitochondrial presequence proteases, upon entry into the mitochondrial matrix. Two cleavages of a specific subset of octapeptide-containing precursor proteins are sequentially carried out by the Mitochondrial processing peptidase MPP and mitochondrial intermediate peptidase MIP (Mitochondrial Intermediate Peptidase)/Oct1 (Gakh et al. 2002; Vogtle et al. 2011). The human MIP protein is encoded by the encoded by the *MIPEP* gene. Recessive mutations in *MIPEP* cause left ventricular non-compaction, developmental delay, seizures, and severe hypotonia and were shown to lead to accumulation of processing intermediates of respiratory chain proteins (Eldomery et al. 2016).

TXN2 (Thioredoxin 2) is a member of the thioredoxin family, a group of small multifunctional redox-active proteins. The encoded protein may play important roles in the regulation of the mitochondrial membrane potential and in protection against oxidant-induced apoptosis (Lu and Holmgren 2012). Mutations in a single patient have been reported to date; the patient suffered from an infantile-onset neurodegenerative disorder with severe cerebellar atrophy, epilepsy, dystonia, optic atrophy, and peripheral neuropathy (Holzerova et al. 2016). TXN2 deficiency in patient cells resulted in increased ROS levels, disturbed ROS homeostasis and impaired function of the OXPHOS system (Holzerova et al. 2016).

**Fig. 4.1** (continued) (**e**) Nuclear genes encoding for ribosomal proteins and genes involved in impaired mitochondrial translation are *MRPL3*, *MRPS16*, *MRPS22*, *MRPL12* and *MRPL44***.** (**f**) Genes responsible for mitochondrial translation initiation, elongation and termination: *RMND1*, *TUFM*, *TSFM*, *GFM1*, *C12orf65*. Additional nuclear genes such as translational activators and mRNA stability factors (*LRPPRC*, *TACO1* and *MTPAP*) also involved in impaired mitochondrial protein synthesis

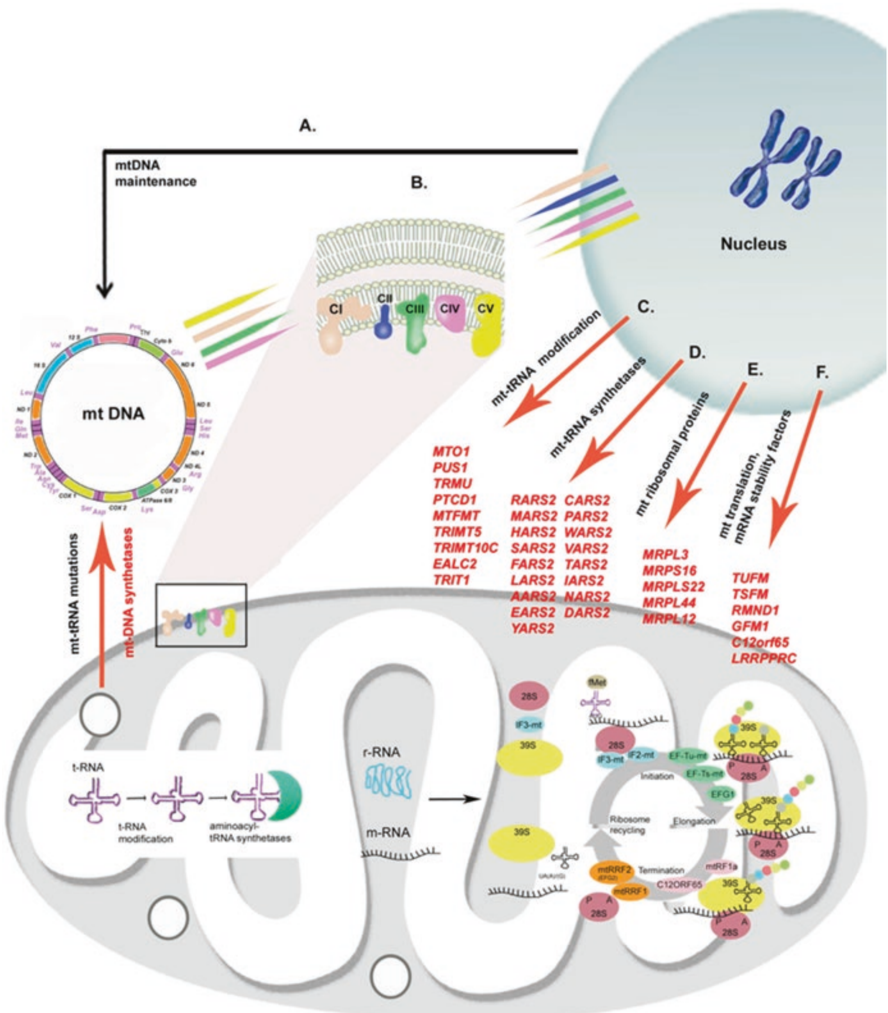


**Fig. 4.1 Schematic overview of human genes involved in mitochondrial protein synthesis defects.** (**a**) Prior to mitochondrial protein synthesis the mtDNA needs to be maintained and correctly replicated and transcribed under the influence of several nDNA-encoded proteins. Mutations within nDNA-encoded genes responsible for these functions lead to mtDNA deletion(s) and depletion. Numerous other nDNA-encoded proteins have to be imported into the mitochondria for accurate mitochondrial translation processes. These nuclear encoded genes are categorised into different groups based on their role in the translational machinery. (**b**) nDNA-encoded RC subunit genes and RC assembly factors need to be synthetized, transported to the mitochondrial matrix and assembled into functional enzyme complexes with the 13 mDNA-encoded proteins. These 13 proteins are represented within complexes I, III, IV and V (CI: ND1, ND2, ND3, ND 4 L, ND4, ND5, ND6; CIII: CYTB; CIV: COX1, COX2, COX3; CV: ATPase6, ATPase8). (**c**) Nuclear genes involved in mt-tRNA modification are: *MTO1*, *PUS1*, *TRMU*, PTCD1, *MTFMT, TRIMT5, TRIMT10C, ELAC2 and TRIT1*. (**d**) Mitochondrial ARS enzymes: *RARS2, MARS2, HARS2, SARS2, FARS2, LARS2, AARS2, EARS2, YARS2, CARS2, PARS2, WARS2, VARS2, TARS2, IARS2, NARS2, DARS2*.

## 4.11 Conclusions and Future Directions

The wide range of tissue specific clinical presentations of abnormal mitochondrial protein synthesis highlight that neurons, skeletal muscle and heart are the most frequently affected organs. Studying the specific disease mechanism leading to tissue damage in these diseases may help us to understand basic pathological pathways and hopefully reveal some treatable targets for future applications (Figs. 4.1).

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# Chapter 5
# Mitochondria Dynamics: Definition, Players and Associated Disorders

**Maria Eugenia Soriano, Marta Carro Alvarellos, Giovanni Rigoni, and Luca Scorrano**

**Abstract** The mitochondrial ultrastructure, morphology, and interactions with other organelles are dynamic features that mirror mitochondrial function. These characteristics change and adapt depending on the metabolic state, pluripotency capacity and cell type. On the other hand, the manipulation of mitochondrial dynamics might regulate metabolism, stemness, and differentiation. The key players in the control of mitochondrial dynamics are known as mitochondrial shaping proteins that mainly regulate the fusion and fission events that control mitochondrial interactions. The chronic alteration in any of these events leads to cell dysfunction and the onset of degenerative disorders. This chapter summarizes the current knowledge on mitochondrial dynamics, the main players, and their pathophysiological relevance.

## 5.1 Definition and Relevance of Mitochondrial Dynamics

The term mitochondrial dynamics refers to the processes that regulate mitochondrial communication through fusion and fission events that determine their network state, fragmented or elongated, and the remodeling of the cristae compartment. It also refers to the mitochondrial communication with other organelles, which impact on mitochondrial morphology, and implies the participation of the mitochondrial motility mechanisms inside the cytoplasm. Mitochondria undergo continuous and frequent fusion and fission episodes to exchange metabolites and mtDNA, contributing to the buffering of harmful metabolites as reactive oxygen species (ROS) and mutated mtDNA. Theoretically, an equilibrium between fusion and fission is an

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ideal condition to maintain inter-mitochondrial communication and to promote molecule exchange, resulting in the presence of the characteristic interconnected mitochondrial network. Still, the frequency of fusion and fission events, more than the presence of an elongated network, might be considered an indicator of mitochondrial health. Many studies associate a defect in fusion with the presence of a fragmented network, and vice-versa, a defect in fission with superelongated mitochondria. However, depending on the cell type, the mitochondrial network might appear superelongated or fragmented, phenotypes that are not necessarily associated with a dysfunctional status.

Changes in mitochondrial dynamics occur during metabolic adaptations such as nutrients deprivation, a condition that induces mitochondrial network superelongation accompanied by an increase in cristae biogenesis, respiratory supercomplexes (RSCs) formation, and ATP synthesis (Fig. 5.1). On the other hand, fragmentation has been observed in some tumoral cell lines, it has been proposed to slow down mitochondrial motility through kinesins, to precede apoptosis, and it is a necessary event in the elimination of dysfunctional mitochondria during mitophagy. In the latter situation, mitochondrial bioenergetic activity is reduced, likely to preserve cell survival. The mitochondrial network status change to adapt to energy

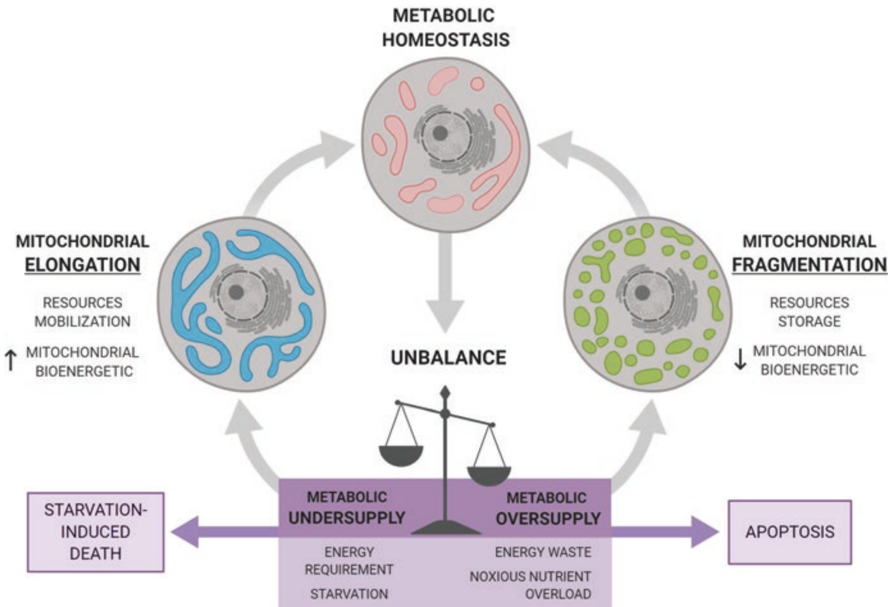


**Fig. 5.1 Impact of mitochondrial dynamics on metabolism**. The balance between mitochondrial fusion and fission is crucial in the adaptation to the cellular metabolic requirements. During starvation, the mitochondrial network fuses and elongate, raising ATP synthesis and recovering the metabolic homeostasis. Under an energetic oversupply, the increase of mitochondrial fission preserves cell survival by declining energetic production. If mitochondrial dynamics are impaired, cells cannot adapt and undergo cell death

requirements and maintain cell homeostasis. It is well-accepted that the prolonged interruption or unbalance of the fusion-fission events has detrimental effects because of the cell's inability to deal with energetic requirements; therefore, the use of the term "dynamic" is crucial to understand mitochondrial function and homeostasis.

The impact of the alteration of mitochondrial fusion or fission in cell and organism viability has been demonstrated *in vivo* by using mouse models knockout for proteins regulators of mitochondrial dynamics, known as "mitochondrial shaping proteins". The existence of human disorders caused by mutations in the same proteins has further supported the crucial role of mitochondrial dynamics in cell function and viability. In this chapter, we illustrate the last findings in fusion and fission machinery, their role in the known molecular mechanism, and the impact of mutations on cell viability and human-associated disorders.

## 5.2 The Fusion and Fission Players: "Mitochondrial Shaping Proteins"

Mitochondria fusion requires the participation of four different membranes: an outer (OMM) and inner membrane (IMM) from two different mitochondria. The fusion might follow an ordinated sequence of events articulated by the underlying molecular mechanism: the fusion of the OMM is the first step that allows the proceeding of the IMM fusion. Less clear are the molecular interactions that dictate the closure of the OM and IM of each "daughter mitochondria" after fission. Consistently, the proteins involved in fusion and fission are localized in both the outer and inner membranes. Up to date, three profusion proteins have been identified: OPA1 at the IMM, MFN1 and MFN2 at the OMM. The fission protein DRP1 appears as the main component binding to different adaptor proteins on the OMM. It is still controversial if Dynamin 2 is required or not in the final steps of mitochondrial division collaborating with DRP1; therefore, its possible involvement is not discussed in this chapter. Ablation of profusion proteins results in a fragmented mitochondrial network, while ablation of fission proteins leads to formation of a hyper-elongated network. These events can respond to specific physiological conditions or correlate with the presence of a pathological condition. Noteworthy, other not canonical fusion and fission regulators such as YME1L (Anand et al. 2014; Chrzanowska-Lightowlers and Lightowlers 2019), MSTO-1 (Donkervoort et al. 2019; Gal et al. 2017) or FBXL4 (Emperador et al. 2019; Sabouny et al. 2019) have been described, likely modulating the canonical players or by affecting parallel pathways that lately impact on the mitochondrial network status.

## *5.2.1 Profusion Proteins and Related Disorders*

### 5.2.1.1 Optic Atrophy 1 (OPA1)

OPA1 is a nuclear-encoded mitochondrial protein located in the IMM; ubiquitary expressed but with diverse expression levels among tissues. The OPA1 gene contains 29 exons that in human encode for eight mRNA splicing variants with different combinations of the exons 4, 4b, and 5b (Fig. 5.2). All the splicing variants contain an MPP (mitochondrial processing peptidase) cleavage site that generates a long-isoform (L-OPA1), and one or two additional cleavage sites: S1 (exon 5) and S2 (in isoforms containing exon 5b) that generate the short forms (s-OPA1). All isoforms contain the exon 5 with the S1, and only some isoforms conserve the S2, meaning that all isoforms generate an L- and at least one s-OPA1 form. Different proteases can mediate the processing at the S1 and S2 sites such as YME1L, OMA1, and PARL, indicating the tight regulation in the process (Macvicar and Langer 2016;

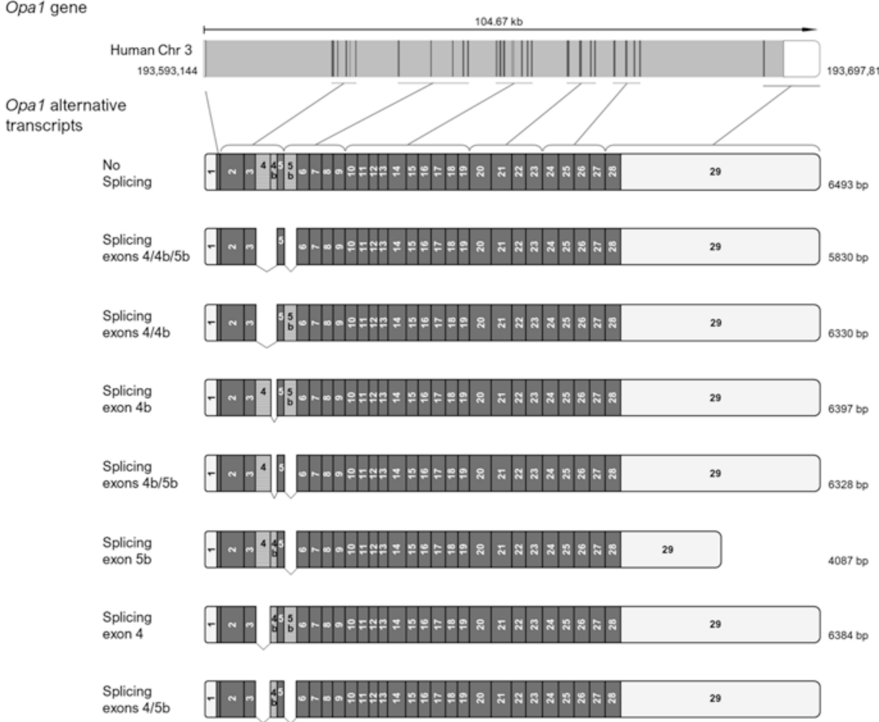


**Fig. 5.2 OPA1 gene and transcripts.** The OPA1 gene is located in human chromosome 3. It is composed of 29 exons (1–29 lines in the gene, and grey in the transcripts representation), that generates an RNA precursor in which alternative processing gives rise to 8 different splicing variants in human. The splicing variants differ in the alternative presence of exons 4,4b and 5b. Exons 1 and 29 (in white) are transcribed and contain not coding sequences useful for ribosomal recognition and translation termination

Pellegrini and Scorrano 2007). Although all the isoform transcripts are expressed in all tissues, the expression levels vary among them. Transcripts containing [3/4/5/6-29] and [3/5/5b/6-29] are highly expressed in the retina, fetal brain, heart, skeletal muscle, lung, and ovary; transcripts containing [3/4/5/5b/6-29] are highly expressed in kidney, liver, and colon; and transcripts containing [3/4b/5/5b/6-29] and [3/4/4b/5/6-29] were enriched in the skeletal muscle (Delettre et al. 2001; Del Dotto et al. 2018a).

The OPA1 amino acidic sequence contains, in addition to the mitochondrial targeting sequence (MTS) and the transmembrane domain (TM), which are localized at the N-terminal domain, a heptad-repeated domain (HR1) with a coiled-coil (CC0/CC1), a GTPase domain, a middle domain, and a GTPase effector domain (GED) harboring another CC motif (CC2) (Fig. 5.3). The long isoforms conserve the TM, GTPase, middle, and GED domains, while the short isoforms lack the TM, reason why they are not membrane integrated. Although the co-presence of short and long isoforms is physiological, in some specific conditions like loss of mitochondrial membrane potential and cell death, the ratio L-OPA1/s-OPA1 decreases, supporting a role for both isoforms in cell function. A crucial player in the equilibrium is OMA1, which is activated after membrane depolarization and thus induces L-OPA1 proteolysis at S1 site, increasing s-OPA1 levels.

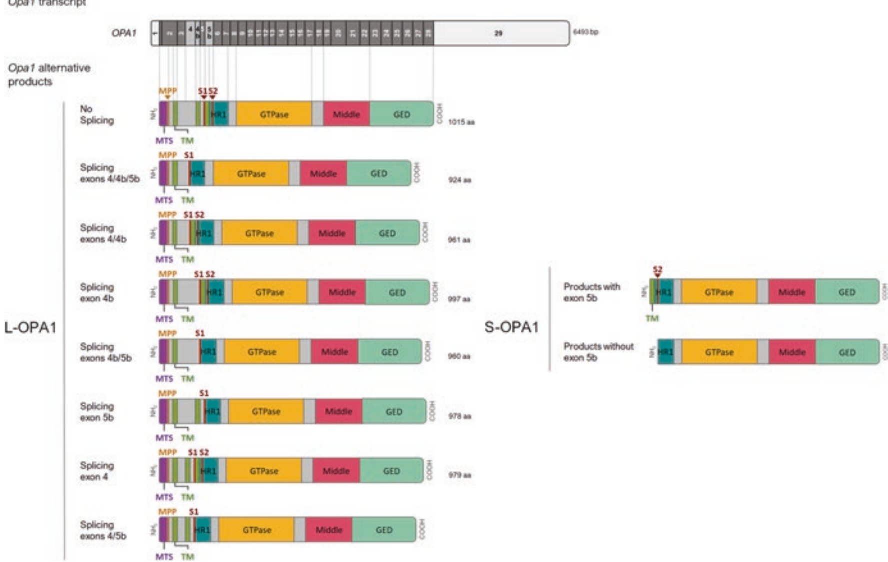


**Fig. 5.3 OPA1 isoforms and proteolytic products.** The translation of the OPA1 transcripts can generate the protein products described as L-OPA1. All L-OPA1 contain the MTS that will be eliminated by the processing at the MPP site. Since exons 5 and 5b contain two different proteolytic sites: S1 and S2, respectively, each L-OPA1 produces at least one proteolytic product lacking the TM and is generally known as s-OPA1 isoforms. The different OPA1 protein domains are highlighted in different colors

The OPA1 role in fusion depends on the GTPase and GED domains since mutations in any of them lead to the onset of a fragmented network, decreased fusion activity, and increased cell death (Cipolat et al. 2004; Frezza et al. 2006). Interestingly, most of the pathological OPA1 mutations in humans are retrieved in the GTPase or GED domains in heterozygosis, with the result of the premature translation termination and the generation of a truncated peptide (Del Dotto et al. 2018b). OPA1 fusion activity requires MFN1, functionally correlated by a molecular mechanism that is not known. The primary clinical symptom observed in patients affected by OPA1 mutations is optic atrophy due to retinal ganglion cell degeneration, a condition that is known as "ADOA" (Autosomal Dominant Optic Atrophy). However, in some cases, the typical ADOA clinical phenotype is accompanied by deafness, ophthalmoplegia, myopathy, ataxia, and neuropathy, a more severe syndrome that is called "ADOA plus". Few of these patients show a typical mitochondrial DNA depletion syndrome with encephalopathy and cardiomyopathy, which molecular trigger is the absence of fusion (El-Hattab et al. 2017). The phenotype can be genetically explained as a haploinsufficiency defect or dominant missense mutations. However, few patients with homozygous mutations are characterized by multisystemic syndromes. The lack of fusion appears as the primary molecular defect, as confirmed in experiments performed in patient's cells and biopsies (Amati-Bonneau et al. 2009; Lenaers et al. 2012; Zanna et al. 2008; Chevrollier et al. 2008; Spinazzi et al. 2008).

Besides the role in fusion, OPA1 has an additional molecular function in the maintenance of cristae ultrastructure, which is independent of the profusion activity. The OPA1 ablation induces the widening of the cristae and cristae junctions (CJ), allowing the cytochrome c (cyt c) redistribution from the cristae lumen to the intermembrane space (IMS) and eventually to the cytoplasm after the permeabilization of the OMM (MOMP), triggering apoptosis (Frezza et al. 2006). Indeed, pathological OPA1 mutations induce a higher sensitivity to cell death when expressed in murine cell lines. The effect of OPA1 in the maintenance of cristae morphology and resistance to cell death correlates with a higher assembly efficiency of respiratory chain complexes (RCs) in supercomplexes (RSCs), an event that is favored in thinner cristae respect to wider ones (Cogliati et al. 2013). Since supercomplexes present a higher respiratory capacity than the individual complexes, mitochondria with thinner cristae show improved respiratory performance. Because OPA1 levels modulate cristae width, the alteration of the OPA1 function is expected to modify also mitochondrial respiration. Consistently, murine cells overexpressing OPA1 show thinner cristae and increased respiratory rate (Cogliati et al. 2013; Varanita et al. 2015), while it decreases in OPA1 ablated mitochondria that present wider cristae (Cogliati et al. 2013). Noteworthy, more physiological models have confirmed the impact of cristae shape on supercomplexes assembly (Balsa et al. 2019). These studies raise the question of whether the detrimental set of ultrastructural alterations due to loss of OPA1 function is also observed in patients, thus further contributing to the pathologic phenotype. Some studies on ADOA patients show a modest decrease of CI enzymatic activity with respect to control cells, but with no differences in CIII or CIV (Mayorov et al. 2008). In contrast, other studies show a

significant reduction of ATP synthesis driven through CI in galactose medium, forcing mitochondrial Oxphos activity (Zanna et al. 2008), and reduced activity of CI, CII and CIV in an ADOA plus patient (Finsterer and Laccone 2019). Still, the results obtained after OPA1 ablation *in vivo* in mice support the defect on Oxphos activity associated with a RSCs assembly defect, but not to a lower complexes protein synthesis (Cogliati et al. 2013; Varanita et al. 2015; Tezze et al. 2017). From these studies, it can be concluded that both lack of fusion and loss of ultrastructure might contribute to the severity of the disease (Fig. 5.4).

Additional molecular phenotypes observed in some ADOA patients are mtDNA depletion and deletions. This feature could be due to a suggested direct interaction of mtDNA with OPA1 (Elachouri et al. 2011), or as a consequence of the fusion dysregulation (Silva Ramos et al. 2019; Chen et al. 2010). Indeed, it has been shown that fusion is necessary for mtDNA segregation, stability, and dilution of mutated mtDNA among the mitochondrial network. In the absence of fusion, mtDNA mutations and deletions accumulate, generating not enough mtDNA encoding proteins to maintain proper Oxphos capacity. Overall, it can be concluded that OPA1 mutations

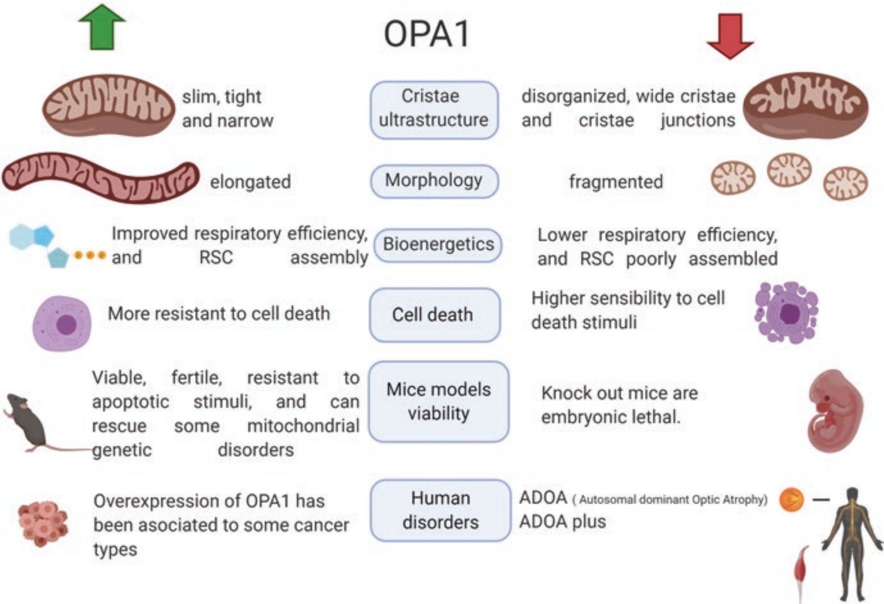


**Fig. 5.4 Effects of OPA1 up- or down-regulation.** OPA1 downregulation or ablation (right side) induces an increase of cristae and cristae junctions width, less efficient respiratory capacity due to the deficient RSCs assembly, and higher sensibility to cell death. *In vivo*, *Opa1* KO mice are embryonic lethal, and conditional *Opa1* KO mice have confirmed the increased sensitivity to induced tissue injury. On the other hand, OPA1 transgenic mice (left side) with a mild OPA1 overexpression show thinner mitochondrial cristae with the expected improved respiratory capacity due to the more efficient assembly of RSCs. These mice are resistant to different apoptotic insults, and OPA1 overexpression improves the phenotype of mice models for mitochondrial disorders. High OPA1 protein levels have been also observed in some types of tumors

induce a mitochondrial dysfunction consequence of the combination of fusion activity defects and ultrastructure cristae alteration as primary events, resulting in the mild or more severe pathological condition, ADOA or ADOA plus, respectively.

#### 5.2.1.2 Mitofusin 1 and Mitofusin 2 (MFN1 and MFN2)

Mitofusins 1 and 2 are integral outer membrane GTPases that mediate mitochondrial fusion and mito-Endoplasmic reticulum (ER) tethering. From the structural point of view, both proteins are very similar, showing a GTPase domain at the N-terminal region facing the cytosol, two TM domains, and two heptad-repeated domains (HR1 and HR2) containing coiled-coil domains, CC1 and CC2, respectively (Fig. 5.5). A distinctive proline-rich domain (PR) is present only in MFN2, which likely favors the mobility and interactions of the HR1 and GTPase domain in the cytosol. MFN1 and MFN2 mediate mito-mito interaction and fusion by trans-interacting with other MFNs, 1 or 2, of a different mitochondrion and thus creating homo- or hetero-oligomers (Fig. 5.6) (Ishihara et al. 2004). Preliminary topological studies indicating a cytosolic location for HR2 led to hypothesize this domain to be crucial for MFN-MFN interaction. Recently, the MFNs topology has been questioned since bioinformatic studies evidenced the presence of a unique TM domain that localizes the HR2 in the IMS, and not back to the cytosol as previously proposed by similarity with bacterial MFNs (Mattie et al. 2018). This new localization raises doubts on the different models proposed to regulate MFNs oligomerization by which HR1 and HR2, both in the cytosolic side, might interact (closed state) or not (extensive state), to release and expose HR2 and allow the trans-interaction with the HR2 from other MFNs 1 or 2 in a different mitochondrion (Franco et al. 2016). Currently, substantial evidence supports the HR2 localization in the IMS acting as a redox sensor that likely modulates the stress-induced hyperfusion of mitochondria (Mattie et al. 2018), remaining unclear the trans-interaction molecular mechanism of MFNs (Fig. 5.5).

Besides the MFN2 role in mitochondrial fusion, it also has a crucial role in the mito – ER tethering regulating calcium homeostasis and lipid transport through specialized membrane regions known as MAMs (mitochondria-associated ER membranes) (Phillips and Voeltz 2016) (Fig. 5.6). Mito-ER contacts and mitochondrial morphology are functionally related since PINK1-Parkin dependent phospho-ubiquitination of MFN2 mediates its degradation, thus reducing mito-ER contacts, decreasing the fusion activity and increasing fragmentation, which is a requirement to facilitate the elimination of dysfunctional mitochondria (Mclelland et al. 2018). The fact that only mutations in MFN2 and not MFN1 cause a pathological phenotype in humans denotes the different protein's function and suggests the interaction with the ER as a primary molecular mechanism involved in the pathology. Indeed, more than 100 mutations, mainly localized in the GTPase, have been identified in MFN2 to cause an inherited neurological disorder known as Charcot-Marie-Tooth axonal type 2 (CMT2A) (Stuppia et al. 2015). CMT2A is an autosomal-dominant peripheral neuropathy, which symptoms are progressive muscle weakness, motor

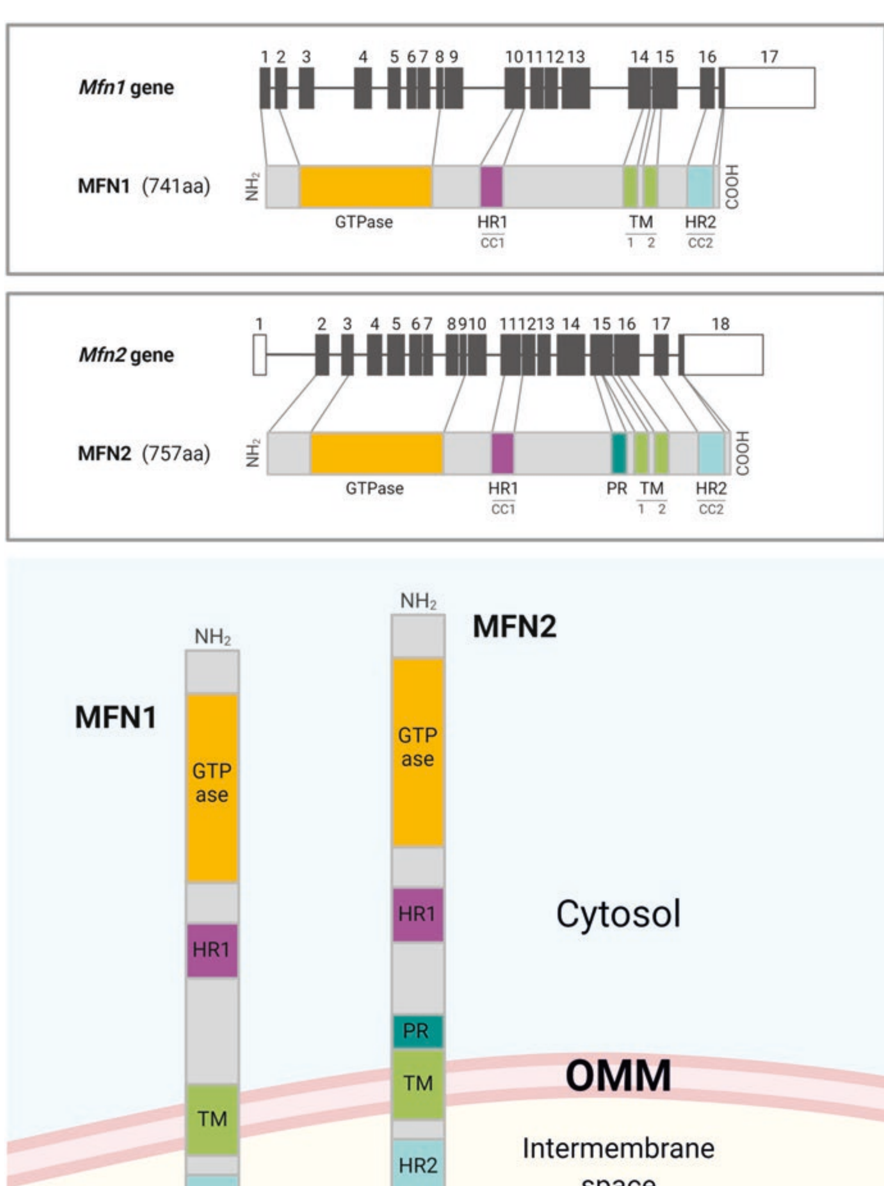


**Fig. 5.5 MFN 1 & 2 genes, protein domains and topology.** The *MFN1* and *2* genes contain 17 or 18 exons, respectively. The main difference between the two proteins is the presence of a proline-rich domain (PR) in MFN2, which is suggested to regulate the HR1 and GTPase domains interaction with other proteins. Recent studies support the localization of the MFN2 – HR2 domain in the IMS, acting as a redox sensor that modulates MFN2 conformational changes. The different protein domains are highlighted in different colors. OMM: outer mitochondrial membrane

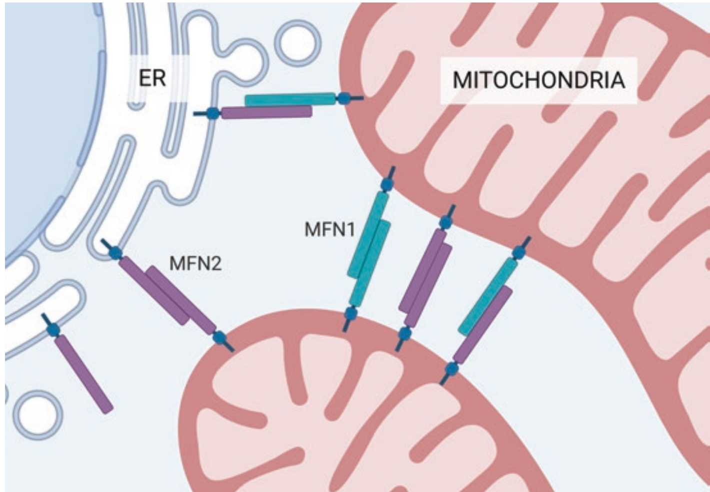


**Fig. 5.6 MFN1 and/or MFN2 dimers regulate mito-mito and mito-ER tethering.** MFN1 in green; MFN2 in violet

deficits mainly at distal lower limbs, sensory loss, atrophy and scoliosis, with a different severity degree. The mitochondrial phenotype characterization of CMT2A patients cells and mice models revealed unsettle mitochondrial morphology and axonal distribution, in some cases accompanied with fragmentation and defective oxidative phosphorylation (Larrea et al. 2019). The causes of the variable CMT2A phenotypes are still subject of study; however, it is hypothesized that the level of MFN2 expression, mutated vs. wild-type, could be a crucial determinant factor.

The *Mfn1* or *Mfn2* KO mice die at mid-gestation, which evidences the essential role of these proteins during embryonic development (Chen et al. 2003). In the case of *Mfn2* KO, defects in the trophoblast giant cells of the placenta significantly compromise mice viability. Indeed, the MFN2 induced-ablation after placenta formation allows the survival of two-third of individuals after birth, which show severe motor defects with feeding problems and succumbing after a couple of weeks. On the contrary, MFN1 ablation after placenta formation gives rise to viable and fertile mice. The divergent phenotypes observed have been attributed to the tissue-specificity of the proteins expression. Indeed, while both proteins are highly expressed in heart and adrenal glands, MFN1 is well expressed also in the liver, pancreas, and testis (Eura et al. 2003; Santel et al. 2003), whereas MFN2 is highly expressed in skeletal muscle, brain, nervous system, and brown adipose tissue, which are the main tissues affected in CMT2A (Detmer and Chan 2007). Still, MFN1 or MFN2 share a role in mitochondrial fusion since the ablation of one or the other leads to the lack of mitochondrial fusion resulting in a very fragmented mitochondrial network. Moreover, OPA1 requires MFN1 but not MFN2 to induce fusion, as demonstrated by the fact that MFN2 does not influence OPA1 fusion activity, data that further support the diverse molecular mechanism of both MFNs to regulate mitochondrial fusion. Remarkably, the fragmented phenotype due to the ablation of

one of MFNs can be rescued with the overexpression of the other, indicating that not only hetero-oligomerization but also homo-oligomerization of MFNs regulate mitochondrial fusion (Ishihara et al. 2004).

A recent study has identified the MFN2 critical residues that participate in the fusion event. Such residues are localized in the HR1 domain (374–384) and dictate the conformational changes versus the MFN2 "open state" that favors mitochondrial fusion. Based on this finding, there have been synthesized mini-peptides that act as MFN2 agonists, inducing fusion. These peptides were able to rescue the mitochondrial defects and axonal transport in MFN2-mutant cells and mice models (Rocha et al. 2018). Whether mitofusin agonists can be used as a therapeutic tool in patients affected by CMT2A or other neurological disorders requires further investigation.

A growing number of studies have identified additional proteins which contribution to fusion requires validation. Among these, MSTO-1 (misato homolog-1) and FBXL4 (F-box leucine-rich repeat 4) appear the most interesting, with a phenotype similar to mitochondrial depletion syndromes. MSTO-1 is a soluble protein, likely cytoplasmic, with a partial mitochondrial colocalization. Patients affected by MSTO-1 mutations show neurodevelopmental impairment, myopathy, ataxia, and pigmentary retinopathy. Patient's fibroblasts are characterized by a decreased fusion activity, fragmented mitochondrial network and mtDNA depletion, which could be consequence of the fusion defects (Gal et al. 2017; Iwama et al. 2018; Donkervoort et al. 2019). The protein FBXL4 is located in the IMS protein, and patients with homozygous mutations show encephalopathy, developmental delay and hypertrophic cardiomyopathy, with a life expectancy that goes from days to a few years (Sabouny et al. 2019; Emperador et al. 2019). The fibroblasts from patients evidenced mitochondrial network fragmentation, bioenergetic defects, and mtDNA depletion, a phenotype that was recovered after the forced induction of mitochondrial fusion. Although nothing is known about the molecular mechanisms by which these proteins regulate fusion, the published studies support an intriguing functional correlation with their profusion activity and mtDNA stability. We wait for future studies to elucidate how these two events are molecularly related.

## *5.2.2 Fission Proteins and Related Disorders*

### 5.2.2.1 DRP1

The dynamin GTPase DRP1 is a cytosolic protein that generally locates in the cytosol. After specific post-translational modifications, DRP1 translocates onto the OMM where it polymerizes creating a constriction ring around a mitochondrion to induce the division in two independent organelles. Still, DRP1 is necessary but not enough to induce the process since it requires the presence of protein adaptors located on the mitochondria surface that, by an unclear mechanism, regulate DRP1 polymerization and mitochondrial constriction in a GTP-dependent manner (Fig. 5.7a).

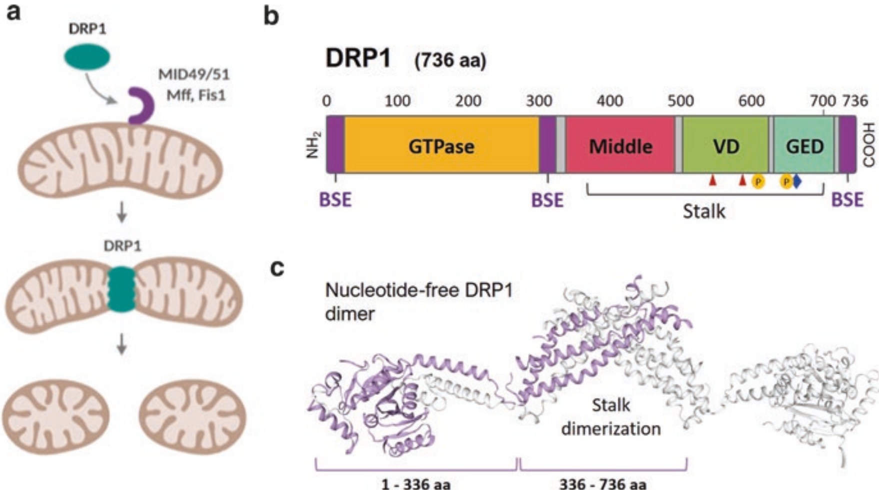


**Fig. 5.7** (**a**). **DRP1 translocation onto mitochondria.** DRP1 translocates onto mitochondria through the interaction with adaptor proteins located in the OMM. (**b**). **DRP1 protein domains and sites of post-translational modification**; phosphorylation (yellow circles), ubiquitination (red arrows), S-nitrosylation (blue rhombus). (**c**). **DRP1 dimer model.** Bioinformatic model of the DRP1 dimers interacting through the stalk region

DRP1 is 736 aa long and is composed of a GTPase domain at the N-term followed by the middle domain, a variable region (VD), and a GTPase effector domain (GED). At the C- and N-terminal sides of the GTPase domain, and C-term of the GED, there are three different bundle signaling elements (BSE) (Fig. 5.7b). The BSE elements transmit the GTP-dependent conformational changes from the GTPase domain to the middle and GED domains, which are implicated in DRP1 oligomerization and assembly. The region formed by the middle and the GED domains is called stalk, and contains a hydrophobic region known as VD. The VD favors the interaction of the protein with the lipids in the membrane and, by inducing conformational changes, also regulates DRP1 assembly. The stalk is crucial for dimerization and the stability of DRP1 polymers (Ramachandran et al. 2007), even though GTP containing-DRP1 tetramers seem to be the required conformation to form the ring structure that will constrict the mitochondrion (Fig. 5.7c). Recent reports based on *in vitro* data have hypothesized a crucial role of the adaptor proteins for DRP1 tetramers-competent-oligomerization and curvature, events that are altered in the presence of DRP1 pathological mutations (Kalia et al. 2018).

The regulation of DRP1 activity has not been fully described, but a growing number of studies point to post-translational modifications such as phosphorylation (Kashatus et al. 2011; Taguchi et al. 2007), SUMOylation (Figueroa-Romero et al. 2009; Braschi et al. 2009; Choi et al. 2017), S-nitrosylation (De Palma et al. 2010), and ubiquitination as important molecular events (Nakamura et al. 2006; Yonashiro et al. 2006; Karbowski et al. 2007). Up to date, only a few residues concentrated in the VD and GED have been identified to be targets of such modifications.

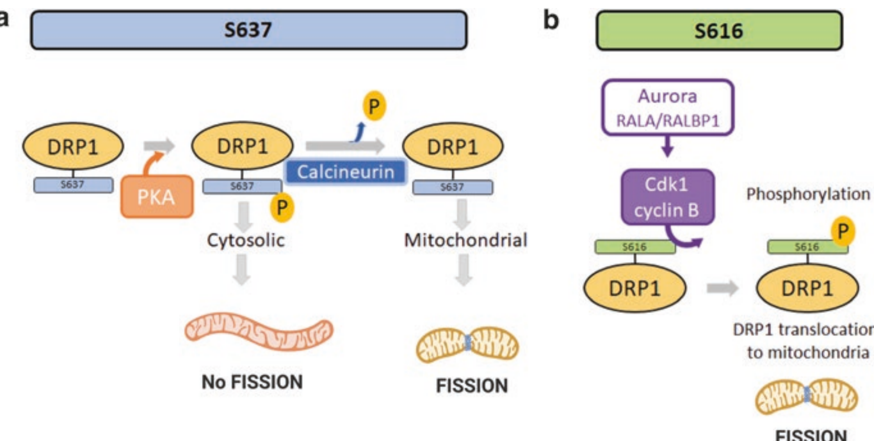


**Fig. 5.8 Effects of DRP1 phosphorylation events on fusion and fission**. (**a**). The Protein Kinase A (PKA) phosphorylates DRP1 on Ser637, inhibiting DRP1 translocation onto mitochondria and fission. The dephosphorylation of the same residue by calcineurin induces the DRP1 translocation onto the OMM to interact with the receptors and inducing fission. (**b**). The phosphorylation of DRP1 on Ser616 provokes the opposite effect and induces the translocation onto mitochondria. This is mediated by cyclin B and requires the pathway involving Aurora, RalA and RalBP1, the latter colocalizing with CyclinB and DRP1 in the OMM

PKA-dependent phosphorylation of $Ser^{637}$ inhibits DRP1 translocation onto OMM and fission (Gomes et al. 2011). On the contrary, calcineurin-dependent dephosphorylation induces DRP1 translocation to mitochondria (Fig. 5.8a) (Cereghetti et al. 2008). Other phosphorylation events have been described, such as at $Ser^{616,}$ which induces fission during mitosis (Fig. 5.8b) (Taguchi et al. 2007). A different modification is given by ubiquitination that usually targets proteins to be degraded or mediates protein-protein interactions. In the case of DRP1, its ubiquitination by the ubiquitin ligase MITOL mediates the translocation to mitochondria, increasing fission (Karbowski et al. 2007; Nakamura et al. 2006). Also, SUMOylation (by MAPL)- deSUMOylation (by SENP3) of DRP1 might regulate its localization in the cytoplasm or mitochondria, respectively (Prudent et al. 2015; Guo et al. 2017; Anderson and Blackstone 2013). Overall, post-translational modifications appear as the main events regulating DRP1 pro-fission activity in physiological conditions.

The interruption of fission by mutation or ablation of DRP1 results in the formation of a hyper-elongated mitochondrial network and accumulation of metabolites, proteins and mtDNA (mutated and not). Interestingly, it has been proposed that the ER somehow marks fission sites where DRP1 also interacts with nucleoid (protein-nucleoid acid complexes containing mtDNA) during mitochondrial division to ensure an equal mtDNA-nucleoid distribution (Lewis et al. 2016; Friedman et al. 2011; Westermann 2011). In DRP1 KO cells, nucleoids cluster in mito-bulbs, which has been interpreted as an inability to distribute in the IMM, resulting in mtDNA instability (Ishihara et al. 2015a; Parone et al. 2008). How nucleoid aggregation and

mtDNA depletion are coordinated is unknown, but it appears clear the involvement of fusion and fission events in mtDNA stability and nucleoid dynamics.

The absence of DRP1 in mice is embryonic lethal, showing smaller body size, delayed cardiac and liver development, and thinner neural tube layer. The use of conditional KO mice led to describe the crucial role of DRP1 in polarized cells such as neurons, since DRP1 depletion induces mitochondria aggregation impeding efficient energy distribution in neurites and synapses. Heart-specific *Drp1* conditional KO mice develop cardiac dysfunction that results in heart failure (Kageyama et al. 2014; Ishihara et al. 2015b; Ikeda et al. 2015). On the other hand, mouse embryonic fibroblasts and stem cells show less evident phenotype with respect to neurons, with no alteration in membrane potential, mitochondrial mass, respiration, ATP levels, or mtDNA, probably due to compensating mechanisms (Ishihara et al. 2009). Still, the presence of an elongated mitochondrial network is a shared feature in all DRP1 ablated cells.

Few clinical reports confirm the pathophysiological relevance of DRP1 in humans with the finding of mutations in the middle, the GED, or the GTPase domain associated with neurological syndromes. All of them are characterized by abnormal brain development, encephalopathy, epilepsy, developmental delay or regression, and optic atrophy in some cases. *Drp1 de novo* mutations in heterozygosis affecting the middle domain were the first described, showing a phenotype from mild (A403C) to lethal (A395D) depending on critical amino acids, and characterized by a dominant-negative effect (Waterham et al. 2007; Vanstone et al. 2016). Pathogenic variants have also been described in the GTPase domain in homo and heterozygosis, the latter showing a dominant-negative effect and resulting in the onset of neurological disorders and optic atrophy (Nasca et al. 2016; Yoon et al. 2016; Hogarth et al. 2018; Gerber et al. 2017). Recently, a novel *de novo* heterozygous variant in the GED domain (T691C) has been described in a 27-yr-old woman with static encephalopathy, a history of seizures, and nystagmus (Assia Batzir et al. 2019). In all cases, mitochondrial appear abnormally elongated. Unfortunately, because of the low cohort of patients described and the phenotypical variability observed, it has not been possible to establish a phenotype-genotype correlation. Studies in cellular models with pathological mutations point to the DRP1 inability of oligomerizing as the probable cause (Fahrner et al. 2016).

#### 5.2.2.2 Fission Receptors: FIS1, MFF, MID49 and MID51

As mentioned above, DRP1 is necessary but not enough to induce fission since its translocation from the cytoplasm to mitochondria is mediated by adaptor proteins inserted in the OMM through a transmembrane domain. Four main receptors have been described: FIS1, MFF, MID49 and MID51, even though the role of mammalian FIS1 as an adaptor is debated. Several studies have demonstrated that receptors can recruit DRP1 to the fission point independently, suggesting that they are functionally redundant. Moreover, the interaction with the receptors might induce

conformational changes to the DRP1 tetramers, determining and allowing the formation of the constriction DRP1-ring.

The first receptor described in mammals based on yeast homology studies was FIS1 (James et al. 2003; Yoon et al. 2003). The involvement of FIS1 in the regulation of mitochondrial network morphology is based on the fact that its overexpression induces fragmentation, and the downregulation leads to a highly elongated mitochondrial network. The observed effects were explained through the interaction of FIS1 and DRP1, as demonstrated by immunoprecipitation experiments. Still, the DRP1 dependent fragmentation was also present in the absence of FIS1, a result that was explained by the existence of other DRP1 receptors. Interestingly, a recent study has suggested that FIS1 induces fragmentation by inhibiting the profusion activity of OPA1 and MFNs by a mechanism that is DRP1-independent, data that open a new molecular perspective (Yu et al. 2019). Although mammalian FIS1 KO cells show mild or no fission defects, its ablation in mice is lethal (Otera et al. 2010; Loson et al. 2013). Muscle-specific KO mice present mitochondrial hyperfusion and functional impairment, and abnormal mitophagy (Zhang et al. 2019). To date, there are no human disorders associated with FIS1 mutations.

The protein MFF (Mitochondrial Fission Factor) was identified by a high-throughput screen of siRNAs (Gandre-Babbe and Van Der Bliek 2008) in which its silencing induced a hyper-elongated mitochondrial network and resistance to cell death, similarly to the phenotype observed in DRP1 or FIS1 siRNA treated cells. On the other hand, overexpression of MFF increased mitochondrial fission in a FIS1-independent manner and mediated by the interaction with DRP1 on the OMM (Otera et al. 2010). Such interaction requires the MFF N-terminal region that harbors two repeated motifs (R1 and R2) and the DRP1 stalk domain, being favored when DRP1 is organized in oligomers (Liu and Chan 2015). *In vivo*, mice lacking MFF die due to cardiomyopathy 13 weeks after birth, with a phenotype characterized by neuromuscular deficiencies, decreased fertility, and immunological alterations (Chen et al. 2015). Heart mitochondria from these mice show defects in oxygen consumption due to the reduced activity of complex I, II, and IV of the respiratory chain, a phenotype that is shared with *Drp1* KO mice (Kageyama et al. 2014; Ishihara et al. 2015a), and thus might be considered a typical trait of fission-defective mitochondria. Fibroblasts from *Mff* KO mice show the elongated mitochondrial network expected after the ablation of fission-related proteins, supporting the MFF role as part of the fission machinery. In neurons, MFF ablation induces elongation of the axonal mitochondria, a feature that does not seem to modify mitochondrial function. Instead, the accumulation of elongated mitochondria at the presynapsis increases calcium uptake capacity of mitochondria, thus altering neurotransmitters release (Lewis et al. 2018). In humans, only few patients have been identified with mutations in MFF, all characterized by clinical features similar to Leigh-like encephalopathy, and the presence of hyper-elongated mitochondria in fibroblasts from these patients (Nasca et al. 2018; Koch et al. 2016; Shamseldin et al. 2012).

A leading role as DRP1 receptors has been attributed to MiD49 (mitochondrial dynamics protein of 49 kDa) and MiD51 (mitochondrial dynamics protein of

51 kDa), which are exclusively present on mitochondria and not on peroxisomes like the other DRP1 receptors. It has been proved that MiD49 and MiD51 recruit more DRP1 molecules than FIS1 or MFF, conferring to the MiD proteins the primary role in this event (Palmer et al. 2013). Moreover, the presence of FIS1 or MFF is not required for the DRP1 recruitment by MiD proteins (Otera et al. 2016). Data showing the colocalization of MFF and MiD51 in foci together with DRP1 suggests their collaboration in the recruitment of the protein and fission event (Osellame et al. 2016; Richter et al. 2014). Moreover, MiD51 appears as the only receptor that mediates fission coupled to cristae remodeling and cytochrome c release by a mechanism mediated by the first 14 aa in the N-terminal domain of the protein (Otera et al. 2016). Recent crystallographic studies have proposed that even if DRP1 requires a receptor to associate to the OMM, in the case of MiD49, the DRP1 polymer must then leave the receptor to form the constriction ring. Whether this is a unique feature of MiD49 or it is shared with other receptors is not known. Some of the pathological DRP1 mutations described up to date alter the MiD49-DRP1 interaction hampering the ring formation (Kalia et al. 2018).

Currently, no mice models KO for MiD49 or MiD51 have been published, and only one study has described a patient affected by a homozygous nonsense mutation (p.Q92*) in the *MIEF2* gene encoding MID49. The patient presented progressive muscle weakness and exercise intolerance at 6 years of age, imbalanced mitochondrial dynamics, mitochondrial myopathy with numerous ragged red and cytochrome *c* oxidase (COX) negative fibers and combined respiratory chain complex I and IV deficiency (Bartsakoulia et al. 2018). This clinical phenotype is very similar to the one observed in patients affected by DRP1 and MFF mutations, supporting their involvement in the same process. Although the growing knowledge about the regulation of fusion and fission, many open questions remain to be addressed regarding the molecular mechanism and the apparent functional redundancy of the DRP1 receptors.

## 5.3 Physiopathological Relevance of the Fusion-Fission Unbalance

The mitochondria homeostasis requires an equilibrium between fusion and fission events that must be dynamic to efficiently adapt and respond to the different cellular requirements. When either fusion or fission is hold up or blocked, mitochondria cannot exchange metabolic compounds, molecules and mtDNA, events that are required for mitochondrial well-functioning and proper quality control. In physiological conditions as during cell cycle progression, higher fission *vs.* fusion is observed during mitosis (Mitra et al. 2009), likely contributing to a more efficient mitochondrial segregation. This event needs the recruitment onto the mitochondria of phosphorylated DRP1 at Ser616, which is mediated by Aurora-dependent RalA phosphorylation on S194, and RalBP1, the latter located on the mitochondria

surface that stimulates Cyclin B kinase to phosphorylates DRP1 (Fig. 5.8b) (Kashatus et al. 2011). The players of the phosphorylation events might change depending on the cell type (Merrill et al. 2011). Moreover, fission precedes physiological or pathological apoptosis and it is also required for the elimination of dysfunctional mitochondria by mitophagy (Pernas and Scorrano 2016). All the processes just described are necessary for cell survival and tissue homeostasis, and requires the re-activation of fusion after their completion. Indeed, if fission is maintained and not reverted versus fusion, mitochondria increase ROS generation and accumulation, leading to oxidative stress, mtDNA mutations and replication defects, and a lower respiratory efficiency with consequent mitochondrial dysfunction and higher sensitivity to cell death.

On the other hand, the equilibrium unbalanced versus fusion is observed during the G1-S phase of the cell cycle that requires higher oxidative capacity (Mitra et al. 2009). Fusion is also forced during nutrient deprivation, so there is an increase in ATP levels and allows cell survival. This response is due to the DRP1 phosphorylation on Ser637 by PKA that impedes DRP1 translocation onto mitochondria and therefore inhibits fission (Gomes et al. 2011). A prolonged fusion unbalance results in very elongated mitochondria, that not being able to divide, accumulate metabolites and ROS. This provokes the arrest of cell cycle and accumulation of damaged mitochondria because mitophagy cannot progress for their elimination. Therefore, for the fusion-fission unbalanced to be pathologic, it must be maintained in time interfering or hampering the normal function of the cell that finally will result in cellular and organism dysfunction.

Noteworthy, the concomitant disruption of both fusion and fission, and therefore the adynamic mitochondrial condition is less deleterious in mice than the disruption of only one of them. Indeed, a beautiful study by Song et al. (2017) genetically dissects the consequences of disrupting only fusion, only fission or both in heart-specific KO mice. Surprisingly *Mfn1*$^{-/-}$ *Mfn2*$^{-/-}$ *Drp1*$^{-/-}$ triple KO mice display increased life spam respect to *Mfn1*$^{-/-}$ *Mfn2*$^{-/-}$ double KO or *Opa1*$^{-/-}$ KO mice, and also respect to *Drp1*$^{-/-}$. Still, the triple KO mice show different cardiac phenotype with respect to the "only-fusion" or "only-fission" KO mice. These results evidence that the lack of dynamism is better than the unbalance and further support the essential role of fusion and fission in cell and tissue homeostasis.

In general, in physiological conditions, there is a direct correlation between fusion or fission and the cell energy requirements, which change in the different tissues and cell types. An interesting example is the difference between stem cells and differentiated that display a characteristic mitochondrial dynamics profile that also regulates their stemness potential (Chen and Chan 2017; Zhong et al. 2019). Indeed, stem cells are characterized by a glycolytic metabolism associated with increased phosphorylated DRP1 on Ser616 localized in mitochondria, and the presence of fragmented perinuclear mitochondria (Xu et al. 2013; Wang et al. 2014). On the contrary, differentiated cells usually contain higher levels of MFN1, MFN2 and OPA1, which results in the formation of an interconnected and elongated

mitochondrial network. This phenotype is accompanied by an increase in the expression of mitochondrial biogenesis markers (e.g. PGC1a and TFAM), high ATP levels, and the prevalence of oxidative metabolism (Kasahara et al. 2013; Cho et al. 2006; Bahat et al. 2018). The stemness vs. differentiation capacity is influenced by the fusion-fission balance; indeed, fission inhibition leads to embryonic stem cells to differentiate and induce the metabolic switch from glycolysis to oxidative. Less clear is whether pro-fission activity may regulate the reprogramming process *vs.* pluripotency without inducing cell death (Prieto et al. 2016a, b). Regulation of fusion and fission also influence longevity since it has been shown that fusion stimulation prolongs lifespan in *C. elegans* (Chaudhari and Kipreos 2017).

Similarly, in pathological conditions such as several cancer cell types, mitochondrial fragmentation is often due to high DRP1 levels and lower MFN2 protein levels with respect to controls (Kashatus et al. 2015; Zhao et al. 2013; Chen and Chan 2017). This phenotype impinges on cell replication, pushing cancer cell to enter in mitosis, and increasing proliferation. Accordingly, when DRP1 levels decrease and MFN2 increases, there is an impairment of cancer cell growth (Xu et al. 2017). Tumor invasiveness is also stimulated by fission, and expression of mitochondrial biogenesis factors as PGC1α (Valcarcel-Jimenez et al. 2019). Many other human disorders have been associated with the fusion and fission unbalance as the cause (e.g., DOA, CMT2A) or just an exacerbating contribution to the pathological traits such as Alzheimer's and Huntington's disease (Fig. 5.9). Future studies will shed light on the therapeutic potential of fusion and fission regulators.

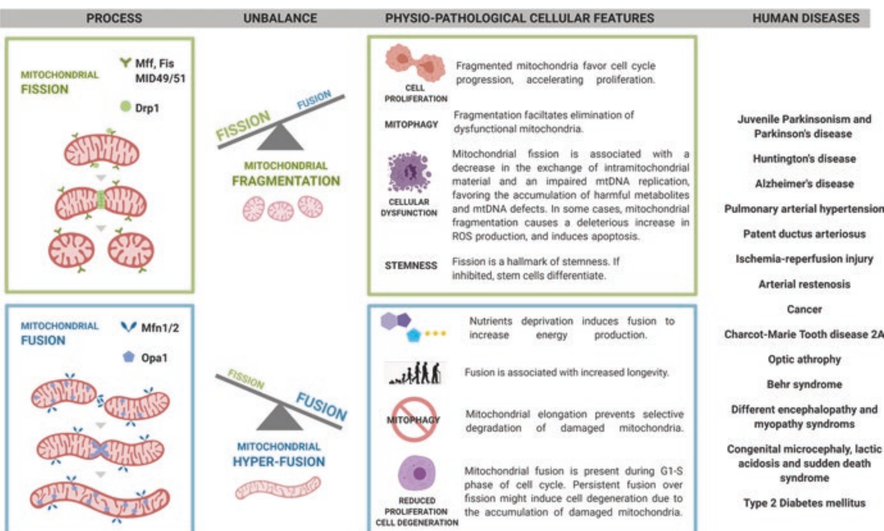


**Fig. 5.9** Overview of the physiopathological conditions affected by the fusion-fission unbalance

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# Chapter 6
# Coenzyme Q Biosynthesis Disorders

**Gloria Brea-Calvo, María Alcázar-Fabra, Eva Trevisson, and Plácido Navas**

**Abstract** Coenzyme Q (CoQ) is a lipidic molecule that transfers electrons between complexes I and II to complex III in the mitochondrial respiratory chain. It is also essential for processes mediated by other mitochondrial dehydrogenases, such as those involved in pyrimidine nucleotides biosynthesis, beta-oxidation and sulfide biosynthesis. A nuclear-encoded multiprotein complex at the inner mitochondrial membrane drives CoQ biosynthesis, which requires at least 13 proteins, leastways in yeasts. Mutations in the genes (*COQ* genes) coding for these proteins cause a decrease of CoQ biosynthesis rate leading to primary CoQ deficiency, a very heterogeneous group of mitochondrial diseases affecting different tissues and organs, and showing variable severity and age of onset. In general, this primary condition shows a good response to the supplementation with high doses of CoQ, but early diagnosis is compulsory to limit tissue damage. However, sometimes effectiveness is reduced, possibly due to its low bioavailability and, probably, difficulties crossing the blood-brain barrier. Secondary CoQ deficiency is a more common condition, in which defects of diverse mitochondrial processes induce an adaptive CoQ decrease. Secondary deficiency can be caused by oxidative phosphorylation (OXPHOS) defects, such as complex III dysfunction or mitochondrial DNA (mtDNA) depletion, or even non-OXPHOS mitochondrial defects. Here, we review the current knowledge of CoQ biosynthesis pathway, the genetic defects leading to primary deficiency and those conditions in which mitochondrial defects cause secondary deficiency.

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## 6.1 CoQ Structure and Functions

Coenzyme Q (CoQ) is a lipidic molecule formed by a benzoquinone ring attached to an isoprenoid chain at position 3, whose length is species-specific (Fig. 6.1a). The chain has 6 isoprenoid groups in the yeast *Saccharomyces cerevisiae* ($CoQ_6$), eight in *Escherichia coli* ($CoQ_8$), nine and ten in mice and rats ($CoQ_9$, $CoQ_{10}$) and mostly 10 in humans. The diversity in the side chain length is interpreted as a requirement for its stability inside the bilayer of different phospholipids composition (Bentinger et al. 2010). CoQ oxidized form (ubiquinone) is an electron carrier that can be reduced to ubiquinol with two electrons. This reduction can occur directly by one reaction of two electrons, or it can pass through two steps of one electron each, producing the semiquinone intermediate (Mitchell 1975; Wang and Hekimi 2016). CoQ is present in all cells, being mainly located in the mitochondrial inner membrane as a component of the respiratory chain, driving electrons from complex I and complex II, to complex III (Fig. 6.1b) (Alcázar-Fabra et al. 2016; Crane et al. 1957; Kozlov et al. 1998; Lenaz et al. 2007). Mitochondrial CoQ can also be reduced by other different dehydrogenases, such as the electron transfer flavoprotein dehydrogenase (ETFDH), a component of the β–oxidation of fatty acids (Watmough and Frerman 2010); the mitochondrial dihydroorotate dehydrogenase, responsible of

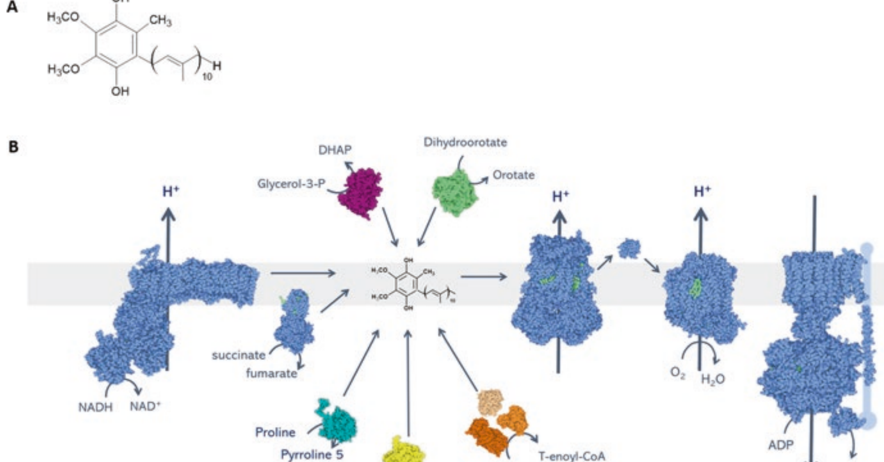


**Fig. 6.1** (**a**) Chemical structure of Coenzyme Q; (**b**) OXPHOS system showing complexes I and II, and other dehydrogenases that reduce CoQ in the inner mitochondrial membrane. Depicted using Illustrate (Goodsell et al. 2019). *CI* NADH:CoQ oxidoreductase, *CII* succinate dehydrogenase, *CIII* CoQ: cytochrome c oxidoreductase, *CIV* Cytochrome c oxidase, *CV* ATP synthetase, *DHODH* Dihidroorotate dehydrogenase, *GPDH* Glycerol 3 phosphate dehydrogenase, *ProDH* Proline dehydrogenase, *ACAD* Acyl-CoA dehydrogenase, *ETF-FAD* Electron Transfer Flavoprotein, *ETFDH* Electron Transfer Flavoprotein Coenzyme Q reductase, *SQOR* sulfide: quinone oxidoreductase, *Cyt c* cytochrome *c*, *IMS* inter membrane space, *MIM* mitochondrial inner membrane

pyrimidine nucleotides biosynthesis (Evans and Guy 2004) and the glycerol-3-phosphate dehydrogenase, an enzyme involved in lipid metabolism (Harding et al. 1975). Additionally, CoQ is a substrate for the sulfide: quinone oxidoreductase (SQOR), which oxidizes hydrogen sulfide ($H_2S$) in the first step of its catabolism in the mitochondria. SQOR levels and activity have been recently reported to be severely decreased in CoQ deficiency (Luna-Sánchez et al. 2017; Quinzii et al. 2017; Ziosi et al. 2017).

CoQ has also been shown to be a structural component of Complex III–at least in yeasts- contributing to both its assembly and stability. This integral location optimizes the efficiency of the electron transport from $CoQH_2$ throughout the CoQ cycle, which is an essential step for proton translocation and hence energy conservation (Cramer et al. 2011; Santos-Ocaña et al. 2002). Complex III can be associated with complexes I and IV in a super assembly manner, constituting several types of respiratory chain supercomplexes (Letts and Sazanov 2017; Lobo-Jarne and Ugalde 2018; Schägger and Pfeiffer 2000). One of these assemblies is the so-called respirasome that contains complexes I, III and IV, which can transfer electrons from NADH to $O_2$ (Enríquez 2016; Gu et al. 2016; Guo et al. 2016, 2017). CoQ has been proposed to be an essential component in respiratory-active respirasome and other associations (Acín-Pérez et al. 2008), being organised in different pools according to the complexes involved in the superassembly (Enriquez and Lenaz 2014; Lapuente-Brun et al. 2013). However, it has been recently suggested that CoQ constitutes one single pool that can be reduced either by complexes I or II. CoQ can be experimentally reoxidized more rapidly by alternative quinol oxidases outside the supercomplexes than by complex III inside these superstructures. This would indicate that substrate channeling does not occur, but instead CoQ diffuses freely (Fedor and Hirst 2018; Hirst 2018). Thus, the existence of a single or various CoQ pools in the mitochondrial respiratory chain is still controversial.

Being an integral part of the system, CoQ biosynthesis rate must be linked to balanced electron transport chain components. Thus, secondary deficiencies can be derived from defects in OXPHOS components (Yubero et al. 2016). Mutations in other genes, such as *ETFDH*, involved in the fatty acids oxidation pathway (Gempel et al. 2007) could also cause secondary deficiencies due to the functional link between processes. Secondary deficiencies have also been linked to other OXPHOS defects by extensive omics analysis in tissues of several knockout mouse strains defective in essential nuclear DNA-encoded factors involved in mtDNA expression (Kühl et al. 2017). Secondary CoQ deficiency in these cases could indicate that these mtDNA expression defects would provoke an imbalance of the OXPHOS system components that would lead to the downregulation of the CoQ biosynthesis in an attempt to adjust the CoQ levels to the rest of the components of the system.

Mitochondrial respiratory chain is the primary source of reactive oxygen species (ROS) (Murphy 2009) and the structural organization of supercomplexes has been proposed to modulate ROS production (Genova and Lenaz 2015; Lenaz et al. 2016). High levels of ROS cause macromolecular damage, whereas low levels of mitochondrial ROS act as a signal to enhance systemic defense mechanisms by inducing an adaptive response (Gonzalez-Freire et al. 2015). High ROS cause accumulation

of mtDNA damage, leading to a defective mitochondrial protein synthesis, which ultimately impacts on mitochondrial physiology, and may lead to premature senescence and ageing (Larsson 2010; Sgarbi et al. 2014; Teshima et al. 2014). CoQ is also involved in ROS production, which can reconfigure electron transport after complex I disorganization, increasing electron flow through complex II (Guarás et al. 2016). Mutations or knock-down of CoQ biosynthesis genes in *C. elegans* increase longevity associated with lower levels of ROS, despite decreasing mitochondrial function (Asencio et al. 2003; Wong et al. 1995). Thus, CoQ-dependent ROS production would be rather acting as signaling to modulate longevity as it acts in other cellular protection pathways (Scialò et al. 2016; Yee et al. 2014).

CoQ is the only lipidic antioxidant endogenously synthesized by the cell. It can directly avoid lipid peroxidation in membranes (Bentinger et al. 2007; Maroz et al. 2009) and, in plasma membrane, it acts as an antioxidant, recycling ascorbic acid and α-tocopherol (Arroyo et al. 2004; Mukai 2001; Navas et al. 2007). Extramitochondrial CoQ role has recently recovered relevance as part of the plasma membrane antioxidant system that inhibits ferroptosis, a form of regulated cell death induced by the iron-dependent peroxidation of lipids, through the NAD(P)H/CoQ oxidoreductase FSP1 (Bersuker et al. 2019; Doll et al. 2019).

Participation of CoQ in all these functions would pleiotropically influence the cellular physiology leading to mitochondrial health or disease depending on its concentration and/or biosynthesis rate under genetic or environmental conditions. Here, we review the underlying mechanisms modulating cellular CoQ levels and those that affect the optimum concentration leading to CoQ deficiency syndrome.

## 6.2 Primary CoQ Deficiency

CoQ deficiencies are characterized by reduced levels of CoQ in tissues due to either impairment of it biosynthesis or as a consequence of defects not directly involved with this process, the so called primary and secondary deficiencies, respectively.

Primary CoQ deficiencies are rare autosomal recessive conditions caused by mutations in any of the genes directly involved in the CoQ biosynthesis pathway at the enzymatic or the regulatory level. CoQ, which is structurally composed by a redox-active benzoquinone ring and a polyisoprenoid tail that anchors the molecule to the membrane (Fig. 6.1a), can be incorporated by the diet, but it is mainly endogenously synthesized in mitochondria and subsequently distributed to other membranes (Fernandez-Ayala et al. 2005).

The information available on CoQ biosynthesis mainly derives from studies of intermediates of the pathway in bacteria and mutant yeasts. In *Saccharomyces cerevisiae*, the synthesis of CoQ depends on at least 13 nuclear-encoded genes called coq genes (coq1–coq11, Yah1 and Arh1) that are evolutionarily highly conserved. Some of the products of these genes assemble in a multienzymatic complex (the CoQ Synthome) located in the mitochondrial inner membrane (Allan et al. 2015; Belogrudov et al. 2001; Gonzalez-Mariscal et al. 2014; Marbois et al. 2005, 2009).

Currently, the exact enzymatic role of the different components of the route is only partially known. Orthologues of most of the yeast coq genes have been identified in humans (Awad et al. 2018), but the assembly and regulation of the CoQ Synthome have not yet been wholly demonstrated in humans, although a more complete evidence of the existence of a complex by BN-PAGE experiments has recently been reported (Floyd et al. 2016; Lohman et al. 2014; Nguyen et al. 2014; Reidenbach et al. 2018; Stefely et al. 2016; Yen et al. 2016; 2020). In yeast, the CoQ biosynthetic complex is spatially and functionally related to ERMES (endoplasmic reticulum-mitochondria encounter structure) complex, also known as ER-mitochondria contacts, and the loss of this structure impairs respiration through reduction of CoQ levels (Eisenberg-Bord et al. 2019). This complex requires the CoQ lipid intermediates for its formation, and it seems to be conserved from yeast to human cells (Subramanian et al. 2019).

CoQ main head precursor is 4-Hydrozybenzoate (4-HB). Yeast and bacteria can synthetize it *de novo* through the shikimate pathway (Clarke 2000), while mammals and yeast can use tyrosine to produce 4-HB by a yet poorly known pathway (Payet et al. 2016; Stefely and Pagliarini 2017; Stefely et al. 2016). Mammals can also use phenylalanine, which is converted to tyrosine, and then to 4-HB. Interestingly, yeast but not mammals or bacteria can use para-aminobenzoic acid (pABA) as an alternative ring precursor for $CoQ_6$ synthesis (Marbois et al. 2010; Pierrel et al. 2010). Resveratrol and para-coumarate can also be used as alternative ring precursors by bacteria, yeast and mammals, probably through 4-HB transformation (Xie et al. 2015). The isoprene units are synthesized through the mevalonate pathway in extra-mitochondrial membranes and then condensed by Coq1p (PDSS1/PDSS2 heterotetramer in mammals) in mitochondria (Ashby and Edwards 1990). Next, head and tail are conducted to the mitochondria inner membrane by unknown mechanisms, and there are linked together by Coq2p (COQ2 in mammals) (Ashby et al. 1992; Forsgren et al. 2004). Additional modifications of the head are performed by different Coq proteins in mitochondria (Fig. 6.2) Coq3p (COQ3 in mammals) is an O-methylase which modifies C5 and C6 (Hsu et al. 1996; Jonassen and Clarke 2000; Poon et al. 1999); Coq5p (COQ5 in mammals) methylates C2 (Barkovich et al. 1997; Nguyen et al. 2014) and Coq6p (COQ6 in mammals) (Gin et al. 2003; Ozeir et al. 2011) and Coq7p (COQ7 in mammals) (Marbois and Clarke 1996; Tran et al. 2006) are hydroxylases modifying C5 and C6 respectively. It has been shown that Coq6p acts as a deaminase in yeast as well, whenever pABA is used as a precursor (Ozeir et al. 2015). The yeast mitochondrial ferredoxin and ferredoxin reductase (Yah1 and Arh1) are required for CoQ synthesis by transferring electrons to Coq6p (Pierrel et al. 2010). Still, there is no evidence that it occurs in humans as well. CoQ synthesis requires a C1-decarboxylation and hydroxylation catalyzed by enzymes that have not yet been identified. Coq4p (COQ4 in mammals) does not have any catalytic activity assigned, but it has been proposed to participate in the stabilization of the CoQ Synthome (Belogrudov et al. 2001; Marbois et al. 2009). In yeasts, Coq8p (COQ8A (or ADCK3/CABC1) and COQ8B (or ADCK4) in mammals) has been proposed to regulate CoQ biosynthesis by phosphorylation of Coq3p, Coq5p

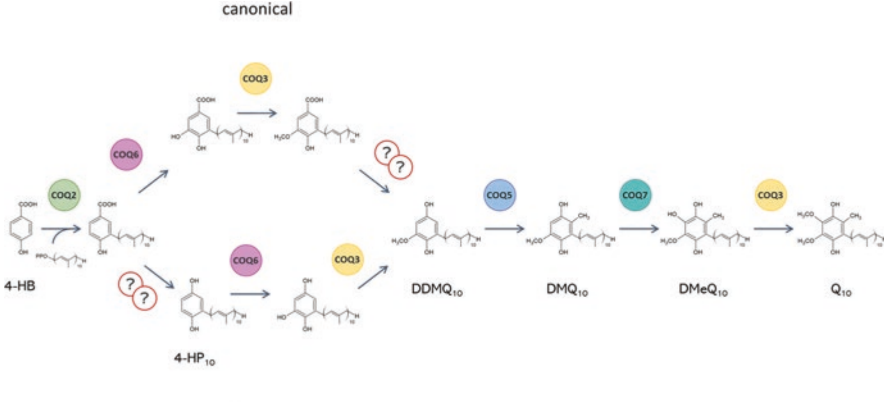


**Fig. 6.2** Human CoQ biosynthesis pathway. The classical and alternative routes (suggested in (Acosta-López et al. 2019)) are depicted

and Coq7p (Stefely et al. 2015; Tauche et al. 2008; Xie et al. 2011, 2012). Recently, however, an ATPase activity has been assigned to COQ8A/ADCK3, whose role in CoQ biosynthesis pathway still needs to be further clarified (Reidenbach et al. 2018). Coq9p (COQ9 in humans) is a lipid-binding protein that binds to Coq7p. It would enable CoQ synthesis probably by lipid presentation of the substrate (He et al. 2017; Hsieh et al. 2007; Lohman et al. 2014, 2019). Coq10p (COQ10A and COQ10B in humans) probably chaperones CoQ to the sites where it is needed within the mitochondrial membranes (Barros et al. 2005; Cui and Kawamukai 2009; Tsui et al. 2019). Coq11p is essential for CoQ synthesis in yeast, but a clear human orthologue is still lacking (Allan et al. 2015; Bradley et al. 2020). Moreover, three other genes of the *ADCK* family (*ADCK1*, *ADCK2* and *ADCK5*) have been suggested to be involved in the biosynthetic process, although currently, there is no experimental evidence for this (Doimo et al. 2014; Stefely et al. 2015; Vázquez-Fonseca et al. 2019). The phosphatase Ptc7 has been demonstrated to regulate CoQ biosynthesis by activating Coq7 in yeast (Martín-Montalvo et al. 2013; González-Mariscal et al. 2018). In mammals, its orthologue PPTC7 is thought to regulate mitochondrial biogenesis and metabolism, by a process linked to CoQ content modulation. The specificity of this link is controversial, though (González-Mariscal et al. 2018; Niemi et al. 2019).

The exact order of reactions involved in the modifications of the aromatic ring of CoQ is still unclear in eukaryotes. The accepted model starts with a C5 hydroxylation of the head group performed by COQ6 (Fig. 6.2) (Kawamukai 2016). However, yeast and human *COQ6* knockout cells accumulate 4-HP, a compound that is decarboxylated and hydroxylated in position C1 of the ring. Therefore, these reactions (which are catalyzed by still unidentified enzymes) must occur before or independently on C5 hydroxylation by COQ6 (Acosta-López et al. 2019; Ozeir et al. 2011).

### 6.2.1 Clinical Manifestations of Primary CoQ Deficiencies

Primary deficiencies are very rare conditions. In the last years, next-generation sequencing advances have allowed the identification of an increasing number of pathogenic variants in *COQ* genes. Approximately 277 patients from 184 families with primary CoQ deficiency caused by homozygous or compound heterozygous pathogenic variants of some but not all *COQ* genes (*PDSS1*, *PDSS2*, *COQ2*, *COQ4*, *COQ5*, *COQ6*, *COQ7*, *COQ8A*, *COQ8B* and *COQ9*) have been identified up to date (Alcázar-Fabra et al. 2018) (Table 6.1). Primary CoQ deficiencies are mainly of early-onset, ranging from birth to early childhood (*PDSS1*, *PDSS2*, *COQ2*, *COQ4*, *COQ5*, *COQ6*, *COQ7* and *COQ9*), or from childhood to adolescence (*COQ8A* and *COQ8B*). However, there are reports for some adult-onset cases harboring mutations in *COQ2* (Mitsui et al. 2013), *COQ8A* (Horvath et al. 2012; Terracciano et al. 2012) or *COQ8B* (Atmaca et al. 2017) (Table 6.2).

Symptoms of CoQ deficiencies have been traditionally grouped into five categories: encephalomyopathy, cerebellar ataxia, severe infantile multisystemic disease, nephropathy, and isolated myopathy (Emmanuele et al. 2012). However, this classification is currently dismissed since a growing number of patients have been studied lately and show a broader and overlapping clinical spectrum (Acosta-López et al. 2019; Salviati et al. 2017). Also, it has become more evident that only patients affected by secondary CoQ deficiency have isolated myopathy (Salviati et al. 2017; Trevisson et al. 2011).

Impairment of CoQ biosynthesis caused by mutations in *COQ* genes are clinically highly diverse and manifest with defects in different tissues and systems, mainly skeletal muscle, central and peripheral nervous system, kidney and heart (Fig. 6.3). Not only the clinical manifestations, but also the specific phenotypic consequences of mutations in *COQ* genes are very heterogeneous. Mutations in some *COQ* genes affect particular tissues (e.g. *COQ8A* (Mignot et al. 2013) and *COQ8B* (Ashraf et al. 2013)), while others' pathological variations are more pleiotropic (e.g. *COQ2* (Desbats et al. 2016) and *COQ4* (Brea-Calvo et al. 2015)). Also, mutations in the same *COQ* gene can cause very variable clinical phenotypes with different age of onset, as it occurs with *COQ2* or *COQ4* patients. Genotype-phenotype correlations are currently difficult to establish, mainly because of the low number of patients identified and the highly complex set of symptoms associated with them. The diversity of clinical manifestations suggests that different pathomechanisms may exist. Genetic background, compensation mechanisms, maternal effect and maybe, environmental factors, could determine the moment and the tissues affected during development and thus, influence the outcome of each genetic defect in each individual.

The central nervous system (CNS) is very often affected in primary CoQ deficiency patients. CNS symptoms may be present in individuals with mutations in any of the *COQ* genes, but they are less frequent in patients with pathogenic variants of *COQ6* and *COQ8B*, in whom the central phenotype is renal involvement (Table 6.3). **Encephalopathy**, defined as a broad spectrum of brain manifestations,

**Table 6.1** Pathogenic variants of *COQ* genes found in primary CoQ deficiency patients reported in the literature

| Gene | Length (AA) | Exons | RefSeq[1] | Pathogenic Variants | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | F (P) | cDNA Mutation | AA Modification | Exon | Reference |
| PDSS1 | 415 | 12 exons | NM_014317.5 NP_055132.2 | 1 (1) | c.661_662insT | p.Arg221Leufs* | 7 | Vasta (2012) |
| | | | | 1 (2) | c.924T > G | p.Asp308Glu | 10 | Mollet (2007) |
| | | | | 1 (1) | c.1108A > C | p.Ser370Arg | 12 | Vasta (2012) |
| PDSS2 | 399 | 8 exons | NM_020381.4 NP_065114.3 | 1 (1) | c.485A > G | p.His162Arg | 3 | Iványi (2018) |
| | | | | 1 (1) | c.964C > T | p.Gln322* | 6 | López (2006) |
| | | | | 1 (1) | c.1042_1148-2816del | p.? | 8 | Iványi (2018) |
| | | | | 2 (2) | c.1145C > T | p.Ser382Leu | 8 | López (2006) and Sadowski (2015) |
| | | | | 1 (1) | c.1151C > A | p.Ala384Asp | 8 | Sadowski (2015) |
| | | | | 1 (3) | NK | NK | NK | Rötig et al. (2000) and Rahman et al. (2012) |
| COQ2[2] | 371 | 7 exons | NM_001358921.2 NP_001345850.1 | 1 (1) | c.26dupT | p.Ala10Argfs*33 | 1 | Starr (2018) |
| | | | | 1 (1) | c.232A>G | p.Met78Val | 1 | Mitsui (2013) and Desbats et al. (2016) |
| | | | | 5 (7) | c.287G > A[4] | p.Ser96Asn | 2 | Diomedi-Camassei et al. (2007), Dinwiddie (2013), Desbats et al. (2016), Scalais (2013) and Eroglu (2018) |
| | | | | 2 (3) | c.368G > A | p.Arg123His | 2 | Sadowski (2015) and Xu (2018) |
| | | | | 1 (1) | c.395T > G | p.Met132Arg | 2 | Desbats et al. (2015a, b) |
| | | | | 1 (1) | c.440G > A | p.Arg147His | 3 | Diomedi-Camassei et al. (2007) and Desbats et al. (2016) |
| | | | | 8 (9) | c.533A > G | p.Asn178Ser | 3 | Diomedi-Camassei et al. (2007), Mccarthy (2013), Desbats et al. (2016), Sadowski (2015), Starr (2018) and Bezdíčka et al. (2018) |
| | | | | 1 (1) | c.551delT | p.Leu184fs*14 | 4 | Mccarthy (2013) and Desbats et al. (2016) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 1 (1) | c.682T > C[5] | p.Cys228Arg | 5 | Wu (2019) |
| | | | | 1 (1) | c.706C > T | p.Leu236Phe | 5 | Sadowski (2015) |
| | | | | 1 (1) | c.731C > T | p.Thr244Ile | 5 | Starr (2018) |
| | | | | 2 (3) | c.740A > G | p.Tyr247Cys | 5 | Diomedi-Camassei et al. (2007), Salviati (2005), Quinzii (2006), Desbats (2016) and Sadowski (2015) |
| | | | | 1 (2) | c.755C > T | p.Ala252Val | 5 | Jakobs (2013) and Desbats (2016) |
| | | | | 2 (3) | c.823A > G | p.Thr275Ala | 6 | Starr (2018) and Xu (2018) |
| | | | | 2 (2) | c.1009C > T[4] | p.Arg337* | 7 | Dinwiddie (2013), Desbats (2016) and Starr (2018) |
| | | | | 1 (2) | c.1019G > C | p.Gly340Ala | 7 | Gigante (2017) |
| | | | | 1 (2) | c.1047delT | p.Asn351Ilefs*15 | 7 | Mollet (2007) |
| COQ4 | 265 | 7 exons | NM_016035.5 NP_057119.3 | 1 (1) | c.23_33delTCCTCCGTCGG | p.Val8Alafs*19 | 1 | Sondheimer (2017) |
| | | | | 1 (2) | c.155T > C | p.Leu52Ser | 2 | Brea-Calvo (2015) |
| | | | | 1 (2) | c.164G > T | p.Gly55Val | 2 | Caglayan (2019) |
| | | | | 1 (1) | c.190C > T | p.Pro64Ser | 2 | Brea-Calvo (2015) |
| | | | | 1 (2) | c.197_198delGCinsAA | p.Arg66Gln | 2 | Chung (2015) |
| | | | | 2 (3) | c.202G > C | p.Asp68His | 2-3 | Chung (2015) and Helbig (2016) |
| | | | | 1 (2) | c.230C > T | P.Thr77Ile | 3 | Bosch (2018) |
| | | | | 1 (2) | c.245T > A | p.Leu82Gln | 3 | Chung (2015) |
| | | | | 1 (1) | c.311G > T | p.Asp111Tyr | 4 | Sondheimer (2017) |
| | | | | 1 (1) | c.356C > T | p.Pro119Leu | 4 | Sondheimer (2017) |
| | | | | 12 (16) | c.370G > A[6] | p.Gly124Ser | 4 | Lu (2019), Ling (2019) and Yu (2019) |

(continued)

**Table 6.1** (continued)

| Gene | Length (AA) | Exons | RefSeq[1] | Pathogenic Variants | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | F (P) | cDNA Mutation | AA Modification | Exon | Reference |
| | | | | 2 (2) | c.371G > T | p.Gly124Val | 4 | Ling (2019) and Yu (2019) |
| | | | | 4 (5) | c.402+1G > C | ? | Intron 4 | Yu (2019) |
| | | | | 1 (1) | c.421C > T | p.Arg141* | 5 | Brea-Calvo (2015) |
| | | | | 1 (1) | c.433C > G | p.Arg145Gly | 5 | Brea-Calvo (2015) |
| | | | | 1 (1) | c.469C>A | p.Gln157Lys | 5 | Helbig (2016) |
| | | | | 1 (2) | c.473G > A | p.Arg158Gln | 5 | Chung (2015) |
| | | | | 1 (2) | c.521_523delCCA | p.Thr174del | 5 | Brea-Calvo (2015) |
| | | | | 1 (1) | c.533G > A | p.Gly178Glu | 6 | Ling (2019) |
| | | | | 1 (1) | c.550T > C | p.Trp184Arg | 6 | Yu (2019) |
| | | | | 3 (3) | c.718C > T | p.Arg240Cys | 7 | Brea-Calvo (2015) and Chung (2015) |
| | | | | 1 (1) | 3.9 Mb deletion of chromosome 9q34.13, including COQ4 gene | | | Salviati (2012) |
| COQ5 | 327 | 7 exons | NM_032314.4 NP_115690.3 | 1 (3) | 9590 pb tandem duplication of the last 4 exons of COQ5 after 1Kb of 3′UTR (modifies the 3′UTR) (base pair positions on Chr 12: 120,940,150-120,949,950/hg19) | | | Malicdan (2018) |
| COQ6 | 468 | 12 exons | NM_182476.3 NP_872282.1 | Isoform 1 | | | | |
| | | | | 1 (1) | c.145G > T | p.Ala49Ser | 1 | Schoonen (2019) and Louw (2018) |
| | | | | 6 (6) | c.189_191delGAA | p.Lys64del | 2 | Park et al. (2017a, b) |
| | | | | 1 (1) | c.484C > T[3] | p.Arg162* | 5 | Heeringa (2011) |
| | | | | 1 (1) | c.564G > A[3] | p.Trp188* | 5 | Heeringa (2011) |
| | | | | 1 (1) | c.686A > C | p.Gln229Pro | 6 | Park et al. (2017a, b) |
| | | | | 2 (7) | c.763G > A | p.Gly255Arg | 7 | Heeringa (2011) and Doimo (2014) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 6 (6) | c.782C > T | p.Pro261Leu | 7 | Gigante (2017) and Park et al. (2017a, b) |
| | | | | 1 (1) | c.804delC | p.Leu269Trpfs*13 | 8 | Stańczyk et al. (2018) |
| | | | | 5 (8) | c.1058C > A | p.Ala353Asp | 9 | Heeringa (2011), Doimo (2014), Sadowski (2015), Koyun (2018) and Yuruk Yildirim et al. (2019) |
| | | | | 3 (3) | c.1078C > T | p.Arg360Trp | 9 | Cao (2017), Li (2018) and Stańczyk et al. (2018) |
| | | | | 1 (1) | c.1154A > C | p.Asp385Ala | 11 | Sadowski (2015) |
| | | | | 2 (2) | c.1235A>G[3] | p.Tyr412Cys | 11 | Doimo (2014) and Sadowski (2015) |
| | | | | 1 (1) | c.1341G > A | p.Trp447* | 11 | Heeringa (2011) and Doimo (2014) |
| | | | | 1 (1) | c.1383delG | p.Gln461fs*478 | 12 | Heeringa (2011) and Doimo (2014) |
| | | | | Isoform 2 | | | | |
| | | | | 2 (2) | c.41G > A[3] | p.Trp14* | 1 | Schoonen (2019), Louw (2018) and Song et al. (2018) |
| COQ7 | 217 | 6 exons | NM_016138.5 NP_057222.2 | 1 (1) | c.319C > T | p.Arg107Trp | 3 | Kwong (2019) |
| | | | | 1 (1) | c.332T > C (and c.308C > T)[7] | p.Leu111Pro (and p. Thr103Met) | 3 | Wang (2017) |
| | | | | 1 (1) | c.422T > A | p.Val141Glu | 4 | Freyer (2015) and Wang (2017) |
| | | | | 1 (1) | c.599_600delinsTAATGCATC | p.(Lys200Ilefs*56 | 6 | Kwong (2019) |
| COQ9 | 318 | 9 exons | NM_020312.4 NP_064708.1 | 1 (1) | c.384delG | p.Gly129Valfs*17 | 4 | Kaya Ozcora et al. (2017) |
| | | | | 1 (1) | c.521 + 1delG | p.Ser127_Arg202del | Intron 5 | Danhauser (2016) |
| | | | | 1 (4) | c.521 + 2T>C | p.Ser127_Arg202del | Intron 5 | Smith (2018) |
| | | | | 1 (4) | c.711 + 3G>C | p.Ala203_Asp237del | Intron 7 | Smith (2018) |
| | | | | 1 (1) | c.730C > T | p.Arg244* | 7 | Rahman et al. (2001) and Duncan (2009) |

(continued)

**Table 6.1** (continued)

| Gene | Length (AA) | Exons | RefSeq[1] | Pathogenic Variants | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | F (P) | cDNA Mutation | AA Modification | Exon | Reference |
| COQ8A/ ADCK3 | 647 | 15 exons | NM_020247.5 NP_064632.2 | 1 (1) | c.500_521del22insTTG | p.Gln167Leufs*36 | 3 | Lagier-Tourenne et al. (2008) |
| | | | | 4 (5) | c.589-3C > G[8] | p.Leu197Valfs*20 | Intron 3 | Mignot (2013), Schirinzi (2019) and Galosi (2019) |
| | | | | 1 (2) | c.637C > T | p.Arg213Trp | 4 | Mollet (2008) and Mignot (2013) |
| | | | | 1 (3) | c.685-690delCTGGCA | p.Leu229_Ala230del | 5 | Hajjari (2019) |
| | | | | 2 (3) | c.811C > T | p.Arg271Cys | 6 | Horvath (2012), Mignot (2013) and Sun (2019) |
| | | | | 1 (2) | c.815G > T | p.Gly272Val | 6 | Mollet (2008) and Mignot (2013) |
| | | | | 1 (1) | c.815G>A | p.Gly272Asp | 6 | Mollet (2008) and Mignot (2013) |
| | | | | 1 (1) | c.827A > G | p.Lys276Arg | 6 | Pronicka (2016) |
| | | | | 1 (2) | c.830T > C | p.Leu277Pro | 6 | Jacobsen (2017) |
| | | | | 5 (7) | c.895C > T | p.Arg299Trp | 7 | Horvath (2012), Mignot (2013) and Hikmat (2016) |
| | | | | 3 (3) | c.901C > T | p.Arg301Trp | 7 | Sun (2019) |
| | | | | 1 (2) | c.910G > A | p.Ala304Thr | 7 | Horvath (2012) |
| | | | | 1 (1) | c.911C > T | p.Ala304Val | 7 | Horvath (2012) |
| | | | | 1 (1) | c.913G > T | p.Asp305Tyr | 7 | Chang (2018) |
| | | | | 1 (1) | c.993C > T[9] | p.Lys314_Gln360del | 8 (del of exon8?) | Lagier-Tourenne et al. (2008), Anheim (2010) and Mignot (2013) |
| | | | | 1 (1) | c.1000C > T | p.Arg334Trp | 8 | Sun (2019) |
| | | | | 1 (1) | c.1013C > T | p.Ala338Val | 8 | Kaya Ozcora et al. (2017) |
| | | | | 3 (6) | c.1027C > T | p.Gln343* | 8 | Shalata (2019) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 5 (8) | c.1042C > T | p.Arg348* | 8 | Gerards (2010), Terracciano (2012), Sun (2019) and Galosi (2019) |
| | | | | 1 (1) | c.1081-1_1082dupGTA | p.Gln360_ Tyr361ins* | Intron 8/ Exon 9 | Mignot (2013) |
| | | | | 1 (2) | c.1136T > A | p.Leu379* | 9 | Gerards (2010) |
| | | | | 1 (2) | c.1228C > T | p.Arg410* | 10 | Mignot (2013) |
| | | | | 1 (1) | c.1229G > A | p.Arg410Gln | 10 | Sun (2019) |
| | | | | 1 (2) | c.1286A > G[3] | p.Tyr429Cys | 11 | Horvath (2012) |
| | | | | 3 (3) | c.1331_1332insCACAG | p.Glu446Alafs*33 | 11 | Schirinzi (2019) and Galosi (2019) |
| | | | | 1 (1) | c.1334–1335del[3] | p.Thr445Argfs*52 | 11 | Sun (2019) |
| | | | | 1 (1) | c.1358delT | p.Leu453Argfs*24 | 11 | Mignot (2013) |
| | | | | 3 (4) | c.1396delG | p.Glu466Argfs*11 | 11 | Mutlu-Albayrak et al. (2020) |
| | | | | 1 (4) | c.1398 + 2T > C[10] | p.Asp420Trpfs*40; p.Ile467Alafs*22 | Exons 11-12 | Lagier-Tourenne et al. (2008) |
| | | | | 1 (1) | c.1399-3_1408del | Exon 12 skipping? | Exons 11-12 | Chang (2018) |
| | | | | 1 (2) | c.1506 + 1G>A | p.Val503Metfs*21 | Intron 12 | Jacobsen (2017) |
| | | | | 1 (1) | c.1511_1512delCT | p.Ala504fs* | 13 | Barca et al. (2016a) |
| | | | | 1 (1) | c.1523T > C | p.Phe508Ser | 13 | Mignot (2013) |
| | | | | 2 (2) | c.1532C > T | p.Thr511Met | 13 | Sun (2019) and Chang (2018) |
| | | | | 1 (1) | c.1534C > T[11] | p.Arg512Trp | 13 | Nair (2018) |
| | | | | 1 (1) | c.1541A > G | p.Tyr514Cys | 13 | Lagier-Tourenne et al. (2008) |
| | | | | 1 (1) | c.1645G > A | p.Gly549Ser | 14 | Lagier-Tourenne et al. (2008), Anheim (2010) and Mignot (2013) |

(continued)

**Table 6.1** (continued)

| Gene | Length (AA) | Exons | RefSeq[1] | Pathogenic Variants | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | F (P) | cDNA Mutation | AA Modification | Exon | Reference |
| | | | | 2 (2) | c.1651G > A | p.Glu551Lys | 14 | Mollet (2008) and Sun (2019) |
| | | | | 1 (1) | c.1702delG | p.Glu568Argfs* | 15 | Pronicka (2016) |
| | | | | 1 (2) | c.1732T > G | p.Phe578Val | 15 | Hikmat (2016) |
| | | | | 1 (1) | c.1749_1751delCAC | p.Thr584del | 15 | Chang (2018) |
| | | | | 3 (4) | c.1750_1752delACC | p.Thr584del | 15 | Lagier-Tourenne et al. (2008), Blumkin et al. (2014) and Sun (2019) |
| | | | | 1 (2) | c.1805C > G | p.Pro602Arg | 15 | Blumkin et al. (2014) |
| | | | | 1 (1) | c.1813dupG | p.Glu605Glyfs*125 | 15 | Mollet (2008) and Mignot (2013) |
| | | | | 2 (3) | c.1823C > T | p.Ser608Phe | 15 | Shalata (2019) |
| | | | | 3 (4) | c.1844G > A[8] | p.Gly615Asp | 15 | Mignot (2013), Schirinzi (2019) and Galosi (2019) |
| | | | | 1 (2) | c.1844dupG | p.Ser616Leufs*114 | 15 | Liu (2014) |
| | | | | 1 (1) | 27.6 kb deletion of 1q42.3 including Exons 1–2 | | | Galosi (2019) |
| | | | | 1 (1) | 29 kb partial deletion of the gene including exons 3 to 15 (base pair position: 227,150,977-227,195,656, hg19) | | | Mignot (2013) |
| | | | | 1 (1) | 2.9Mb duplication at chromosome region 1q42.11q42.13 (including ADCK3) | | | Malgireddy (2016) |
| COQ8B/ ADCK4 | 544 | 15 exons | NM_024876.4 NP_079152.3 | 1 (1) | c.101G > A | p.Trp34* | 2 | Ashraf (2013) |
| | | | | 4 (8) | c.293T > G | p.Leu98Arg | 5 | Korkmaz (2016) and Atmaca (2017) |
| | | | | 1 (2) | c.449G > A | p.Arg150Gln | 6 | Park et al. (2017a, b) |
| | | | | 3 (5) | c.532C > T | p.Arg178Trp | 7 | Ashraf (2013), Korkmaz (2016), Vazquez-Fonseca et al. (2017) and Feng (2017) |
| | | | | 3 (4) | c.645delT | p.Phe215Leufs*14 | 8 | Ashraf (2013), Korkmaz (2016) and Vazquez-Fonseca et al. (2017) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 1 (1) | c.649G > A | p.Ala217Thr | 8 | Lolin (2017) |
| | | | | 6 (7) | c.737G > A | p.Ser246Asn | 9 | Feng (2017), Park et al. (2017a, b) and Kakiuchi et al. (2019) |
| | | | | 1 (2) | c.748G > A | p.Asp250Asn | 9 | Korkmaz (2016) |
| | | | | 1 (1) | c.748G > T | p.Asp250Tyr | 9 | Lolin (2017) |
| | | | | 4 (5) | c.748G > [12] | p.Asp250His | 9 | Zhang (2017), Feng (2017) and Yang et al. (2019) |
| | | | | 2 (3) | c.759C > A | p.Asn253Lys | 9 | Park et al. (2017b) |
| | | | | 1 (3) | c.857A > G | p.Asp286Gly | 10 | Ashraf (2013) and Vazquez-Fonseca et al. (2017) |
| | | | | 1 (1) | c.929C > T | p.Pro310Leu | 10 | Korkmaz (2016) |
| | | | | 1 (1) | c.954_956dupGAC | p.Thr319dup | 11 | Ashraf (2013) |
| | | | | 1 (2) | c.958C > T | p.Arg320Trp | 11 | Ashraf (2013) and Vazquez-Fonseca et al. (2017) |
| | | | | 1 (2) | c.1027C > T | p.Arg343Trp | 11 | Ashraf (2013) |
| | | | | 1 (1) | c.1041G > T | p.Cys347* | 12 | Yang et al. (2019) |
| | | | | 5 (13) | c.1199dupA | p.His400Glnfs*11 | 13 | Ashraf (2013), Korkmaz (2016), Vazquez-Fonseca et al. (2017) and Atmaca (2017) |
| | | | | 7 (20) | c.1339dupG | p.Glu447Glyfs*10 | 15 | Korkmaz (2016), Atmaca (2017) and Vazquez-Fonseca et al. (2017) |
| | | | | 1 (2) | c.1356_1362delGGGCCCT | p.Gln452Hisfs* | 15 | Ashraf (2013) |
| | | | | 2 (3) | c.1430G > A | p.Arg477Gln | 15 | Ashraf (2013), Vazquez-Fonseca et al. (2017) and Atmaca (2017) |
| | | | | 1 (3) | c.1447G > T | p.Glu483* | 15 | Ashraf (2013) and Vazquez-Fonseca et al. (2017) |
| | | | | 1 (1) | c.1468C > T | p.Arg490Cys | 15 | Park et al.(2017b) |
| | | | | 1 (1) | c.1493_1494CC > AA | p.Ala498Glu | 15 | Korkmaz (2016) |

(continued)

**Table 6.1** (continued)

[1]Reference sequences correspond to longest transcript

[2]COQ2: Several initiation codons. The last proposed nomenclature is used, corresponding to a 371 aa length protein (Desbats et al. 2016)

[3]These pathogenic variants were found in simple heterozygosis in at least one patient(Doimo et al. 2014; Heeringa et al. 2011; Horvath et al. 2012; Song et al. 2018; Sun et al. 2019)

The patient with these two mutations also carries a novel mutation in *MT-ND1* (3754C > A) with 22% of heteroplasmy in peripheral blood, which may contribute to the disease (Dinwiddie et al. 2013)

[5]The patient with this mutation in homozygosis also carries an additional homozygous mutation in *ARSB* gene (c.1213 + 1G > A), which may contribute to the disease (Wu et al. 2019)

[6]c.370G > A, p.Gly124Ser mutation in *COQ4* has been described as a founder mutation in southern Chinese population, being found in 16 patients of 12 Chinese families (Ling et al. 2019; Lu et al. 2019; Yu et al. 2019)

[7]*COQ7* c.308C > T polymorphism seems to increase COQ7 protein instability and intensify the effect of the mutation. The patient also has a 1555A>G mutation in mtDNA, not previously reported, which may contribute to the disease (Wang et al. 2017)

[8]A patient with these two mutations in heterozygosis in *COQ8A* also carries two compound heterozygous mutations in *PAH* gene, which may contribute to the disease (Galosi et al. 2019)

[9]Pathogenic variant c.993C > T of *COQ8A* shows a conflicting protein change. Based on the sequence, it should be a synonymous change (p.Phe331=) (Lagier-Tourenne et al. 2008)

[10]*COQ8A* c.1398 + 2T > C6 variant affects a splice donor site, different splice variants are expressed: p.Asp420Trpfs*40 and p.Ile467Alafs*22 (Lagier-Tourenne et al. 2008)

[11]The patient with this *COQ8A* mutation in homozygosis also carries an additional homozygous mutation in *MED25* gene (c.518T > C; p.Ile173Thr), which may contribute to the disease (Nair et al. 2018)

[12]Two siblings with this *COQ8B* variant in homozygosis also had an homozygous mutation in *NPHS1* gene (c.1339G > A; p.Glu447Lys) (Zhang et al. 2017)

Abbreviations: *F* number of families with each mutation, *P* number of patients with each mutation, *AA* amino acid

**Table 6.2** Age of onset, biochemical findings and CoQ treatment response of primary CoQ deficiency patients in the literature

| | | | | Age | | Biochemical Findings | | CoQ Treatment | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Gene | Families | Patients | Sex | Onset | Last Examination (*death) | Lactic Acidosis | CoQ Deficiency | CoQ Treatment | Effect | References |
| *PDSS1* | 2 | 3 | F (2) | <2yo (3) | 1,5yo* (1) | 3 | F (2) | | | Mollet et al. (2007) and Vasta et al. (2012) |
| | | | M (1) | | 14–22yo (2) | | WBC (1) | | | |
| *PDSS2* | 5 | 7 | F (2) | <1yo (6) | 8mo* (2) | 2 | M (1) | Yes (2) | No benefits (2) | Iványi et al. (2018), López et al. (2006), Rahman et al. (2012), Rötig et al. (2000) and Sadowski et al. (2015) |
| | | | M (5) | 2yo (1) | 8yo* (1) | | F (1) | | | |
| | | | | | 8-12yo (2) | | | | | |
| | | | | | NK (2) | | | | | |
| *COQ2* | 23 | 31 | F (16) | <3yo (26) | <3yo* (15) | 12 | M (5) | Yes (14) | Neuromuscular functions restored (2) | Bezdíčka et al. (2018, 2020), Desbats et al. (2015, 2016), Dinwiddie et al. (2013), Diomedi-Camassei et al. (2007), Eroglu et al. (2018), Gigante et al. (2017), Jakobs et al. (2013), McCarthy et al. (2013), Mitsui et al. (2013), Mollet et al. (2007), Quinzii et al. (2006), Sadowski et al. (2015), Salviati et al. (2005), Scalais et al. (2013), Starr et al. (2018), Wu et al. (2019) and Xu et al. (2018) |
| | | | M (15) | 10yo (1) | 2-4yo (5) | | F (3) | | No benefits (2) | |
| | | | | 16–18yo (2) | 12yo (1) | | | | No benefits on neuromuscular function (3) | |
| | | | | 70yo (1) | 23-37yo (2) | | | | No benefits on renal function (1) | |
| | | | | NK (1) | 71yo (1) | | | | No deterioration (2) | |
| | | | | | NK (7) | | | | Renal function restored (6) | |

(continued)

**Table 6.2** (continued)

| Gene | Families | Patients | Sex | Age | | Biochemical Findings | | CoQ Treatment | | References |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Onset | Last Examination (*death) | Lactic Acidosis | CoQ Deficiency | CoQ Treatment | Effect | |
| *COQ4* | 26 | 35 | F (20) | Birth (19) | <4do* (7) | 24 | M (7) | Yes (20) | No benefits (7) | Bosch et al. (2018), Brea-Calvo et al. (2015), Caglayan et al. (2019), Chung et al. (2015), Helbig et al. (2016), Ling et al. (2019), Lu et al. (2019), Salviati et al. (2012), Sondheimer et al. (2017) and Yu et al. (2019) |
| | | | M (14) | <1yo (11) | 1mo-3yo* (12) | | F (10) | | Improvement of Sz or Ep (3) | |
| | | | NK (1) | 4–10yo (4) | 9mo-4yo (8) | | | | Improvement in development (2) | |
| | | | | NK (1) | 7yo* (1) | | | | Improvement of cardiac function (2) | |
| | | | | | 8–28yo (6) | | | | Stable condition (2) | |
| | | | | | NK (1) | | | | Subjective response improvement (2) | |
| | | | | | | | | | Improvement of ataxia (1) | |
| | | | | | | | | | Improvement of lactic acidosis (1) | |
| | | | | | | | | | Muscle improvement (1) | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *COQ5* | 1 | 3 | F (3) | Early childhood (3) | 14–22yo (3) | | WBC (3) | Yes (3) | Improvement of ataxia (3) | Malicdan et al. (2018) |
| | | | | | | | M (1) | | | |
| *COQ6* | 24 | 32 | F (13) | <3yo (19) | 5–6yo* (2) | | M (1) | Yes (10) | Improved proteinuria (6) | Cao et al. (2017), Doimo et al. (2014), Gigante et al. (2017), Heeringa et al. (2011), Koyun et al. (2018), Li et al. (2018), Louw et al. (2018), Park et al. (2017a), Sadowski et al. (2015), Schoonen et al. (2019), Stańczyk et al. (2018) and Yuruk Yildirim et al. (2019) |
| | | | M (10) | 4–10yo (8) | 17yo* (1) | | | | Improved renal function (3) | |
| | | | NK (12) | NK (5) | NK* (2) | | | | Improved growth retardation (2) | |
| | | | | | 0.5–17yo (20) | | | | Improved SNHL (1) | |
| | | | | | NK (7) | | | | | |
| *COQ7* | 3 | 3 | F (1) | <1yo (3) | 1yo*(1) | 3 | M (1) | Yes (3) | No deterioration (2) | Freyer et al. (2015), Kwong et al. (2019) and Wang et al. (2017) |
| | | | M (2) | | 6–9yo (2) | | F (3) | | No benefits (1) | |
| *COQ9* | 4 | 7 | F (2) | Birth (6) | Birth*(2) | 5 | M (1) | Yes (4) | No benefits (3) | Danhauser et al. (2016), Duncan et al. (2009), Olgac et al. (2020), Rahman et al. (2001) and Smith et al. (2018) |
| | | | M (5) | 9mo (1) | 12ho*(1) | | F (3) | | Plasma lactate reduced (1) | |
| | | | | | 3do*(1) | | | | | |
| | | | | | 18do* (1) | | | | | |
| | | | | | 2yo* (1) | | | | | |
| | | | | | 3yo (1) | | | | | |

(continued)

**Table 6.2** (continued)

| Gene | Families | Patients | Sex | Age | | Biochemical Findings | | CoQ Treatment | | References |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Onset | Last Examination (*death) | Lactic Acidosis | CoQ Deficiency | CoQ Treatment | Effect | |
| *COQ8A* | 55 | 77 | F (41) | 1–4yo (35) | 22yo*(1) | 7 | M (15) | Yes (29) | No benefits (13) | Anheim et al. (2010), Barca et al. (2016a, b), Blumkin et al. (2014), Chang et al. (2018), Galosi et al. (2019), Gerards et al. (2010), Hajjari et al. (2019), Hikmat et al. (2016), Horvath et al. (2012), Jacobsen et al. (2017), Kaya Ozcora et al. (2017), Lagier-Tourenne et al. (2008), Liu et al. (2014), Malgireddy et al. (2016), Mignot et al. (2013), Mollet et al. (2008), Mutlu-Albayrak et al. (2020), Nair et al. (2018), Pronicka et al. (2016), Schirinzi et al. (2019), Shalata et al. (2019), Sun et al. (2019) and Terracciano et al. (2012) |
| | | | M (33) | 5–11yo (19) | 26yo*(1) | | F (4) | | Improvement of ataxia (9) | |
| | | | NK (3) | 13–27yo(11) NK (12) | 3–11yo (14) | | WBC (1) | | Improvement of tremor and myoclonus (5) | |
| | | | | | 13–25yo (22) | | | | Improvement in motor abilities (5) | |
| | | | | | 26–54yo (32) | | | | Slight improvement of cerebellar signs (2) | |
| | | | | | 81yo (1) | | | | Stabilization of the ataxia (2) | |
| | | | | | NK (6) | | | | Improvement in fatigue and speech (1) | |
| | | | | | | | | | Improvement of cognitive abilities (1) | |

| *COQ8B* | 41 | 79 | F (32) | <1yo-10yo (32) | 13–15yo* (2) | | F (5) | Yes (29) | No benefits (7) | Ashraf et al. (2013), Atmaca et al. (2017), Feng et al. (2017), Kakiuchi et al. (2019), Korkmaz et al. (2016), Lolin et al. (2017), Park et al. (2017b), Vazquez-Fonseca et al. (2017), Yang et al. (2018), Yang et al. (2019) and Zhang et al. (2017) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | M (31) | 11yo-21yo (39) | 25yo* (1) | | | | Improvement of proteinuria (18) | |
| | | | NK (16) | 23–32yo (7) | 29yo* (1) | | | | Better physical fitness and reduced fatigue (1) | |
| | | | | NK (1) | 1–9yo (6) | | | | Improvement of edema (1) | |
| | | | | | 10–20yo (54) | | | | Stabilization of renal function (1) | |
| | | | | | 21–39yo (14) | | | | | |
| | | | | | NK (1) | | | | | |

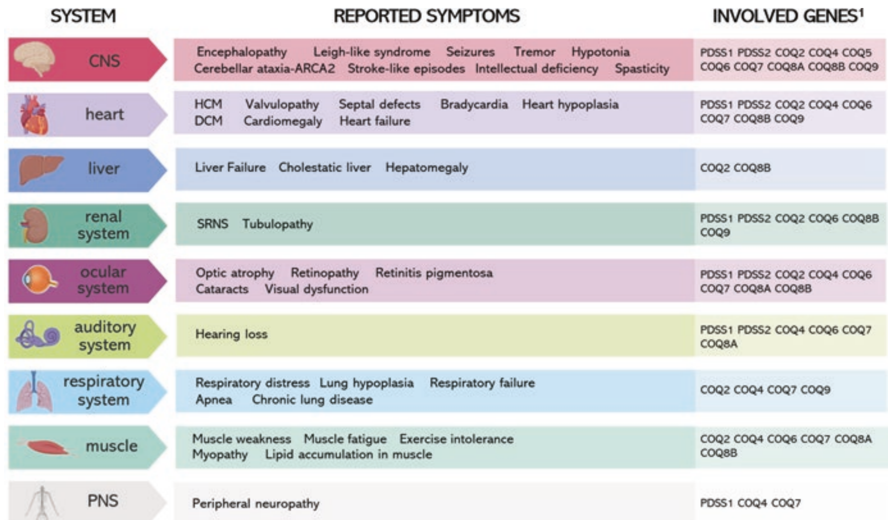


**Fig. 6.3** Main tissues affected in individuals with primary CoQ deficiency. Compilation of the more common clinical manifestations and the genes whose mutations have been associated with one or several of the referred symptoms. For the number of patients showing each symptom refer to Table 6.3. Abbreviations are also indicated in Table 6.3. [1]Symptoms are reported at different frequencies for patients harboring pathogenic variants of the different *COQ* genes

which are often not described in detail in case reports, has been mainly found in *COQ2* (Dinwiddie et al. 2013; Diomedi-Camassei et al. 2007; Eroglu et al. 2018; Mollet et al. 2007), *COQ4* (Brea-Calvo et al. 2015; Chung et al. 2015; Ling et al. 2019; Lu et al. 2019; Salviati et al. 2012; Sondheimer et al. 2017; Yu et al. 2019), *COQ5* (Malicdan et al. 2018) and *COQ9* (Duncan et al. 2009; Olgac et al. 2020; Smith et al. 2018) patients. However, it has also been observed in some *PDSS1* (Mollet et al. 2007), *PDSS2* (Iványi et al. 2018), *COQ6* (Louw et al. 2018; Schoonen et al. 2019), *COQ7* (Kwong et al. 2019), *COQ8A* (Mignot et al. 2013; Nair et al. 2018) and *COQ8B* (Korkmaz et al. 2016) probands. Most of the *COQ2* patients presented early-onset nephrotic syndrome, accompanied in one-third of the cases with encephalopathy and seizures (Bezdíčka et al. 2018; Desbats et al. 2015a, b, 2016; Dinwiddie et al. 2013; Diomedi-Camassei et al. 2007; Eroglu et al. 2018; Gigante et al. 2017; Jakobs et al. 2013; McCarthy et al. 2013; Mollet et al. 2007; Quinzii et al. 2006; Sadowski et al. 2015; Salviati et al. 2005; Scalais et al. 2013; Starr et al. 2018; Xu et al. 2018). *COQ4* cases generally present encephalopathy accompanied by seizures, hypotonia and cerebellar hypoplasia, and almost half of the diagnosed patients had a fatal outcome with death in the postnatal period (Brea-Calvo et al. 2015; Chung et al. 2015; Helbig et al. 2016; Ling et al. 2019; Lu et al. 2019; Salviati et al. 2012; Sondheimer et al. 2017). Likewise *COQ2*, one third of the *COQ8A* patients also suffered **seizures** (Galosi et al. 2019; Hikmat et al. 2016; Horvath et al. 2012; Mignot et al. 2013; Mollet et al. 2008; Sun et al. 2019; Terracciano et al. 2012)**,** and it has also been reported in cases of *PDSS2* (López

**Table 6.3** Clinical manifestations and number of patients affected by symptom in primary CoQ deficiency

| Affected Gene | | *PDSS1* | *PDSS2* | *COQ2* | *COQ4* | *COQ5* | *COQ6* | *COQ7* | *COQ9* | *COQ8A* | *COQ8B* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Total Number of Patients | | 3 | 7 | 31 | 35 | 3 | 32 | 3 | 7 | 77 | 79 |
| CNS | Autosomal recessive cerebellar ataxia 2 (ARCA2) | – | 3 | – | 4 | 3 | 1 | – | – | 70 | – |
| | Basal ganglia lessions (BGL) | – | 1 | 1 | 5 | – | – | 1 | 1 | – | – |
| | Cerebellar atrophy (CAt) | – | – | 1 | 8 | 3 | – | – | 1 | 63 | – |
| | Cerebellar hypoplasia (CHyp) | – | – | – | 10 | – | – | – | 1 | 2 | – |
| | Cerebral atrophy | – | – | – | 6 | – | – | 1 | – | – | – |
| | Cerebral Hypoplasia | – | – | – | 1 | – | – | – | – | – | – |
| | Cerebral palsy | – | 1 | – | – | – | – | – | – | – | – |
| | Chorea | – | – | – | – | – | – | – | – | 2 | – |
| | Depression | – | – | – | – | – | – | – | – | 3 | – |
| | Deteriorated ambulation (DAmb) | – | – | – | 6 | – | – | – | – | – | – |
| | Developmental delay(DD) | 1 | 1 | 2 | 18 | 2 | 3 | 3 | 6 | 11 | 1 |
| | Dysarthria (Dy) | – | – | – | 2 | 3 | – | – | – | 26 | – |
| | Dysdiadochokinesia | – | – | – | 2 | – | – | – | – | 8 | – |
| | Dysmetria | – | – | – | 2 | 2 | – | – | – | 20 | – |
| | Dystonia | – | 1 | 2 | 6 | – | – | – | 1 | 15 | – |
| | Encephalopathy | 2 | 1 | 10 | 11 | 3 | 1 | 1 | 3 | 2 | 1 |
| | Epilepsy (Ep) | – | – | 4 | 9 | 1 | – | – | – | 15 | 2 |
| | Gait instability (GI) | – | – | – | – | – | – | – | – | 12 | – |
| | Hypotonia (Ht) | – | 2 | 6 | 19 | – | 1 | 3 | 2 | 7 | – |
| | Impaired Handwriting (IH) | – | – | – | – | – | – | – | – | 7 | – |
| | Intellectual deficiency (ID) | 2 | 3 | – | 6 | 3 | 1 | 1 | – | 35 | 4 |

(continued)

**Table 6.3** (continued)

| Affected Gene | | *PDSS1* | *PDSS2* | *COQ2* | *COQ4* | *COQ5* | *COQ6* | *COQ7* | *COQ9* | *COQ8A* | *COQ8B* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Total Number of Patients | | 3 | 7 | 31 | 35 | 3 | 32 | 3 | 7 | 77 | 79 |
| | Leigh-like Syndrome (LS) | – | 1 | – | 2 | – | – | – | 2 | – | – |
| | Migraine | – | – | – | – | – | – | – | – | 5 | – |
| | Multifocal global ischemic events (MGIE) | – | – | – | – | – | – | – | 1 | – | – |
| | Muscle stiffness (MS) | – | – | 1 | – | – | – | – | 1 | – | – |
| | Myoclonus (My) | – | – | 2 | 1 | 2 | – | – | – | 14 | – |
| | Non-visual pursuit (nVP) | – | – | 1 | – | – | – | 1 | – | – | – |
| | Nystagmus (Ny) | – | 1 | 3 | 1 | 3 | 1 | – | – | 9 | – |
| | Opisthotonus | – | – | – | – | – | – | – | 1 | – | – |
| | Ptosis (Pt) | – | – | – | – | – | 1 | 1 | – | 3 | – |
| | Pyramidal syndrome | – | 1 | – | – | – | – | – | – | 1 | – |
| | Saccadic eye movements (SEM) | – | – | – | 1 | 1 | – | – | – | 14 | – |
| | Seizures (Sz) | – | 1 | 11 | 19 | 2 | 2 | – | 3 | 17 | 4 |
| | Slow ocular pursuit | – | – | – | – | – | – | – | – | 1 | – |
| | Spams | – | – | – | 2 | – | – | 1 | – | – | – |
| | Spasticity (Sp) | – | 1 | – | 9 | 1 | – | 1 | – | 8 | – |
| | Sporadic Multisystem Atrophy (MSA) | – | – | 1 | – | – | – | – | – | – | – |
| | Strabismus | – | – | – | – | – | – | – | – | 4 | – |
| | Stroke-like lessions (SLL) | – | – | 2 | 2 | – | – | – | – | 6 | – |
| | Thalamic hypoplasia (THyp) | – | – | – | 1 | – | – | – | – | – | – |
| | Tremor (Tr) | – | – | 1 | 2 | 1 | – | – | – | 33 | – |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PNS/ sensory organs | Astigmatism | – | – | – | – | – | – | – | – | – | 2 |
| | Cataracts | – | 1 | – | – | – | – | – | – | 1 | – |
| | Delayed visual maturation | – | – | – | 1 | – | – | – | – | – | – |
| | Hearing impairment | – | – | – | 2 | – | – | – | – | – | – |
| | Hearing Loss (HL) | – | – | – | 1 | – | – | – | – | – | – |
| | Hypermetropia | – | – | – | – | – | – | – | – | – | 2 |
| | Myopia | – | 2 | – | – | – | – | – | – | – | – |
| | Optic Nerve Atrophy (OA) | 2 | 1 | 1 | – | – | 1 | – | – | – | – |
| | Peripheral Neuropathy (PNSN) | 2 | – | – | 1 | – | – | 2 | – | – | – |
| | Retinitis Pigmentosa (RP) | – | 2 | 1 | – | – | – | – | – | – | 1 |
| | Retinopathy (Rp) | – | – | 2 | – | – | – | – | – | – | – |
| | Rod-Cone dysfunction | – | – | – | – | – | – | – | – | 1 | – |
| | Sensorineural hearing loss (SNHL) | 2 | 4 | – | – | – | 18 | 3 | – | 2 | – |
| | Visual aureas | – | – | – | – | – | – | – | – | 1 | – |
| | Visual dysfunction | – | 2 | – | 6 | – | – | 2 | – | 1 | 1 |
| Kidney | Chronic kidney disease (CKD) | – | – | 1 | – | – | 2 | – | – | – | 18 |
| | Dysplastic kidneys | – | – | – | – | – | – | 1 | – | – | – |
| | End-stage Renal Disease (ESRD) | 1 | 2 | 10 | – | – | 16 | – | – | – | 47 |
| | Enlarged kidneys | – | – | – | – | – | – | – | 1 | – | 1 |
| | Hematuria | – | – | – | – | – | – | – | – | – | 8 |
| | Kidney dysfunction (KD) | – | – | 2 | – | – | – | 1 | – | – | 1 |
| | Medullary nephrocalcinosis (MNC) | – | – | – | – | – | – | – | – | – | 6 |
| | Only Proteinuria | – | – | 1 | – | – | 1 | – | – | – | 9 |
| | Renal cysts | – | – | – | – | – | – | 1 | 1 | – | – |
| | Steroid resistant nephrotic syndrome (SRNS) | 1 | 7 | 25 | – | – | 25 | – | – | – | 66 |
| | Tubulopathy (Tp) | – | – | 1 | – | – | – | – | 1 | – | – |

(continued)

**Table 6.3** (continued)

| Affected Gene | | *PDSS1* | *PDSS2* | *COQ2* | *COQ4* | *COQ5* | *COQ6* | *COQ7* | *COQ9* | *COQ8A* | *COQ8B* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Total Number of Patients | | 3 | 7 | 31 | 35 | 3 | 32 | 3 | 7 | 77 | 79 |
| Muscle | Exercise intolerance (EI) | – | – | – | – | – | – | – | – | 14 | – |
| | Lipid accumulation in muscle (LAM) | – | – | 1 | – | – | – | – | – | 3 | – |
| | Muscle fatigue (MF) | – | – | – | – | – | – | – | – | 3 | 1 |
| | Muscle weakness (MW) | – | – | 1 | – | – | 3 | 3 | – | 13 | 1 |
| | Myopathy | – | – | – | 2 | – | – | 1 | – | 1 | – |
| Heart | Bradycardia | – | – | – | 5 | – | – | – | 2 | – | – |
| | Cardiomegaly | – | – | – | 3 | – | – | – | 1 | – | – |
| | Cardiomyopathy | – | – | – | – | – | – | – | – | 1 | 2 |
| | Heart Failure(HF) | – | – | – | 2 | – | – | 1 | – | – | 1 |
| | Heart hypoplasia (HHyp) | – | – | – | 1 | – | – | – | – | – | – |
| | Hypertrophic Cardiomyopathy (HCM) | – | 2 | 3 | 13 | – | – | 2 | 1 | – | 2 |
| | Isolated ventricular non-compactation (IVNC) | – | – | – | – | – | – | – | 1 | – | – |
| | Pericardial effusion | – | – | – | 1 | – | – | – | – | – | 1 |
| | Septal defects | – | – | – | 1 | – | 1 | – | – | 1 | 2 |
| | Tachycardia | – | – | – | 2 | – | – | – | – | – | – |
| | Valvulopathy (Vp) | 2 | – | – | – | – | – | – | – | – | – |
| | Diastolic dysfunction | – | – | – | 1 | – | – | – | – | – | – |
| Other clinical features | Dysmorphic features | – | – | – | 1 | – | 1 | 1 | 1 | 1 | 1 |
| | Edema | – | 2 | 11 | – | – | 2 | – | – | – | 16 |
| | Diabetes Mellitus (DM) | – | – | 6 | – | – | – | – | – | – | – |
| | Obesity | 2 | – | – | – | – | – | – | – | – | – |
| | Hypercholesterolemia | – | – | 2 | – | – | 1 | – | – | 1 | 1 |
| | Goiter | – | – | – | – | – | – | – | – | – | 2 |
| | Hypothyroidism | – | – | – | – | – | – | – | – | – | 1 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Respiratory distress (RD) | – | – | 2 | 14 | – | – | 2 | 2 | – | – |
| Neonatal Pneumonia | – | 1 | – | – | – | – | – | – | – | – |
| Respiratory failure (RF) | – | – | 5 | 5 | – | – | – | – | – | – |
| Apnea | – | – | 2 | 9 | – | – | – | 2 | – | – |
| Chronic Lung Disease (CLD) | – | – | 1 | – | – | – | – | – | – | – |
| Recurrent Respiratory infections | – | – | 1 | 1 | – | – | – | – | 1 | – |
| Lung Hypoplasia (LHyp) | – | – | – | – | – | – | 1 | – | – | – |
| Cyanosis | – | – | – | 2 | – | – | – | 1 | – | – |
| Hypertension (HT) | 1 | 1 | 1 | – | – | 1 | 1 | – | – | 10 |
| Hypotension | – | – | – | 2 | – | – | – | – | – | – |
| Livedo Reticularis (LR) | 2 | – | – | – | – | – | – | – | – | – |
| Dyspnea | – | – | – | – | – | – | – | – | – | 1 |
| Noctural enuresis | – | – | – | – | – | – | – | – | – | 2 |
| Polyuria | – | – | – | – | – | – | – | – | – | 1 |
| Polidypsia | – | – | – | – | – | – | – | – | – | 1 |
| Cholestatic Liver | – | – | 1 | – | – | – | – | – | – | – |
| Hepatomegaly | – | – | – | – | – | – | – | – | – | 1 |
| Hepatosteatosis | – | – | – | – | – | – | – | – | 1 | – |
| Liver failure (LF) | – | – | 3 | – | – | – | – | – | – | – |
| Reduced hematopoiesis in liver | – | – | – | – | – | – | – | 1 | – | – |
| Coma | – | – | – | 1 | – | – | – | – | – | – |
| Hypospadias | – | – | – | – | – | – | – | – | – | 1 |
| Lupus-like symptoms | – | – | – | – | – | – | – | – | – | 1 |
| Oligohydramnios | – | – | 3 | 1 | – | – | 1 | 3 | – | – |
| Recurrent Otitis | – | – | – | – | – | – | – | – | – | 1 |
| Splenomegaly | – | – | – | – | – | – | – | – | – | 1 |
| Crohn's Disease (CD) | – | – | – | – | – | – | – | – | – | 1 |

et al. 2006), *COQ5* (Malicdan et al. 2018), *COQ6* (Heeringa et al. 2011), *COQ9* (Danhauser et al. 2016; Duncan et al. 2009; Olgac et al. 2020) and *COQ8B* (Korkmaz et al. 2016; Lolin et al. 2017). Again, **hypotonia** is a feature that has been reported mainly in *COQ4* (Brea-Calvo et al. 2015; Chung et al. 2015; Ling et al. 2019; Salviati et al. 2012; Sondheimer et al. 2017), some *COQ2* patients (Diomedi-Camassei et al. 2007; Eroglu et al. 2018; Jakobs et al. 2013; Scalais et al. 2013) and the three published cases of *COQ7* (Freyer et al. 2015; Kwong et al. 2019; Wang et al. 2017). However, it has been observed in several other *COQ* patients (*PDSS2* (Iványi et al. 2018; López et al. 2006), *COQ9* (Danhauser et al. 2016) and *COQ8A* (Jacobsen et al. 2017; Mollet et al. 2008; Nair et al. 2018; Shalata et al. 2019)) with lower frequency. **Dystonia** has been observed in some *COQ8A* patients (Chang et al. 2018; Gerards et al. 2010; Horvath et al. 2012; Liu et al. 2014; Mignot et al. 2013; Mollet et al. 2008; Sun et al. 2019), but also in a lower proportion in *PDSS2* (Rahman et al. 2012; Rötig et al. 2000), *COQ2* (Jakobs et al. 2013), *COQ4* (Yu et al. 2019) and *COQ9* ones (Duncan et al. 2009). **Cerebellar ataxia** and **cerebellar atrophy** seem to be hallmarks for *COQ8A* patients since they have been reported in 70 and 63 out of the 77 identified subjects respectively. In almost half of the described cases these symptoms have been associated with intellectual disability and tremor, or seizures, dysarthria, dysmetria, saccadic eye movements, dystonia, dysdiadochokinesia or spasticity in other instances (Anheim et al. 2010; Barca et al. 2016a; Blumkin et al. 2014; Chang et al. 2018; Gerards et al. 2010; Hajjari et al. 2019; Hikmat et al. 2016; Horvath et al. 2012; Jacobsen et al. 2017; Kaya Ozcora et al. 2017; Lagier-Tourenne et al. 2008; Liu et al. 2014; Malgireddy et al. 2016; Mignot et al. 2013; Mollet et al. 2008; Nair et al. 2018; Pronicka et al. 2016; Shalata et al. 2019; Sun et al. 2019; Terracciano et al. 2012). The only family affected by a mutation in *COQ5* up to now also shows a cerebellar ataxic phenotype similar to *COQ8A* patients (Malicdan et al. 2018). Cerebellar ataxia has also been observed in some patients harboring pathological variations of *PDSS2* (Rahman et al. 2012; Rötig et al. 2000), *COQ4* (Bosch et al. 2018; Caglayan et al. 2019; Yu et al. 2019) and *COQ6* (Heeringa et al. 2011) genes. **Cerebellar hypoplasia** has been annotated in one third of the *COQ4* reported probands (Brea-Calvo et al. 2015; Chung et al. 2015; Yu et al. 2019), in one *COQ9* (Olgac et al. 2020) and in two *COQ8A* patients (Shalata et al. 2019). It should be noted that sometimes it could be challenging to differentiate cerebellar hypoplasia from cerebellar atrophy, especially if the progression of the latter cannot be proven by repeated MRI. **Epilepsy** is another CNS symptom associated with CoQ primary deficiency that has been mainly found in *COQ8A* (Gerards et al. 2010; Hikmat et al. 2016; Horvath et al. 2012; Malgireddy et al. 2016; Mignot et al. 2013; Mollet et al. 2008; Pronicka et al. 2016; Schirinzi et al. 2019; Terracciano et al. 2012), *COQ4* (Brea-Calvo et al. 2015; Caglayan et al. 2019; Chung et al. 2015; Ling et al. 2019) and *COQ2* (Diomedi-Camassei et al. 2007; Gigante et al. 2017; Scalais et al. 2013), but also in *COQ5* cases (Malicdan et al. 2018) and some *COQ8B* patients (Korkmaz et al. 2016; Vazquez-Fonseca et al. 2017). In some *COQ8A* (Hikmat et al. 2016; Horvath et al. 2012; Mignot et al. 2013; Mollet et al. 2008), *COQ2* (Diomedi-Camassei et al. 2007; Eroglu et al. 2018) and *COQ4* (Bosch et al. 2018) cases, epilepsy was accompanied by **stroke-like**

**episodes**, which contributed significantly to the deterioration of the neurological status and could explain the heterogeneity of the functional outcome among affected siblings (Mignot et al. 2013). **Spasticity** has been observed in some *COQ8A* (Anheim et al. 2010; Gerards et al. 2010; Horvath et al. 2012; Lagier-Tourenne et al. 2008; Mignot et al. 2013; Mollet et al. 2008) and *COQ4* (Bosch et al. 2018; Brea-Calvo et al. 2015; Caglayan et al. 2019; Ling et al. 2019; Yu et al. 2019) cases and a few *PDSS2* (Rahman et al. 2012; Rötig et al. 2000), *COQ5* (Malicdan et al. 2018) and *COQ7* (Wang et al. 2017) patients. **Intellectual disability** is a feature found in a variable number of cases but in patients of almost all the *COQ* genes (*PDSS1*, *PSDD2*, *COQ4* to *COQ7*, *COQ8A* and *B*). Some studies predict that specific *COQ2* variants increase susceptibility to adult-onset **multisystem atrophy** (MSA), particularly in East Asian population, but not in the Caucasian one (Katzeff et al. 2019; Mitsui et al. 2013; Ogaki et al. 2014; Procopio et al. 2019).

Peripheral nervous system (PNS) and sensory organs manifestations are less common, but related symptoms have been reported in several cases of primary deficiencies (Table 6.1). **Sensorineural hearing loss** (SNHL) is the most frequent PNS phenotype in *COQ6* patients, in whom it was associated with Steroid Resistant Nephrotic Syndrome (SRNS) in all cases (Cao et al. 2017; Gigante et al. 2017; Heeringa et al. 2011; Koyun et al. 2018; Park et al. 2017a, b; Yuruk Yildirim et al. 2019). Some probands with *PDSS1* (Mollet et al. 2007), *PDSS2* (Iványi et al. 2018; Rahman et al. 2012; Rötig et al. 2000), *COQ7* (Freyer et al. 2015; Kwong et al. 2019; Wang et al. 2017) and *COQ8A* (Lagier-Tourenne et al. 2008) pathological variants presented SNHL as well. Two siblings with *PDSS1* pathological variations were reported to suffer **peripheral neuropathy**, associated with **optic atrophy** and early-onset SNHL (Mollet et al. 2007). All the three *COQ7* patients presented SNHL, and in two cases, they also showed peripheral **polyneuropathy** and/or **visual dysfunction** (Freyer et al. 2015; Kwong et al. 2019; Wang et al. 2017). **Optic nerve atrophy** was reported in very few individuals with *PDSS1* (Mollet et al. 2007), *PDSS2* (Rötig et al. 2000), *COQ2* (Diomedi-Camassei et al. 2007) and *COQ6* (Park et al. 2017a) pathogenic variants. Other visual impairments such as **cataract** (Horvath et al. 2012; Rötig et al. 2000), **retinopathy** (Diomedi-Camassei et al. 2007; Jakobs et al. 2013), **retinitis pigmentosa** (Iványi et al. 2018; Korkmaz et al. 2016; Mitsui et al. 2013; Rötig et al. 2000) or **delayed visual maturation** (Sondheimer et al. 2017) have been observed in several cases of *COQ* patients.

Renal involvement has also been reported for several of the *COQ* patients (Table 6.1). S**teroid resistant nephrotic syndrome** (SRNS) is often found in primary CoQ deficiency patients, specifically in those with pathogenic variants of *PDSS2*, *COQ2*, *COQ6* and *COQ8B*. It has generally been reported in patients starting as proteinuria that, when untreated, evolved to **end-stage renal disease** (ESRD) during childhood (Sadowski et al. 2015).The majority of the identified *COQ2* patients displayed early-onset nephrotic syndrome, isolated or associated with encephalopathy and seizures (Bezdíčka et al. 2018; Diomedi-Camassei et al. 2007; Eroglu et al. 2018; Gigante et al. 2017; McCarthy et al. 2013; Mollet et al. 2007; Quinzii et al. 2006; Sadowski et al. 2015; Salviati et al. 2005; Scalais et al. 2013; Starr et al. 2018; Wu et al. 2019; Xu et al. 2018). Of note, the hallmark of *COQ6*

pathogenic variants is childhood-onset SNRS associated with SNHL (Cao et al. 2017; Gigante et al. 2017; Heeringa et al. 2011; Park et al. 2017a, b; Yuruk Yildirim et al. 2019). *COQ8B* patients mainly presented with an adolescence-onset SRNS due to focal segmental glomerulosclerosis, associated with oedema and hypertension, which generally progressed to ESRD (Ashraf et al. 2013; Atmaca et al. 2017; Feng et al. 2017; Hughes et al. 2017; Korkmaz et al. 2016; Lolin et al. 2017; Park et al. 2017b; Vazquez-Fonseca et al. 2017; Yang et al. 2018; Zhang et al. 2017). All the 7 reported patients of *PDSS2* (Iványi et al. 2018; López et al. 2006; Rahman et al. 2012; Rötig et al. 2000; Sadowski et al. 2015) and 1 of the three reported *PDSS1* patients (Vasta et al. 2012) presented SNRS as well. Two cases of *COQ2* and *COQ9* patients displayed a tubulopathy (Dinwiddie et al. 2013; Duncan et al. 2009).

Skeletal muscle manifestations are not common in these patients (Table 6.1). **Isolated myopathy** has not been found in patients with molecular confirmation of primary CoQ deficiency (Salviati et al. 2017). Most of the patients with a predominant muscular clinical phenotype are affected by secondary CoQ defects (see next section). **Muscle weakness** and **muscle fatigue** are the most frequent symptoms associated with this tissue in primary deficiencies, but in combination with other affectations and, in any case, reported in very few cases of each of the genes (Alcázar-Fabra et al. 2018).

Heart conditions are, instead, more frequent, being **hypertrophic cardiomyopathy** possibly a hallmark for *COQ4* patients with prenatal-onset, specifically (Brea-Calvo et al. 2015; Chung et al. 2015; Ling et al. 2019; Sondheimer et al. 2017; Yu et al. 2019). Some patients with *PDSS2* (Iványi et al. 2018; Rötig et al. 2000), *COQ2* (Desbats et al. 2015a, b; Dinwiddie et al. 2013; Scalais et al. 2013), *COQ7* (Freyer et al. 2015; Kwong et al. 2019), *COQ9* (Duncan et al. 2009) and *COQ8B* mutations (Atmaca et al. 2017; Vazquez-Fonseca et al. 2017; Zhang et al. 2017) also presented hypertrophic cardiomyopathy. Less frequently found cardiac manifestations include **valvulopathies** (Mollet et al. 2007), **heart hypoplasia** (Brea-Calvo et al. 2015), **septal defects** (Korkmaz et al. 2016; Li et al. 2018; Nair et al. 2018; Park et al. 2017a, b; Salviati et al. 2012), **heart failure** (Brea-Calvo et al. 2015; Chung et al. 2015; Korkmaz et al. 2016; Kwong et al. 2019), **bradycardia** (Brea-Calvo et al. 2015; Chung et al. 2015; Danhauser et al. 2016; Eroglu et al. 2018; Ling et al. 2019; Smith et al. 2018; Sondheimer et al. 2017) or **pericardial effusion** (Atmaca et al. 2017; Vazquez-Fonseca et al. 2017; Yu et al. 2019). It should be taken into account that some of these symptoms could be secondary consequences of a more general defect.

Other symptoms, more heterogeneous, have also been reported in some patients affected by mutations in all the different *COQ* genes. Among them, **respiratory distress** and **apnea** seem to be characteristic of *COQ4* patients (Brea-Calvo et al. 2015; Chung et al. 2015). **Oedema** is always present in cases with nephrotic syndrome, so it is more frequent in pathogenic variants of genes with renal involvement, such as *COQ8B*, *COQ2* and *COQ6*. For more details on this type of affections, refer to Table 6.1 and bibliography (Alcázar-Fabra et al. 2018).

It is essential to note that this non-detailed list of symptoms associated with variants of different *COQ* genes has a limited validity due to the small number of

patients described for each of them (Table 6.1). It should also be considered that the number of patients with pathological variations in the different genes varies widely, so the higher frequency found for some symptoms in some cases can be due to the sampling effect.

### *6.2.2 Biochemical Findings*

Biochemically, primary CoQ deficiency patients, in particular those with neonatal-onset, can show higher levels of lactate in plasma or serum (Table 6.3), although normal lactate levels do not exclude the possibility of a CoQ deficiency (Rahman et al. 2012). Skeletal muscle biopsies typically show decreased CoQ steady-state levels and reduced combined enzymatic activity of complexes I + III and/or II + III. Still, these tests are unable to differentiate between primary and secondary CoQ deficiencies (Salviati et al. 2017). *In vivo* assessment of CoQ biosynthetic rate is possible, by measuring the incorporation of a labelled CoQ precursor in skin fibroblast cultures. This technique allows to biochemically discriminate between primary and secondary deficiencies (Rodríguez-Aguilera et al. 2017). The biochemical determination of CoQ levels is a useful and quick strategy for the identification of primary CoQ deficiencies. However, only a genetic test (using next-generation sequencing (NGS) approaches, either genetic panels or whole-exome and–genome sequencing) will definitively determine the molecular diagnosis of these pathologies. It should bear in mind, that for each new potentially pathogenic variants of the *COQ* genes that are identified by NGS, a molecular validation is necessary.

In general, primary CoQ deficiencies respond quite positively to CoQ supplementation, but it is not always the case (Table 6.3). Still, an early definitive diagnosis is compulsory, in order to start the treatment as soon as possible to limit the damage that the condition could cause to tissues (Montero et al. 2008; Yubero et al. 2015). However, due to its hydrophobicity and possibly low bioavailability, new approaches are being developed to increase CoQ levels. Bypass treatments have been tested in cellular models of *COQ6* and *COQ7* patients. Vanillic acid and 2,4-HB, that are analogues of the head precursors have been shown to induce recovery of endogenous CoQ synthesis in *COQ6* and *COQ7*-defective cells respectively (Acosta-López et al. 2019; Freyer et al. 2015; Wang et al. 2017).

### *6.2.3 Pathogenesis*

The pathogenesis of primary CoQ deficiency is far from being simple, and our understanding is still scarce. It is highly probable that the reduced activity of the OXPHOS system and an increase of ROS would be crucial factors involved in the pathogenesis, but the role of CoQ in other mitochondrial processes, and even in other membranes, might also contribute to explain the origin of some affections in

primary deficiencies, at least partially. For example, de novo pyrimidine synthesis is impaired in CoQ deficiency, further contributing to the development of the disease (López-Martín et al. 2007). Moreover, sulphide oxidation pathway has been described to be tissue-specifically defective in primary CoQ deficiency, leading to an accumulation of $H_2S$, and thus, an alteration of protein S-sulphydrilation promoting changes in vasorelaxation, inflammation and ROS production. These changes have been proposed as another cause of pathogenesis in primary CoQ deficiencies (Quinzii et al. 2017). Besides, the disparity on the age of onset, the different tissues affected and the specificity of some of the symptoms associated to certain genes suggest that *COQ* genes may be involved in other processes and the molecular mechanism of the disease would also be dependent on these yet unknown functions. In any case, it should bear in mind that a complete picture is lacking since there are too few patients harboring mutations in each *COQ* gene.

## 6.3 Secondary CoQ Deficiency

Reduced levels of CoQ can also be found in patients due to conditions not directly related with CoQ biosynthesis malfunctioning, but with oxidative phosphorylation failure, other non-OXPHOS mitochondrial defects or even impairment of other non-mitochondrial processes (Yubero et al. 2016). The classification of patients with either primary or secondary CoQ deficiency strictly depends on their genetic analysis (Salviati et al. 2017).

Secondary CoQ defects are more common than the primary ones (Desbats et al. 2015a, b; Yubero et al. 2016). This fact could be explained by the variety of processes where CoQ is involved and, possibly, the existence of mechanisms for modulating CoQ levels in response to a failure in these processes.

Isolated myopathies presented as muscular weakness, hypotonia, exercise intolerance or myoglobinuria are commonly reported as muscular manifestations in diseases associated with secondary CoQ deficiencies, but also neurological decline and ataxia are also often reported (Sacconi et al. 2010; Salviati et al. 2017).

Analysis of CoQ levels in cohorts of patients affected by diverse OXPHOS pathologies shows that among the different mitochondrial defects, the most common conditions associated with secondary CoQ deficiency are depletion syndromes (Montero et al. 2013; Yubero et al. 2016). However, more studies on broader cohorts of patients affected by different conditions are needed to better understand if certain diseases are more likely to develop secondary deficiencies than others, as well as the underlying molecular mechanism involved. It has been hypothesized that severe mitochondrial deficiencies would cause secondary CoQ deficiency by inhibiting the maturation of COQ proteins in the mitochondria, although further research still needs to be done (Yen et al. 2020). Very interestingly, comparative omic studies performed in mouse models of OXPHOS dysfunction caused by nuclear-encoded essential factors for mtDNA maintenance showed association with secondary CoQ deficiency (Kühl et al. 2017). Other mitochondrial conditions are associated, at a

different degree, with a reduction in CoQ levels. For example, mutations in *MT-TL1*, causing MELAS syndrome, and *MT-TK*, causing MERFF syndrome, were also associated to a secondary CoQ deficiency in skeletal muscle (Cotán et al. 2011; Sacconi et al. 2010). Other examples are: mutations in the mitochondrial chaperone *BSC1L* leading to complex III deficiency, associated with isolated mitochondrial encephalopathy (Fernandez-Vizarra et al. 2007); complex I deficiency caused by mutations in *NDUFS4*, associated to multifocal dystonia and Leigh syndrome (Bris et al. 2017; Ortigoza-Escobar et al. 2016); or combined OXPHOS defects caused by mutations in *EARS2*, which encodes for the mitochondrial aminoacyl-tRNA synthetase specific for glutamate, causing either leukoencephalopathy or multisystem fatal infantile disease (Talim et al. 2013; Taskin et al. 2016).

The study of mouse models is instrumental in predicting secondary CoQ deficiencies (Kühl et al. 2017), which can be exploited for human molecular diagnosis. One example is *Parl*$^{-/-}$ mouse model: *PARL* (coding for Presenilin Associated Rhomboid Like protein) is the only mitochondrial member known from the rhomboid family (Spinazzi and De Strooper 2016), a conserved group of intramembrane proteases. *Parl*$^{-/-}$ mouse model shows a phenotype similar to Leigh syndrome, with a severe complex III defect caused by the disappearance of mature Ttc19, a factor required for complex III stability (Bottani et al. 2017). Moreover, *Parl*$^{-/-}$ brain mitochondria show a significant decrease of CoQ biosynthesis associated with a reduction of Coq4 and other Coq peptides, independent to TTC19 defect. This suggests that the protease could be involved in CoQ biosynthesis complex regulation, compensating for complex III defect (Spinazzi et al. 2019). This adaptive mechanism, which should aim a balanced respiratory chain, would try to increase the survival of cells and tissues in CoQ deficiency conditions (Fernández-Ayala et al. 2013). The same rationale of a compensatory mechanism could be applied to the two cases with pyruvate dehydrogenase complex mutations showing CoQ overproduction (Asencio et al. 2016).

CoQ secondary deficiency has also been associated with non-OXPHOS gene defects, but the mechanisms involved are still elusive (Yubero et al. 2016). A pediatric patient with a defective GLUT1 transporter caused by a heterozygous variant in the *SLC2A1* gene showed significantly reduced CoQ levels (Yubero et al. 2014). However, this is not a common feature in GLUT1 deficiency syndrome (Barca et al. 2016b), indicating that possibly other factors would be involved in the CoQ defect in this patient.

CoQ is reduced by *ETFDH*-encoded flavoprotein-ubiquinone oxidoreductase as a critical step of mitochondrial fatty acid β-oxidation (Bentinger et al. 2010) (Fig. 6.1b). Mutations in this enzyme, and in the electron transfer flavoprotein, cause multiple acyl-CoA dehydrogenation deficiency (MADD)–also known as glutaric acidemia II or glutaric aciduria II- which is associated with decreased levels of CoQ (Buján et al. 2014; Gempel et al. 2007). It has been proposed that CoQ is distributed in specific pools within the mitochondrial inner membrane, which are dedicated to either NADH or $FADH_2$-mediated CoQ reduction in the respiratory chain (Lapuente-Brun et al. 2013). Specific modulation of CoQ levels in response to the decrease of the enzyme that reduces it would explain the secondary deficiency

associated with this condition. This could explain some of the secondary deficiencies associated with *ETFDH* mutations. However, it should be noted that not all MADD patients have CoQ deficiency (Liang et al. 2009; Wen et al. 2013).

Cerebellar ataxia has been found in some cases of primary CoQ deficiency, which can be explained by the high-energy requirement of the cerebellum (Salviati et al. 2017)–see previous section-. Mutations in other genes that cause cerebellar ataxia can also induce secondary CoQ deficiency. Mutations in *APTX* gene, for example, which encodes for the single-stranded DNA repair aprataxin, cause ataxia with ocular motor apraxia (Date et al. 2001; Moreira et al. 2001) and induce CoQ deficiency in muscle and fibroblasts of these patients (Quinzii et al. 2005; Yubero et al. 2016).

*ANO10* gene encodes for a member of the anoctamin family of transmembrane proteins with calcium-activated chloride channel activities, whose mutations cause spinocerebellar ataxia associated to CoQ deficiency in skeletal muscle, plasma and cerebrospinal fluid (Balreira et al. 2014; Chamard et al. 2016; Nanetti et al. 2019).

Also, it has been shown that patients with Friedrich ataxia can show a decrease of CoQ content in skeletal muscle as a consequence of the mutation in *FXN* gene, which encodes for frataxin, a protein regulating iron transport into mitochondria (Yubero et al. 2016).

A secondary reduction of CoQ levels also seems to be associated with ageing (Hernández-Camacho et al. 2018). Some drugs, such as statins for hypercholesterolemia treatment, are reported to induce myopathy with secondary CoQ deficiency, since both cholesterol and CoQ share a part of their biosynthetic pathways (Marcoff and Thompson 2007; Uličná et al. 2012).

The mechanisms explaining CoQ secondary defects are still elusive. Several hypotheses, which depend on the specific primary mitochondrial or non-mitochondrial defect, have been proposed: (i) oxidative stress induced by non-functional respiratory chain could induce an increase in CoQ degradation rate; (ii) interference of the signaling pathways involved in CoQ biosynthesis could cause a decrease in CoQ biogenesis; (iii) a destabilization of the CoQ biosynthetic complex could be induced in response to the primary change or (vi) a general deterioration of mitochondrial function could be responsible for the reduced CoQ levels (Desbats et al. 2015a, b; Yubero et al. 2016).

Of course, the original pathology will influence the particular symptoms associated with secondary CoQ deficiencies and, probably, the lack of CoQ could even potentiate them (Desbats et al. 2015a, b). In fact, many of these patients partially improve their condition by CoQ supplementation, which supports the importance of an early diagnosis also in these cases (Quinzii and Hirano 2011).

## 6.4 Concluding Remarks

Next-generation sequencing approaches have allowed in the last years the genetic diagnoses of an increasing number of patients showing decreased levels of CoQ in tissues due to defects in CoQ biosynthesis pathway. Primary CoQ deficiencies are those caused by mutations in the *COQ* genes. Instead, secondary defects are caused by mutations in genes not directly involved in CoQ synthesis. Biochemically, both primary and secondary deficiencies present with decreased levels of CoQ in tissues. The only reliable way to distinguish primary from secondary CoQ deficiency is genetic analysis (Salviati et al. 2017).

The difficulties in the diagnosis and the proper addressing to the appropriated health service relay in the wide heterogeneity of clinical manifestations and the low number of patients identified. Here we present an overview of the different manifestations associated with the various mutations in the *COQ* genes in primary deficiencies and their frequency to serve as a diagnostic guide for clinicians who face the challenge of diagnosing rare mitochondrial diseases. A more significant coordinated effort at the level of the translational research is necessary to expand the cohorts of patients affected by mutations in the different *COQ* genes in order to better define the clinical spectrum associated with each genetic defect.

The variety of mitochondrial disorders showing a secondary deficiency and the comprehensive studies in mice models associated with secondary CoQ defects, clearly show the complexity of the pathogenic process of these mitochondrial conditions and the wide range of tissues, organs, and cellular functions affected. Specific efforts need to be done to better understand the–probably very diverse- pathomechanisms underlying CoQ reduction in other diseases.

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# Chapter 7
# Cytochrome *c* Defects in Human Disease

**Leonardo Salviati**

**Abstract** Cytochrome *c* is a small soluble heme protein, encoded by the *CYCS* gene, which carries out several crucial functions in the cells. It is one of the two electron carriers in the mitochondrial respiratory chain, it is required for the mitochondrial importation of proteins with the CX3C and CX9C motifs through the MIA40/ERV1 pathway, it can both scavenge ROS or promote ROS production through p66$^{shc}$, it catalyzes the oxidation of cardiolipin, and it is an essential player of the intrinsic pathway of apoptosis. Autosomal dominant mutations of *CYCS* have been reported in four families, and cause a peculiar clinical picture characterized by mild thrombocytopenia, with otherwise normal platelets, and none of the classical manifestations of respiratory chin defects. Functional and structural studies indicate that these mutations, cause a relatively minor respiratory defect, but increase the susceptibility to apoptosis in affected cells. The precise mechanism that links the proapoptotic effect to the abnormal platelet production is not completely clear.

*CYCS* mutations represent a novel paradigm of mitochondrial disease, in which it is the perturbation of the apoptotic pathway, rather than the impairment of energy production, that is crucial in the pathogenesis of the clinical manifestations.

## 7.1 Introduction

The mitochondrial respiratory chain is comprised of five enzymatic complexes and two electron carriers, coenzyme Q and Cytochrome *c* (Cyt*c*). To date, defects of all these components have been reported in human patients, the most recent are mutations in cytochrome *c* (Morison et al. 2008).

Cyt*c* is a small soluble heme protein located in the mitochondrial intermembrane space, loosely associated with the inner membrane (Huttemann et al. 2011). It is highly conserved throughout evolution (the yeast and human proteins share 64% identity and 77% similarity). While it was originally identified as the electron

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carrier between respiratory chain complexes III and IV, subsequent research has shown that it plays essential role in other crucial processes such as apoptosis, mitochondrial protein import, and ROS production and scavenging.

## 7.2 Gene Stucture

Cyt*c* is encoded by the *CYCS* gene on chromosome 7p15.3. The gene spans approximately 7 kb and is divided in three exons. Exon 1 is non-coding, and the 5′ untranslated region (UTR) extends to exon 2, where the initial ATG is found. The open reading frame encodes a 105 amino acid protein. Exon 3 contains a very long 3'UTR, of more than 5 kb, which contains a canonical AAATAA polyadenylation consensus at the end. The mature protein lacks the initial methionine, and has a molecular mass of 11,458 Da (Evans and Scarpulla 1988; Zhang and Gerstein 2003).

This fact has generated some confusion in the nomenclature. The biochemical literature refers to specific amino acid residues as in the mature protein, while recent molecular genetic articles follow the recommendations of the Human Genome Variation Society (HGVS) based on the cDNA sequence. Therefore, glycine 41 of the legacy nomenclature correspond to glycine 42 according to HGVS rules. In this chapter, we will follow the HGVS rules for the nomenclature of aminoacidic residues. Besides the *CYCS* gene, the human genome harbors at least 49 pseudogenes, some of which are related to the murine testis-specific isoform (which has no active counterpart in humans and will not be discussed in this work) (Zhang and Gerstein 2003).

## 7.3 The Protein

Cyt*c* is synthesized in the cytoplasm as apo-cytochrome *c* and is then delivered to the mitochondrial intermembrane space using a non-canonical pathway (Dumont et al. 1988). Compared to other nuclear encoded mitochondrial proteins, it does not contain a classical mitochondrial targeting sequence, nor it requires a membrane potential across the inner mitochondrial membrane (Zimmermann et al. 1981). Apo-cytochrome *c* associates, and reversibly crosses the outer mitochondrial membrane (Mayer et al. 1995). Once inside the intermembrane space, it binds to Holocytochrome *c* Synthase (HCCS – also known as Heme Lyase), which catalyzes the covalent binding of the heme group to Cyt*c*. Apparently, it is the binding to HCCS itself, rather than the attachment of the heme group, that is critical for the mitochondrial import independently from the heme lyase activity (Indrieri et al. 2013). Cyt*c* is one of the first proteins for which the crystal structure was solved (Dickerson et al. 1967). It is comprised of four alpha helixes, a short anti-parallel beta sheet, and three omega loops (Krishna et al. 2003). Cyt*c* contains a characteristic CXXCH heme binding motif (aa. 15–19). The heme group is hosted into a

highly hydrophobic pocket and is covalently bound to cysteine 15 and cysteine 18, while histidine 19 and methionine 81 coordinate the iron ion of the heme group. The redox status of the iron ion does not affect binding, making cytochrome *c* a good electron transporter (Lei and Bowler 2019).

## 7.4 Physiological Functions

### *7.4.1 Bioenergetics*

Cyt*c* plays a central role in several crucial cellular functions (Huttemann et al. 2011) (Fig. 7.1). Its main physiological role (and the first to be identified) is to transfer electrons from respiratory chain complex III to complex IV. Each Cyt*c* molecule accepts a single electron from the $bc_1$ complex and delivers it to Cytochrome *c* Oxidase (COX). COX then transfers this electron to molecular oxygen. Four reduced Cyt*c* molecules are required to reduce one $O_2$ molecule. The $\Delta G^o$ of the reaction is reaction is −100 kJ/mol, making it essentially and irreversible reaction under physiological conditions (Hinkle et al. 1991). It has been proposed by several groups that this could be the rate limiting step of the electron transfer chain (Huttemann et al. 2011).

Besides its direct role in electron transfer, Cyt*c* is also an important regulator of oxidative phosphorylation with different mechanisms. First, it can bind ATP, and binding results in decreased electron transfer (Craig and Wallace 1995). Second, it is the target of both tyrosine and serine/threonine phosphorylation. At least two tyrosines, residues 49 and 98 appear to be phosphorylated in vivo, and this causes an inhibition of electron transfer to COX (Lee et al. 2006). Cyt*c* is also reported to

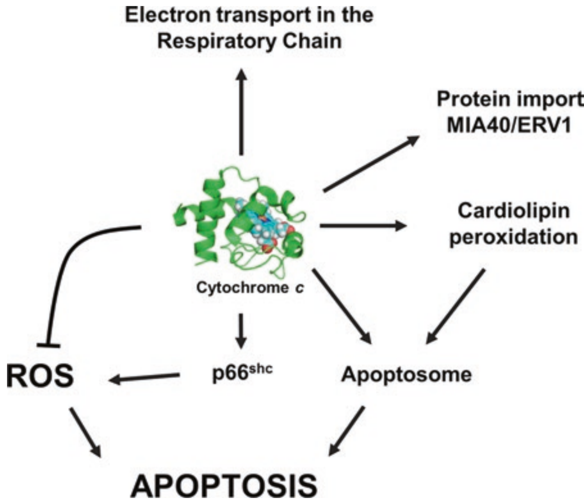


**Fig. 7.1** The diverse (and interconnected) functions of Cytochrome c in cellular homeostasis

be phosphorylated in vivo on threonine 29 and serine 48, and this also results in inhibition of electron transfer and, in the case of serine 48, of caspase3 activation (Mahapatra et al. 2017; Kalpage et al. 2019). Overall, phosphorylation appears to be a protective mechanism because it avoids mitochondrial hyperpolarization, which may trigger ROS production and apoptosis.

### *7.4.2 Mitochondrial Protein Import*

A subset of mitochondrial proteins are not imported through the classical pathway and lack a N-terminal targeting sequence. They are characterized by the presence of the CX3C or CX9C motifs and are imported through the MIA40-ERV1 pathway (Riemer et al. 2011). An example is the COX assembly factor COX17 (Mesecke et al. 2005). CX3C and CX9C proteins are imported into the intermembrane space by outer membrane translocases in the reduced form. MIA40 is an oxidoreductases that catalyzes the formation of disulfide bonds among the conserved cysteine, promoting their correct folding, and effectively trapping these proteins in the intermembrane space (Peleh et al. 2016). Reduced MIA40 is then oxidized by ERV1, which in turn transfers the electrons to Cyt*c* (Bihlmaier et al. 2007).

### *7.4.3 ROS Production and Scavenging*

Cyt*c* can both promote ROS formation an act as ROS scavenger. As a scavenger, it can react with both superoxide anion (Korshunov et al. 1999) and hydrogen peroxide (Wang et al. 2003). Under specific stress conditions, Cyt*c* can also promote ROS formation by transferring electrons to p66$^{shc}$ (Giorgio et al. 2005), a genetic determinant of life span in mammals, that increases production of mitochondrial ROS which may act as signaling molecules for apoptosis induction.

Cytochrome *c* can also specifically catalyze the $H_2O_2$-dependent peroxidation of cardiolipin (Belikova et al. 2006; Kagan et al. 2005), a crucial step in the initiation of apoptosis (see below).

### *7.4.4 Apoptosis*

Cyt *c* plays a fundamental role in programmed cell death through the intrinsic pathway (Liu et al. 1996). Upon proapoptotic stimuli, Cyt*c* is released into the cytoplasm where it binds APAF1 (Zou et al. 1997), leading to the formation of the apoptosome, a dATP-dependent process. The apoptosome then activates the caspases cascade (Li et al. 1997). Cells lacking Cyt*c* are resistant to stimuli that activate the intrinsic (and to a lesser extent the extrinsic) pathway of apoptosis (Vempati

et al. 2007). The cardiolipin peroxidase activity of Cyt*c* is critical for modulating Cyt*c* release. In fact, 15–20% of Cyt*c* molecules are tightly bound to cardiolipin on the mitochondrial inner membrane (Schlame et al. 2000; Belikova et al. 2006). During the initial phases of apoptosis there is an increase in the amount of cardiolipin in leaflets of the inner membrane facing the inter-membrane space which further facilitates the formation of Cyt*c*-cardolipin complexes (Tyurina et al. 2014). Cardioipin-binding in the presence of $H_2O_2$ enhances over 50-fold the peroxidase activity of Cyt*c* (Vladimirov et al. 2006) thus favoring the formation of oxygenated cardiolipin species which have no affinity for Cyt*c*. Thus, Cyt*c* is detached from the membrane and can be released in the cytosol (Kagan et al. 2005).

## 7.5 Cytochrome *c* in Human Disease

### *7.5.1 Clinical Phenotype of CYCS Patients*

Among the components of the mitochondrial respiratory chain, Cyt*c* was the last to be involved in human disease, and it is the rarest defect, with only 4 families reported to date (Morison et al. 2008; De Rocco et al. 2014; Johnson et al. 2016; Uchiyama et al. 2018). The phenotype is remarkably similar in all families and consists a mild thrombocytopenia without increased bleeding tendency, and normal platelet size and morphology. The trait is transmitted with an autosomal dominant fashion and is known as autosomal dominant nonsyndromic thrombocytopenia-4 (THC4) (MIM #612004), or as thrombocytopenia Cargeeg type. Apart from platelets, no other cell type is apparently affected: neither other blood cell types, nor other tissues.

### *7.5.2 Molecular Pathology and Pathophysiology*

The four reported families harbor different mutations (see Table 7.1) which affect residues that are virtually invariant in eukaryotes. It is interesting to note that three out of four affect residues are localized in the 40–57 omega loop, which surrounds the heme group (Fig. 7.2).

**Table 7.1** Naturally occurring *CYCS* mutation in human patients with THC4

| cDNA | Protein (HGVS) | Legacy name |
|---|---|---|
| c.124G > A | p.Gly42Ser | G41S |
| c.145T > C | p.Tyr49Ser | Y48H |
| c.155C > T | p.Ala52Val | A51V |
| c.301_303del | p.Lys101del | K100del |

HGVS nomenclature is based on reference sequence NM_018947.5

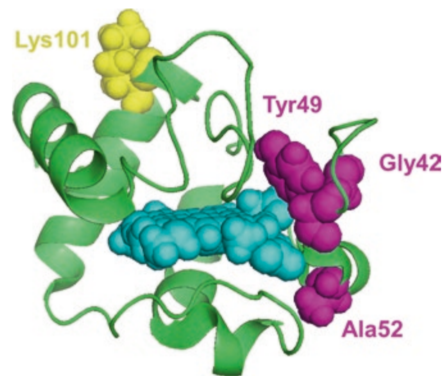


**Fig. 7.2** Three dimensional structure of cytochrome c. Glycine 42, tyrosine 49, and alanine 52, located in the 40–57 omega loop are colored in magenta, lysine 101, located on the C-terminal alpha helix is in yellow, the heme group is in cyan

All mutations have been validated using direct assays on patient platelets, model systems such as yeast or murine *Cycs*$^{-/-}$ cells, or by assays on recombinant proteins. The p.Gly42Ser and p.Tyr49His substitutions appear to affect both respiration (in yeast and murine cells) as well as staurosporine-induced apoptosis (De Rocco et al. 2014). The effect on apoptosis was visible also when these mutants were expressed in wild type cells, confirming a dominant negative or (more likely) a gain of function mechanism, rather than haploinsufficiency. Both mutant proteins appeared to be stable when expressed in cells (De Rocco et al. 2014), although mass spectrometry analysis of patients' platelets showed a reduced ratio between the mutant and wild type protein (Morison et al. 2008). The precise mechanism that determine the enhanced proapoptotic activity of these mutants is still debated. Initially it has been suggested that the p.Gly42Ser mutation alters the electronic structure of the heme cytochrome increasing the electron self-exchange rate and accelerating generation of the oxidized form of cytochrome *c* which promotes apoptosis (Liptak et al. 2011). Subsequent works on recombinant proteins have shown that these two mutants, as well as the p.Ala52Val change, enhance peroxidase activity of affected Cyt*c* molecules by altering the conformation of the 40–57 omega loop and heme coordination (Josephs et al. 2014; Deacon et al. 2017, 2020; Lei and Bowler 2019). The increases spontaneous peroxidase activity could promote apoptosis by increasing cardiolipin oxidation.

The p.Lys101del mutation instead affects the C-terminal domain of the protein and the affected residue is relatively distant from the heme-binding pocket (Fig. 7.2). The information on this allele is scarcer since it has been characterized only by expression in yeast, where it caused reduced respiration and impaired stability of the protein (Uchiyama et al. 2018). The authors concluded for a loss of function effect. However, this hypothesis is unconvincing since the other *CYCS* mutations clearly act through a dominant negative/gain of function mechanism (see above) and the patients display essentially the same phenotype. Further work is required to assess the effects of this mutation in mammalian cells.

Despite all these information, pathophysiology of THC4 is still unclear. The most puzzling issue is the fact that Cyt*c* is a ubiquitous protein, but only the

megakaryocyte lineage seems to affected by the disease. Moreover, we still lack a clear picture of the events that lead to impaired platelet formation.

The reported *CYCS* mutations clearly have pleiotropic effects, and it is difficult to distinguish which among the reported functional alterations identified in mutant Cyt*c* proteins are relevant for the development of clinical manifestations. Given the peculiarity of the clinical manifestations of THC4 compared to other defects of respiratory chain components, the attention of researchers was immediately focused on the perturbation of apoptosis (Morison et al. 2008). Apoptosis is a key event for platelet formation by megakaryocytes. It was initially hypothesized that the mutants induce premature apoptosis in these cells causing proplatelet release in the bone marrow, rather than in the bloodstream, and resulting in ineffective production of otherwise normal platelets (Morison et al. 2008). More recent work has however challenged this view. Cultured megakaryocytes derived from THC4 patients form proplatelets normally. In contrast, these patients display platelet-like structures with altered morphology within the extravascular bone marrow space, suggesting the presence of an abnormal, proplatelet-independent, platelet release process. The failure to recapitulate this mechanism in vitro suggests that pathological platelet release is not an intrinsic property of megakaryocytes carrying the *CYCS* mutation, but depends also on interactions between these cells and the bone marrow environment (Ong et al. 2017).

The actual contribution of the bioenergetics defect to the pathogenesis of this condition is still not clear. The defect in mammalian cells is relatively mild, considering the fact that experiments were conducted in cells "homozygous" for the mutation (they were expressing only the mutant allele) while in patients the mutation is present in the heterozygous state. Moreover, the absence of any of the symptoms related to energy depletion in the patients, also argues against a primary role of impaired electron transfer in the pathogenesis of THC4.

### 7.5.3 HCCS Defects

HCCS is the only known assembly factor for Cyt*c*, and is required for the attachment of the heme group to the apoprotein. However, it is not specific for Cyt*c* but it is required also for the assembly of cytochrome $c_1$, a component of complex III (Indrieri et al. 2013). Mutations in *HCCS* cause microphthalmia with linear skin defects, a rare condition presenting with microphthalmia, microcephaly, characteristic skin lesion consisting of areas of aplastic skin that heal with age to form hyperpigmented areas, and other congenital anomalies (Prakash et al. 2002; Wimplinger et al. 2006). HCCS impairment causes a defect in both Cyt*c* and in complex III, and activates a non-canonical cell death pathway in the brain and eyes that explains the peculiar phenotype of the patients (Indrieri et al. 2013). However, mutations in *NDUFB11,* which encodes a subunit of complex I, and in *COX7B*, which encodes a subunit of complex IV, cause similar phenotypes, suggesting that the abnormal activation of the cell death pathway is not specific for the Cyt*c* defect.

## 7.6 Conclusion

Mutations in *CYCS* expand the clinical spectrum of mitochondrial diseases and underscore the complexity of the pathogenic mechanisms that are associated these conditions, which again reflect the diverse functions of mitochondria in cellular homeostasis. *CYCS* mutations represent a novel paradigm of mitochondrial disease, in which it is the perturbation of the apoptotic pathway, rather than the impairment of energy production, that is crucial in the pathogenesis of the clinical manifestations.

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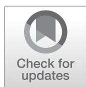
# Chapter 8
# Biochemical Diagnosis of Mitochondrial Disorders




**Delia Yubero, Raquel Montero, and Rafael Artuch**

**Abstract** Despite the enormous advances of the last decade in high throughput technologies, such as in next generation sequencing, diagnosis of mitochondrial oxidative phosphorylation (OXPHOS) diseases is still challenging. Clinical phenotypes are commonly incomplete, especially in paediatric patients. Currently available biomarkers lack both sensitivity and specificity, although the identification of two cytokines (FGF21 and GDF15) as valuable biomarkers has improved diagnostic performance in OXPHOS disease patients.

OXPHOS diseases display unique biological features that have a deep impact on the diagnostic workflow. No accumulation of specific metabolites is expected, and in the milder clinical forms, the biomarkers that are available for diagnosis may show normal results. Furthermore, OXPHOS can be modulated by both genetic and environmental conditions, but in terms of disturbances to biomarkers, the consequences may be indistinguishable. In this chapter, we review the advantages and limitations of the currently available biomarkers for the diagnosis of OXPHOS disease.

## 8.1 Introduction

Diagnosis of mitochondrial disorders is still challenging (Couser and Gucsavas-Calikoglu 2017). Although the advent of next generation sequencing techniques is changing the diagnostic paradigm (Zhang et al. 2012; Cui et al. 2013), a remarkable percentage of patients remain without a diagnosis. This is due to the complex and intricate metabolic pathways that occur in mitochondria. This complexity is at a maximum in the oxidative phosphorylation system (OXPHOS), the final metabolic step that synthesises ATP from the catabolism of most biomolecules. OXPHOS is the only metabolic pathway controlled by two genomes, i.e. the nuclear and the

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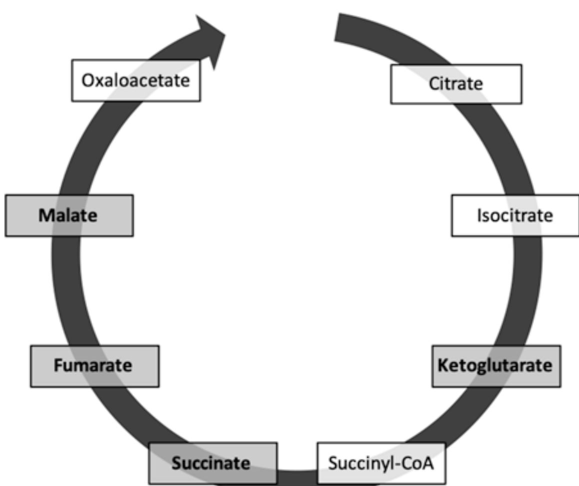


**Fig. 8.1** Schematic representation of Krebs cycle and mitochondrial respiratory chain pathways. Main biomarkers for the study of mitochondrial disorders are highlighted in bold. The imbalance between NAD/NADH causes the accumulation of most metabolites, such as lactate

mitochondrial genomes (Elliott et al. 2008). This two-genome combination has some immediate consequences, such as the lack of specificity and sensitivity of the currently available biomarkers for patient diagnosis and follow-up (Haas et al. 2007). OXPHOS is a metabolic pathway that is critical for life, and thus, complete metabolic blocks are not expected (they would be lethal) and partial deficiencies are responsible for clinical phenotypes. Thus, accumulation of target metabolites in body fluids (which is the first diagnostic tool for hereditary metabolic conditions) is sometimes not present. Moreover, since OXPHOS is a metabolic pathway where most catabolic routes converge, no specific metabolites that could facilitate candidate gene screening are accumulated.

In this chapter, we will review the state of the art of the different biomarkers available for diagnosis of patients with OXPHOS diseases. In Fig. 8.1, the metabolic relationships among the different biomarkers mentioned in this chapter and the OXPHOS system are depicted.

## 8.2 Biochemical Testing

### *8.2.1 First-Line Investigations — Routine Analyses*

When evaluating a patient with suspected OXPHOS disease, it is important to analyse different routine laboratory parameters that will provide key information about the potential involvement of different organs in the disease (Couser and Gucsavas-Calikoglu 2017). Thus, assessment of the acid–base equilibrium with pH and ion measurements will determine if metabolic (lactic) acidosis is present, or if only lactate is increased, with no metabolic acidosis. Muscle, kidney and liver functions are frequently impaired in OXPHOS deficiency patients and they should be

routinely evaluated for a deep definition of the clinical phenotype (AST, ALT, CPK, urea, creatinine, proteins, etc.). Blood count analysis is essential, for example to identify patients with Pearson syndrome, where pancytopenia is present (Sato et al. 2015). Other laboratory parameters can also be analysed depending on the clinical phenotype. In conclusion, these initial tests are essential for the whole diagnostic process, since the above-mentioned biomarker analyses are automated, simple and cost-effective. More importantly, they may be extremely helpful for good phenotypic definition of patients, which is essential for proper interpretation of next generation sequencing (NGS) driven data.

## *8.2.2 Specialised Analyses*

### 8.2.2.1 Lactate and Pyruvate

Lactate determination can be considered as a first-line diagnostic tool, since it is a simple, fully automated biochemical test that is available in all clinical laboratories (Couser and Gucsavas-Calikoglu 2017). The rationale for increased lactate in patient with OXPHOS defects is that the NADH/NAD ratio is high due to a decreased rate of oxidation of NADH in the mitochondrial respiratory chain. Thus, pyruvate is preferentially reduced to lactate in order to recycle NADH to NAD, facilitating glucose oxidation by the anaerobic pathway (Fig. 8.1). Sample collection is critical to avoid misleadingly inflated blood lactate values (citrate tubes should be used to stop glycolysis in erythrocytes after blood withdrawal). An increased blood lactate level is a hallmark of OXPHOS disease and the cut-off value is around 2 mmol/L. However, a finding of increased blood lactate in isolation, especially if moderate, does not necessarily mean that the patient has either lactic acidosis or an OXPHOS disorder. In Table 8.1, different pre-analytical situations and non-OXPHOS diseases that may lead to increased blood lactate values are detailed. As we noted in the introduction, blood lactate concentrations may sometimes be normal in OXPHOS disease patients, especially in those with milder forms. Thus, although lactate is considered a useful diagnostic biomarker, by itself it is neither sensitive nor specific enough to diagnose OXPHOS diseases.

In new-borns, discrimination between environmental and OXPHOS genetic diseases leading to massive hyperlactacidaemia is challenging. For example, it is not always easy to differentiate a severe neonatal asphyxia leading to cerebral palsy and an OXPHOS disease. In our experience, patients with asphyxia, even in the most severe cases, are usually able to restore their lactate values to normal when therapeutic measures are started, while in new-born patients with severe OXPHOS defects, lactic acidaemia is constant and in most cases increases with the evolution of the disease, regardless of the treatments applied.

Lactate can also be measured in urine and cerebrospinal fluid (CSF). While urine lactate determination has the same limitations as in blood samples (lack of sensitivity and specificity), increased CSF lactate levels are usually strongly supportive of

**Table 8.1** Environmental and genetic (non-OXPHOS) conditions leading to increased blood lactate values

| **Pre-analytical factors** |
|---|
| Prolonged tourniquet |
| Inadequate specimen handling (anticoagulants) |
| Crying during blood withdrawal (paediatric patients) |
| **Environmental conditions** |
| Infections/sepsis |
| Seizures |
| Hypoxia/ischaemia/poor perfusion (cardiopulmonary diseases, anaemia, etc.) |
| Diabetes mellitus |
| Neoplasms |
| Liver/renal failure |
| Vigorous exercise |
| Different pharmacological treatments |
| **Genetic conditions (non-OXPHOS diseases)** |
| Pyruvate and Krebs cycle defects |
| Glycogen storage and gluconeogenesis diseases |
| Inborn errors of intermediary metabolism (organic acidaemias) |
| Mitochondrial free fatty acid oxidation defects |
| Genetic diseases of the liver |

an OXPHOS defect, since most of the above-mentioned pre-analytical and environmental conditions do not usually modify CSF lactate values (Morava et al. 2006). Only stroke/epilepsy (although not related to mitochondrial disorders) and central nervous system infections may otherwise increase CSF lactate concentrations. Support for this statement is found in the international criteria for OXPHOS disease diagnosis (Morava et al. 2006), where increased brain CSF lactate (measured spectrophotometrically or by magnetic resonance with spectroscopy (MRS)) is a valuable diagnostic criterion. In fact, MRS-quantified lactate levels in brain tissue is probably a better diagnostic marker for OXPHOS disease than biochemical quantification of lactate in CSF. Thus, it may be that in the future, MRS becomes the standard diagnostic tool for OXPHOS disorders.

As regards pyruvate, its analysis requires special processing and collection tubes containing perchlorate and immediate placement on ice (Artuch et al. 1995). Pyruvate determination is not available in most laboratories, although the analysis is not methodologically complex (spectrophotometric measurement, similar to that of lactate). The blood lactate/pyruvate ratio reflects the NADH/NAD redox status, and when increased, it is indicative of OXPHOS dysfunction. Once again, this type of measurement cannot discriminate between an environmental (for example, hypoxia) or a genetic OXPHOS defect. Probably, the most interesting contribution

of pyruvate as a biomarker is when it is elevated leading to a low lactate/pyruvate ratio. This finding is strongly suggestive of a genetic defect in pyruvate metabolism (pyruvate decarboxylase and dehydrogenase deficiencies). The physiological lactate/pyruvate ratio is usually between 12 and 25 (Couser and Gucsavas-Calikoglu 2017).

#### 8.2.2.2 Amino Acid Profile

Amino acids can be analysed using different chromatographic techniques and their study has the advantage that most of them can be measured simultaneously in a single analytical run. The hallmark amino acid for OXPHOS disease diagnosis is alanine. Alanine is in equilibrium with pyruvate after the aminotransferase reaction catalysed by ALT. Although elevations in alanine concentration greater than 450 mM are suggestive of mitochondrial dysfunction (Couser and Gucsavas-Calikoglu 2017), this is nonspecific and not diagnostic for an OXPHOS defect, as with increased lactate values. However, the simultaneous measurement of lactate and alanine may increase its diagnostic power, since it can rule out situations where lactate is artefactually elevated, such as when blood withdrawal is difficult (a real problem for paediatric care) or other pre-analytical factors. In other words, if a real hyperlactacidaemia exists, alanine values should be concomitantly elevated. Other amino acids can provide useful information for particular diseases, such as the low plasma citrulline values observed in MELAS patients (Naini et al. 2005). As for lactate, increased CSF alanine concentration is a good biomarker that may indicate a mitochondrial disorder with central nervous system involvement. Urinary amino acids are not routinely measured in the diagnostic workflow of OXPHOS defects, but may be of interest if a tubulopathy is suspected as a part of the clinical phenotype.

#### 8.2.2.3 Urine Organic Acid Profile

Complete urinary organic profiling is usually performed by gas chromatography/mass spectrometry analysis (GC-MS). Since organic acids are by-products of amino acids, free fatty acid catabolism or the Krebs cycle, qualitative or quantitative urinary organic acid analysis is a valuable diagnostic tool for most organic acidaemias. Therefore, it is a key laboratory test applied in initial investigations for the differential diagnosis of OXPHOS defects. However, it is not a specific test for the identification of OXPHOS disorders. General markers of global mitochondrial dysfunction such as elevations in lactate, Krebs cycle metabolites or dicarboxylic acids are usually observed in OXPHOS disease patients (Couser and Gucsavas-Calikoglu 2017). Nevertheless, some organic acids, when elevated in urine, may direct investigations towards different aetiologies, being very useful for accurate phenotypic description of patients. For example, massive excretion of fumarate directs the diagnosis

towards fumarase deficiency. Increased oxoglutarate (together with lactate and branched chain ketoacids) is suggestive of a genetic or nutritional thiamine deficiency, or an E3 subunit defect (Ortigoza-Escobar et al. 2016). Increased ethylmalonic or methylmalonic acids should orient the diagnosis towards the presence of mutations in the *ETHE1* or *SUCLA2* genes, respectively (Tiranti et al. 2004; Carrozzo et al. 2007). Increased urinary methylglutaconic acid excretion is an especially interesting finding, since if detected, new diagnostic possibilities are opened for mutations in various candidate genes (Wortmann et al. 2013). There are at least 10 different OMIM entries defining genes that cause methylglutaconic aciduria.

#### 8.2.2.4 Carnitine

There is no acylcarnitine profile that is diagnostic for any of the OXPHOS disorders, although it may be useful to confirm the findings detected by urinary organic acid profiling. Carnitine deficiency is frequently seen in OXPHOS diseases and is thought to be related to compromised energy-dependent carnitine transport in kidney tubules (Campos et al. 1993).

### *8.2.3 Cytokines*

#### 8.2.3.1 FGF21

In recent years, fibroblast growth factor 21 (FGF21) has become a valuable biomarker for the diagnosis of OXPHOS disease. This circulating hormone-like cytokine is a protein with 209 amino acids and is synthesised in the liver, but also in white adipose tissue, brown adipose tissue, pancreas, skeletal muscle and hypothalamus (Badman et al. 2007; Izumiya et al. 2008). ELISA is the gold standard method for analysis of the circulating concentration of FGF21 in human serum/plasma.

FGF21 acts as a regulator of lipid metabolism and the starvation response. In a recent report of a mouse model with late-onset mitochondrial myopathy, levels of FGF21 were overexpressed and correlated with the number of COX-negative fibres as a predictor of disease severity (Suomalainen et al. 2011). Furthermore, FGF21 plays an important role in the regulation of glucose homeostasis and has metabolic effects on insulin resistance and diabetes (Kim and Lee 2014; Gómez-Sámano et al. 2017).

There is a current need for specific mitochondrial biomarkers for diagnosis and to follow the effect of treatment interventions on the course of the disease. For these reasons, the physiological significance of mitochondrial stress-induced FGF21 is still under investigation, although several studies have suggested that muscle FGF21 is overexpressed as a compensatory response to improve mitochondrial function in conditions of cellular stress (Ji et al. 2015). Some authors propose FGF21 as a

specific tool in mitochondrial disease diagnosis, especially in mitochondrial myopathy caused by mtDNA machinery disorders (mutations or changes to imbalance of mtDNA) but not in those caused by deficiencies in structural mitochondrial respiratory chain complex enzymes or assembly (Lehtonen et al. 2016).

#### 8.2.3.2 GDF15

Growth differentiation factor 15 (GDF15) is a cytokine that is regulated by p53 and oxidative stress and belongs to human transforming growth factor β (TGF-β) superfamily (Bootcov et al. 1997). The gene is localised on chromosome 19p12–13.1 and codes for a 40-kDa propeptide that is modified in the endoplasmic reticulum to release a 25-kDa active dimeric protein. It is expressed mainly in placenta, kidney, liver, lung, pancreas and prostate (Eling et al. 2006; Zimmers et al. 2005). GDF15 expression is activated during inflammatory responses caused by pro-inflammatory cytokines or interleukin 6, tumorigenic processes and cardiovascular diseases (Bootcov et al. 1997; Fairlie et al. 2000; Corre et al. 2013). Quantitative commercially available ELISA kits are used for the measurement of human GDF15 levels in serum samples (Montero et al. 2016).

Recently, several authors have identified GDF15 as a potential new biomarker for mitochondrial diseases after a study in patients with *TK2* (thymidine kinase 2) gene mutations found that GDF15 mRNA was overexpressed by skeletal muscle cells (Kalko et al. 2014). Currently, several comparisons are being performed to validate the utility of both factors (FGF21 and GDF15) in mitochondrial disease cohorts. GDF15 seems to be a more sensitive biomarker of OXPHOS disease than FGF21, although a correlation between serum FGF21 and GDF15 circulating levels has been demonstrated (Davis et al. 2016). Furthermore, GDF15 concentrations were found to be elevated in a variety of severe non-mitochondrial disorders (Lehtonen et al. 2016). Although several authors have postulated GDF-15 (and FGF-21) as biomarkers to monitor the progression of OXPHOS diseases or the effect of treatments, recent studies have demonstrated that neither FGF21 nor GDF15 levels are correlated with disease progression (Steele et al. 2016).

These observations suggest that further progress is necessary to elucidate the real diagnostic specificity and sensitivity of FGF21 and GDF15 in investigations for the study of a wide variety of OXPHOS diseases.

#### 8.2.3.3 Coenzyme Q10

Coenzyme $Q_{10}$ ($CoQ_{10}$) is known as a specific biomarker for mitochondrial disease diagnosis. This ubiquitously synthesised lipid is a key component of the mitochondrial OXPHOS pathway but also participates in other biological functions. The value of $CoQ_{10}$ determination in the diagnostic workflow of OXPHOS diseases is mainly associated with a subgroup of diseases that display depleted levels of $CoQ_{10}$. They are caused by genetic defects in the $CoQ_{10}$ biosynthetic pathway (primary

$CoQ_{10}$ deficiencies) or by defects in other pathways that affect directly or indirectly $CoQ_{10}$ levels (secondary $CoQ_{10}$ deficiencies). As primary and secondary $CoQ_{10}$ deficiencies have been demonstrated in different human genetic conditions, the assessment of this molecule is highly advisable for OXPHOS patients since $CoQ_{10}$ deficiency is a potentially treatable condition. $CoQ_{10}$ deficiency is defined by the biochemical diagnosis, but the complexity of $CoQ_{10}$ biosynthesis and the diversity of functions in which it participates make it difficult to predict which gene is causing $CoQ_{10}$deficiency, or even if the clinical outcome after treatment will be good.

$CoQ_{10}$ is found in all tissues, and thus it can be measured in various biological specimens such as blood (Arias et al. 2012), mononuclear cells and platelets (Duncan et al. 2005; Shults et al. 1997; Mortensen et al. 1998), muscle biopsy (Montero et al. 2008), cultured skin fibroblasts, cerebrospinal fluid (Artuch et al. 2004; Duberley et al. 2013), urinary tract cells (Yubero et al. 2015) or buccal mucosa cells (Martinefski et al. 2015), amongst others. One of the difficulties in detecting a $CoQ_{10}$deficiency is that the deficiency may be tissue specific (Ogasahara et al. 1989), and it is thus necessary to investigate endogenous $CoQ_{10}$ biosynthesis in the target organ. First-line $CoQ_{10}$ deficiency diagnosis is traditionally carried out in muscle biopsy tissue and cultured skin fibroblasts. However, the description of a wide variety of phenotypes and the high number of mitochondrial and non-mitochondrial diseases that display a secondary $CoQ_{10}$ deficiency (Yubero et al. 2016) are revealing the importance of analysing the $CoQ_{10}$ status in different biological specimens. Plasma $CoQ_{10}$ assessment may be performed as a routine biochemical test for OXPHOS disease patient investigations, and although it is not thought to reflect endogenous $CoQ_{10}$ cellular levels, it is a useful biomarker for treatment monitoring purposes. Plasma $CoQ_{10}$ levels depend mainly on dietary intake and hepatic biosynthesis (Duncan et al. 2005). Although our experience and that of other groups shows that plasmatic $CoQ_{10}$ levels are quite stable and its reduction is a rare biochemical finding associated with PKU or lysosomal storage diseases (Artuch et al. 1999; Delgadillo et al. 2011; Fu et al. 2010), its role as a diagnostic biomarker for OXPHOS disease patients remains to be established. In other words, it remains unknown at present whether or not plasma $CoQ_{10}$ can be used to indicate deficient tissue $CoQ_{10}$ status in patients with mitochondrial disorders.

The most frequently used laboratory methods for diagnosis of human $CoQ_{10}$ deficiency are based on the analysis of total $CoQ_{10}$ with high-pressure liquid chromatography coupled to ultraviolet (HPLC-UV) (Duncan et al. 2005) or electrochemical (HPLC-ED) (Montero et al. 2008) detection systems. The determination of total tissue $CoQ_{10}$ status is accurate enough to detect human $CoQ_{10}$ deficiencies, and although HPLC-ED methodology is suitable to detect the different naturally occurring forms of $CoQ_{10}$ (oxidised and reduced forms), evaluation of the ratio of reduced and oxidised $CoQ_{10}$ in muscle and other tissues is not better than total CoQ quantification for the identification of $CoQ_{10}$-deficient status (Miles et al. 2008). Other procedures have been applied to examine tissue $CoQ_{10}$ levels, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Barshop and Gangoiti 2007). This method can provide additional information, with the capacity to

estimate the $CoQ_{10}$ biosynthetic rate in fibroblasts incubated with $CoQ_{10}$ precursors (Buján et al. 2014).

The appropriate choice of the biological specimen is a key issue in obtaining the most valuable and accurate information about $CoQ_{10}$ status. Unfortunately, it is not possible to distinguish biochemically between primary and secondary deficiencies, and the selection of candidate genes on the basis of $CoQ_{10}$ analysis alone is not possible. However, this limitation is being overcome with the advent of NGS techniques, although, due to the complexity of OXPHOS diseases, a remarkable proportion of patients with $CoQ_{10}$ deficiency still remain undiagnosed (Desbats et al. 2015; Emmanuele et al. 2012).

#### 8.2.3.4 Other Biomarkers

Other biomarkers can be analysed depending on the clinical indications in OXPHOS disease patients. In some circumstances, they can lead to a rapid and definitive diagnosis. For example, urinary thymidine quantification by a single HPLC run with spectrophotometric detection can lead to the diagnosis of MNGIE, a mitochondrial disorder caused by mutations in the *TYMP* gene that cause a disturbance in the pyrimidine metabolic pathway (Hirano et al. 1994). Especially interesting may be the analyses of some specific biomarkers in CSF, which can also lead to rapid diagnoses. For instance, a deep cerebral folate deficiency is a universal feature in Kearns-Sayre syndrome, even in incomplete paediatric forms, and thus CSF 5-methyltetrahydrofolate is a suitable biomarker (together with increased CSF total proteins) for the investigation of OXPHOS patients in general (Garcia-Cazorla et al. 2008), and for Kearns-Sayre syndrome patients in particular. The biochemical disturbance observed in the CSF of OXPHOS disease patients is probably due to a dysfunction in the choroid plexus, an anatomical structure that generates CSF and where almost all of the folate transport from blood to the brain occurs (Garcia-Cazorla et al. 2008), in an energy-dependent manner. It has been demonstrated that there is an accumulation of mutated mtDNA copies in the choroid plexus of Kearns-Sayre syndrome patients (Tanji et al. 2000), causing a lack of ATP that impairs the active transport of a variety of molecules.

Thiamine in CSF has been demonstrated to be a good biomarker for the diagnosis of genetic defects in thiamine transport caused by mutations in the *SLC19A3* gene, a treatable genetic condition that causes Leigh syndrome (biotin responsive basal ganglia disease) (Ortigoza-Escobar et al. 2016).

### *8.2.4 Definitive Diagnosis*

It is obvious that interpretation of the biomarkers listed here in isolation is of limited use and definitive diagnosis requires a deep phenotypic definition of the patients, especially in the context of the vast array of clinical phenotypes and age of onset of

OXPHOS diseases. Establishing a diagnosis is often challenging, thus a detailed personal and family history and a comprehensive physical examination should be performed. An evaluation in suspected OXPHOS disease cases should include a complete clinical exploration of all organs and systems, and neuroimaging and complementary neurophysiological or other imaging examinations when appropriate.

Regarding laboratory investigations for OXPHOS diseases, traditionally, muscle and fibroblast skin biopsy and analysis was an essential and obligatory step in the study of these disorders. Muscle samples are useful for the measurement of mitochondrial respiratory chain enzyme activities (detailed procedures have been reported elsewhere). Briefly, by using different substrates and inhibitors, individual and combined mitochondrial respiratory chain enzyme activities can be measured (Spinazzi et al. 2012). Although this procedure has been considered one of the gold standard approaches for OXPHOS disease diagnosis, the results may be normal even if disease is present, or conversely, mitochondrial respiratory chain function may be impaired as a secondary effect of a huge list of genetic and environmental conditions. Even more importantly, muscle biopsy is ideal for histopathological studies, and although it is outside of the focus of this chapter, it is worth noting that such studies improve the diagnostic capabilities of laboratory methods. Some histopathological biomarkers are highly specific for particular OXPHOS genetic diseases, such as the presence of ragged red fibres (an optical microscopy finding due to abnormal mitochondrial proliferation), or the observation of COX negative fibres, an enzymohistochemical finding that strongly suggests a global dysfunction of complex IV of the MRC.

Skin fibroblast biopsy is less invasive than muscle biopsy and has the enormous advantage that fibroblasts can stored in tissue banks, and thus biological material is easily available for in vivo experiments. Skin fibroblasts are thus the ideal biological sample for functional studies, protein expression and localisation analysis and others. For example, the oxidation rate of energetic substrates such as labelled pyruvate, or the estimation of $CoQ_{10}$ biosynthesis using labelled precursors, can be measured in this tissue (Buján et al. 2014).

With the advent of newer testing modalities (next generation sequencing (NGS)), the need for the more invasive studies is decreasing. For instance, molecular genetic testing panels are able to identify mtDNA or nDNA mutations at the same time, and can be used for confirmation of a diagnosis as they are becoming more readily accessible. If those results are negative, whole exome (WES) and whole genome (WGS) sequencing techniques are becoming available for the diagnostic purposes, and in the near future will be a commonly-used diagnostic tool in hospitals. However, at least at present and probably in the coming years, muscle and especially skin fibroblast biopsies are still valuable biological specimens for confirming the pathogenicity of mutations detected by NGS. It is common that after a NGS analysis, more than one gene might be a potential candidate responsible of the disease. Considering the lack of specificity and sensitivity of the biomarkers reviewed in this chapter, it is still necessary to confirm the pathogenicity of mutations in candidate genes through functional studies, expression and localisation of the altered protein, and other methods.

## 8.3 Conclusions and Future Directions

The classical biomarkers for the investigation of OXPHOS diseases lack diagnostic sensitivity and specificity. New (but only a few) biomarkers have been identified in recent years, such as the cytokines FGF21 and GDF15, but further investigations seem advisable in this field. Moreover, with the advent of next generation sequencing techniques, unequivocal biomarkers should be identified for the confirmation of the pathogenicity of mutations in OXPHOS diseases.

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# Chapter 9
# Molecular Genetics in the Next Generation Sequencing Era

**Joaquin Dopazo**

**Abstract** The increasingly pervasive adoption of Next Generation Sequencing (NGS) technologies is causing a revolution in biomedical research and, particularly, is transforming health care. This flood of data has had an enormous impact on the discovery biomarkers for diagnosis, prognosis and treatment recommendation. However, the enormous generation of data produced by NGS needs of new methodologies and new strategies to deal with them and to properly interpreting the derived findings. Actually, it has soon be apparent that the concept of biomarker has a limited utility in pathologies or scenarios other than highly penetrant inherited diseases. Approaches to interpret the genetic variation in complex diseases need to take into account the complex system nature of the relationships of the proteins that conforms the mechanism of the disease. Systems biology offers a framework to understand the extensive human genetic heterogeneity revealed by genomic sequencing in the context of complex the network of functional, regulatory and physical interactions Consequently, modeling and computational data analysis will play an increasingly important role in drug discovery along with new emerging applications of Artificial Intelligence in medicine.

## 9.1 Introduction

Since the beginning of this century, the vertiginous development of high-throughput techniques and, especially in the last decade, next generation sequencing (NGS) (Mardis 2017; Goodwin et al. 2016a), has fostered an unprecedented increase in our knowledge of the mutational spectrum of diseases allowing a deeper understanding in their molecular mechanisms.

The vertiginous advance of sequencing technologies has resulted in a sequencing cost for a genome in free fall (See Fig. 9.1).

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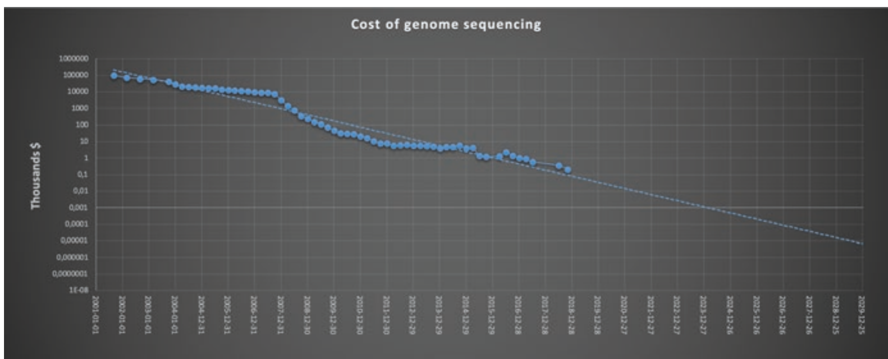


**Fig. 9.1 Cost of genome sequencing**. Until July 2017 data were taken from the NIH webpage (https://www.genome.gov/about-genomics/fact-sheets/DNA-Sequencing-Costs-Data), and completed until 2018 using data in the Wikipedia "1000$ genome entry" (https://en.wikipedia.org/wiki/$1000_genome). Dashed line extrapolates the cost in the future, suggesting a theoretical price of 1$ per genome by the end of 2023

The introduction of NGS in biomedical research and, more recently, in the clinical practice has dramatically changed the way in which this new type of biomedical data must be handled, stored, analyzed and interpreted. While not long ago very simple parameters or discrete variables where the only outcomes of clinical tests now new NGS-based genetic tests generate huge volumes of data that must be managed, interpreted and stored. This technological transformation has turned biomedicine into a Big Data discipline. Actually, genomic data generation has been compared with other major data generators such as astronomy, Twitter and Youtube and prospectively, genomics seem to be either on par with or, possibly even most demanding than the Big Data other domains analyzed in terms of data acquisition, storage, distribution, and analysis of data (Stephens et al. 2015). In fact, large-scale genomic projects such as 1000 Genomes Project (Abecasis et al. 2012), The Cancer Genome Atlas (TCGA) with thousand tumor/normal genome pairs[1] or the Exome Aggregation Consortium (ExAC), with over 60,000 human exomes,[2] just to cite a few ones, have generated publicly available genomic data repositories. Moreover, different active or announced country-level genomic projects will potentially raise the number of available genomes by orders of magnitude. Thus, UK (Turnbull et al. 2018) and Saudi Arabia[3] are currently sequencing 100,000 of their citizens, one-third of Iceland's population will also be sequenced (Sulem et al. 2015), and the

[1] https://tcga-data.nci.nih.gov/docs/publications/tcga/

[2] http://exac.broadinstitute.org/

[3] http://www.bbc.com/news/health-25216135

US,[4] China (Zhu 2012) and the EU[5] have announced plans for sequencing of 1 million genomes in the years coming. This transformative scenario will impact in many areas of research and healthcare with different intriguing derivatives such as the new professional profiles required for managing genomic data or the type of training that will be required in the future. Here we will comment several relevant aspects that include the new challenges posed by the genomics revolution, its impact on healthcare and some innovative analytic approaches, such as Systems Medicine and Artificial Intelligence (AI) that are blooming in this changing, Big Data scenario.

## 9.2 Next Generation Sequencing: A New Genomic Scenario with New Challenges

NGS technologies are characterized by producing several orders of magnitude more sequence data than conventional sequencing technologies at extremely cheaper prices per nucleotide sequenced (Mardis 2017; Goodwin et al. 2016a). Short read technologies are predominantly used for whole exome sequencing (WES) or whole genome sequencing (WGS), being Illumina, after the decline and discontinuation of SOLiD, the most widely used platform (Goodwin et al. 2016b). There are also benchtop versions of some of these technologies, such as MiSeq (Illumina) and Ion Torrent (Life Technologies) although these are more used for targeted sequencing of panels with a reduced number of genes, typically used for diagnostic purposes (Rehm 2013). On the other hand, long read technologies such as Pacific Biosciences (Eid et al. 2009) or Oxford Nanopore Technologies (Clarke et al. 2009), although still affected by a high rate of error, came with the promise of sequencing really long genomic stretches. Actually, it has been recently reported that the ONT MinION produced reads as long as 1 million bp (Sedlazeck et al. 2018). Thus, these long read technologies, in addition to report single nucleotide variants and small indels like short read technologies, could resolve structural variants and the genomic structure of highly repetitive sequences.

The increasing availability of genomes and exomes is depicting an unforeseen scenario of increasing variability (Durbin et al. 2010). About 50k and 60k variants are commonly found in exomes and between 1 and 2 M in genomes (Bamshad et al. 2011), including variants predicted to severely affect the function of human protein-coding genes, known as loss-of-function (LoF) (MacArthur and Tyler-Smith 2010). Actually, an unexpectedly large number of LoF variants are systematically found, even in the genomes of apparently healthy individuals. Conservative estimations point to the existence of, at least, 250 LoF variants per genome, being more than 50

[4] http://news.sciencemag.org/biology/2015/01/white-house-fleshes-out-obama-s-215-million-plan-precision-medicine

[5] https://ec.europa.eu/digital-single-market/en/news/eu-countries-will-cooperate-linking-genomic-databases-across-borders

of them in a homozygous state, which suggests a previously unnoticed level of variation with putative functional consequences (Xue et al. 2012). Actually, such ratios of deleterious variation are not unique to coding regions and can be observed in other non-coding elements of the genome such as miRNAs (Carbonell et al. 2012). This high level of variation along with the pervasive presence of (apparently) deleterious mutations in healthy genomes adds extra complexity to the challenge of discovering candidate genes causal of diseases.

The enormous production of data of NGS technologies has three main associated problems. Firstly, the technical problem of management and storage of such volume of data. Secondly, raw data processing (process by which the primary data produced by the sequencing instrument is converted into a comprehensive description of variation of the samples sequenced) that needs to be fast, especially for clinical applications. And finally, the most complex problem of data interpretation, when variations at genomic level have to be related to phenotypic traits such as diseases or responses to treatments. Although none of these problems has been definitively settled, technical solutions are continuously arising that facilitate data management processes. In the case of genomic data processing there are different algorithms for read mapping in DNA or transcripts and variant calling reviewed elsewhere (Garber et al. 2011; Fonseca et al. 2012). The intrinsic parallel nature of genomic data makes many conventional algorithms, which are mostly sequential, inefficient. Fortunately, new distributed computing solutions such as MapReduce and heterogeneous computational environments, in which conventional CPUs are merged with specialized accelerators such as graphics processing units (GPUs) or field-programmable gate array (FPGA) that can speedup calculations several orders of magnitude, are increasingly available and will be soon common for genomic data analysis.

During the last decade, whole exome sequencing (WES) firstly in research and subsequently in clinical settings has demonstrated to be a powerful tool for discovering new variants and disease genes for rare diseases (RDs) in a matter of months, where previous approaches required of years or even decades. WES has been especially useful for diseases with significant morbidity and mortality and caused by highly penetrant (typically protein-coding) variants (Boycott et al. 2017; Boycott et al. 2013). Moreover, WES has demonstrated a diagnostic utility, with a diagnostic yield in the range of 25–30% among large and heterogeneous rare disease (RD) cohorts (Clark et al. 2018).

However, beyond RDs, the genetic of complex disease remains as a serious challenge, with historically limited success and, as a general rule, fewer therapeutic advances in patient care (Stessman et al. 2014). Unlike most RDs, which are Mendelian disorders, the phenotype of complex diseases is not caused by a single gene mutated but, rather, by many individual gene mutational events, as well as a significant contribution from environmental factors. Typical studies of complex diseases select patients with similar phenotypes and try to identify the common causative mutation for this phenotype using association studies. However, despite the growing number of loci reported over the last decade years, in most cases, much of the heritability of common diseases continues still unresolved (Manolio et al. 2009), remaining the interpretation of the variants found in WES or in WGS still a

challenge. Understanding the mechanisms by which some (among the many) variants shape complex disease phenotypes constitutes a daunting problem that depends on the yet incomplete knowledge of the molecular mechanisms that govern the fate of the cell. Systems biology is deemed to play an increasingly important role in the diagnosis (Fryburg et al. 2014) and, in general, in understanding of common diseases (Hood et al. 2004; Barabasi et al. 2011), as well as other aspects relevant in medicine, such as drug discovery (Dopazo 2014). The construction and use of models that include functional, regulatory and physical protein-protein interaction information to predict the effect of variants and/or differences in gene expression can be computationally very demanding (Schadt et al. 2010).

## 9.3 NGS Fosters the Discovery of New Disease Genes and Variants

The most radical change brought about by the widespread use of NGS is the acceleration of the discovery of new disease determinants. It has been especially successful in the discovery of disease genes in Mendelian disorders (Bamshad et al. 2011; Ng et al. 2010; Boycott et al. 2013) as well as in cancer (Garraway and Lander 2013; Vogelstein et al. 2013). However, although the use of WES in RD diagnosis represents a notable advancement with respect to other genetic tests (Clark et al. 2018), there is still a high ratio (>70%) of patients with a high degree of pre-test suspicion for a monogenic RD for which a molecular diagnosis cannot be provided (Boycott et al. 2019). Therefore, NGS is still extensively used for gene discovery, which contributes to an annual curation of over 10,000 disease variants (Landrum et al. 2017) and about 250 novel disease-gene associations (Boycott et al. 2019). Actually, as a consequence of this continuous cumulation of information, it has been report that reanalysis of negative clinical WES data in the subsequent 1 to 3 years increases diagnostic yield by 10% (Wenger et al. 2017) or even more if this reanalysis is carried out in collaboration with the referring physician (Salmon et al. 2018).

However, with more than 50,000 variants found per exome (or over 1 million per genome) (Bamshad et al. 2011), finding disease-causing variants or genes is still a daunting, time-consuming task that often requires intensive human intervention (Cooper and Shendure 2011). It implies the identification of the most promising genes among the initially enormous pool of candidates by a sequential filtering process known as prioritization. The final objective is maximizing the yield and biological relevance of further downstream validation experiments by focusing on the most promising candidates (Moreau and Tranchevent 2012). Typical filtering strategies involve the use of different knowledge databases and bioinformatic algorithms the allows applying thresholds related with observed frequencies of variants in the normal population, estimation of the potential damaging effect of the mutation in the protein, evolutionary conservation of the region affected by the mutation,

functional role of the affected gene, etc., in order to discard variants with a low potential of being real causal variants.

Although these steps could be applied one by one using directly the different sources of information above mentioned, the high number of variants involved in the prioritization process makes necessary a first step annotation of the variants with all the relevant information so as the subsequent steps of filtering can be carried out in a more interactive way. The most widely used applications that collect information from many heterogeneous sources and annotate lists of variants are Annovar (Wang et al. 2010), the Variant Effect Predictor (McLaren et al. 2016) or the CellBase (Bleda et al. 2012), used in the 100,000 genomes project. Once each variant has been annotated with all the relevant information currently available the prioritization process can be carried out.

Observed variant population frequencies constitute a highly discriminative filter, especially in the case on RDs. Variants with a relatively high frequency in the population are unlikely to be causative of many hereditary disorders. Population variant frequencies are obtained from different repositories such as the 1000 genomes (Durbin et al. 2010), the ExAC (Exome Aggregation Consortium) (Karczewski et al. 2016) or the gnomAD (Genome Aggregation Database) (Lek et al. 2016). However, these databases tend to be biased towards certain ethnic groups or populations. In fact, it the importance of the local component of the genetic variability, even within the same ethnic group (Dopazo et al. 2016) has recently been demonstrated, suggesting a note of caution when using these general-purpose databases.

The potential impact of the variant in the resulting gene product is another important filtering criterion. Thus, the consequence type, as defined in the ENSEMBL repository[6], is used to select variants with a predicted severe effect, (e.g. non-synonymous or stop lost) as the best candidates. For other variants with not so obvious consequences, numerous indexes that predict the potential pathologic effect of an amino acid substitution can be used based on different criteria such as the structural impact, e.g. Polyphen (Ramensky et al. 2002), SIFT (Kumar et al. 2009), etc., evolutionary conservation e.g. PhyloP (Pollard et al. 2010), GERP (Davydov et al. 2010), etc., compendiums of different criteria, such as CADD (Kircher et al. 2014) or, more recently, based on artificial intelligence (Sundaram et al. 2018).

Regional filters are also useful. For example, previous information on regions associated to the disease may be available from linkage disequilibrium studies. Also filters based on genes previously associated with functions, as described in Gene Ontology (Ashburner et al. 2000) or syndromes and phenotypes, as defined in the human phenotype ontology (HPO) (Köhler et al. 2013), that are compatible with the disease studied can be used. Actually, HPO has revealed as an powerful tool for defining previous sets of genes to search for genetic determinants of diseases (Köhler et al. 2009).

Variants with known pathologic effect can be found in different databases, such as HGMD (Stenson et al. 2012), ClinVar (Landrum et al. 2014) or UNIPROT

[6] https://www.ensembl.org/info/genome/variation/prediction/predicted_data.html

(The_Uniprot_Consortium 2014), which can also be used as filtering criteria either by directly finding pathologic variants or for the selection of genes that contain pathological variants as regional filtering options.

Also, in the case of inherited diseases or *de novo* syndromes, the availability of sequencing data of parents, siblings or close relatives can significantly facilitate the process of finding candidate disease genes because these allow filtering out variants that do not segregate coherently with the inheritance model across family pedigrees (Peng et al. 2013; Li et al. 2012).

Although this prioritization process is often carried out with manual intervention, different computer applications can be used to automatize the process to same extent. Applications like Exomizer (Smedley et al. 2015) allow automatizing some of the steps in the prioritization process and proposes a final short list of potential candidates. Alternatively, other applications facilitate the prioritization process by providing users with a user-friendly environment where they keep the control of all the filters used, such as the BiERapp (Aleman et al. 2014b) and other similar programs that inherited this concept (Desvignes et al. 2018; Zia et al. 2019).

## 9.4 NGS Makes Genomic Medicine Mainstream

Thus, with this increasingly fast pace of discovery of disease variants and genes, clinic applications are gaining importance with a foreseeable close horizon in which a diagnosis for all rare diseases will be attained (Boycott et al. 2019). Actually, in current clinical settings, with patients having specific presentations that suggest a genetic cause to the RD, the yield of the WES is likely well over 50% (Beaulieu et al. 2014; Shamseldin et al. 2017). In addition to this increasing availability of knowledge on the determinants of genetic diseases, there is another factor which is even more important for the adoption of NGS in the clinical practices: the cost of genome sequencing. During the last decade, the decrease in the prices of genome sequencing has been so drastic (see Fig. 9.1) that can only be understood by a hypothetical comparison: for example, if the car industry would have experienced the same technologic development, car prices would be nowadays in the range of cents. It is worth noticing that WGS has demonstrated to identify diagnostic variants in 41% of individuals, representing a significant increase over conventional testing results and outpacing WES even in coding regions (Lionel et al. 2017), which suggests that in brief WGS will be the genetic test by default.

In this scenario, the main deterrent to the full exploitation of NGS in the clinic practice is neither the cost nor the information required for the interpretation of the results but, paradoxically, the way in which the genomic data is managed to obtain the final report of the genetic test. Historically, NGS was initially used to sequence patients in a context of research. Unfortunately, the subsequent application of NGS to the clinic has inherited many of the genomic data management procedures that were commonplace in research practices but not necessarily optimal for the clinical regular practice. Thus, patient sequencing is often carried out in facilities external to

the health system and the genomic data produced is studied by teams of bioinformaticians or data scientists using scripting bioinformatic tools in a way that involves much manual management of the data and makes extensive use of web-based resources for the prioritization of the variants. These analysis pipelines result in diagnosis times incompatible with current clinical practices. Thus, typical turn-around time analysis from blood draw to final report are in the range of 15–16 weeks (Swaminathan et al. 2017; Lazaridis et al. 2014) or even longer (Bick et al. 2017). Moreover, most laboratories still return clinical summary reports in PDF files that, in many cases, are manually integrated in the eHR where can be consulted by other physicians (McLaughlin et al. 2014). However, this type of format and the unstructured contents prevents physicians from electronically querying and searching reports for information of interest (Swaminathan et al. 2017). In addition to these problems, another important drawback is the difficulty of finding bioinformaticians or biostatisticians properly trained for these type of analysis (Gómez-López et al. 2017).

Therefore, a completely different strategy must be taken order to make a proper use of genomic data in the clinical setting and scaled them up to the dimensions of a health system. Some strategies that shorten diagnosis times have been proposed, such as the STATseq, which uses an optimized sequencing protocol followed by quick mutational screening to deliver a diagnosis in 50 h in neonatal intensive care units (Saunders et al. 2012; Kingsmore et al. 2015) that was further reduced to 26 h (Miller et al. 2015). Some proposals show a higher degree of automatization providing software applications that tracks patients, samples, genomic results, decisions and produces reports, monitors progress and sends reminders, working with an electronic data capture system (Watt et al. 2013). In addition to automatization, the management of the information, such as secondary findings, is another relevant aspect to be taken into account, as pointed out by the directives of medical colleges (Scheuner et al. 2015; Kalia et al. 2017). The concept of virtual panels, that allows focusing on specific stretches of the genome relevant for the disease, without exploring other areas of the genome where non-actionable mutations could be found is quite useful (Aleman et al. 2014a).

Some general rules must be followed in the future for the proper use and exploitation of patient genomic data at the scale of health systems. Firstly, the data must be managed by geneticists or by clinicians with some training in genomics. Bioinformatician or biostatistician teams must not be doing manually routine diagnosis the same way that a radiography does not need of physicists or engineers to be interpreted. These specialized teams are more useful in the subsequent prospective exploitation of the clinic and genomic big data generated by the health system. Secondly, the software that manages, analyzes and helps with the interpretation of genomic data must be integrated into the rest of corporative informatic systems in a way that ordering a genomic test should have the same complexity that ordering another diagnostic test for the authorized personnel. And third, in addition to the results of the genomic test, the genomic data of the patient must be stored in the health system linked to the eHR of the patient for future prospective clinical studies. The FAIR Principles (Findability, Accessibility, Interoperability, and Reusability)

for data analysis should be extended to the genomic and clinical data (Holub et al. 2018).

## 9.5 Complex Diseases and the Role of Systems Medicine and Artificial Intelligence in the Future of Precision Medicine

Despite success of NGS in finding disease genes in single gene Mendelian disorders (Bamshad et al. 2011; Ng et al. 2010; Boycott et al. 2013), its application to complex diseases has produced more modest results (Stessman et al. 2014). Contrarily to the case of RDs, complex diseases are characterized by disease heterogeneity: patients with similar presentations may have different underlying disease mechanisms caused by a multigenic nature (Gustafsson et al. 2014). For this reason, complex diseases can be better understood as failures of functional modules caused by different combinations of perturbed gene activities rather than by the failure of a unique gene (Oti and Brunner 2007). Actually, the main bottleneck for the interpretation of the significance of gene variants found by NGS in complex diseases is the lack of biological knowledge that allows identifying the functional module affected and, consequently, the damaged functionality, which ultimately account for the disease mechanism.

Recent reports demonstrate that the activity of well-defined functional modules, such as metabolic or signaling pathways, constitute better predictors of complex phenotypes, such as patient survival (Fey et al. 2015; Hidalgo et al. 2017), drug response (Amadoz et al. 2015), etc., than the activity of their constituent genes. Currently, there is a considerable (yet incomplete) knowledge on biological pathways that describe in high detail different functional, regulatory, metabolic and other relationships among genes and molecules (Fabregat et al. 2017; Kanehisa et al. 2016) that can be exploited for the development of mechanistic models that relate biological components of the cell among them according to the known network of functional, regulatory and physical interaction in order to make sense of NGS data in the context of the biological system studied (Auffray et al. 2009; Barabasi et al. 2011).

Therefore, a proper interpretation of the consequences that the genetic variation found by NGS has over phenotypes requires of a detailed understanding of the mechanisms of disease, which can be interpreted by means of mechanistic models of cell signaling (Amadoz et al. 2018) or cell metabolism (Cubuk et al. 2018b). Mechanistic models have been recently used to reveal the disease mechanisms behind different cancers (Hidalgo et al. 2017; Cubuk et al. 2018a), including neuroblastoma (Fey et al. 2015; Hidalgo et al. 2018), breast cancer (Jiao et al. 2017), the shared molecular mechanisms of three cancer-prone genodermatoses (Chacón-Solano et al. 2019) or mechanisms of action of drugs (Amadoz et al. 2015) and other complex scenarios such as the prevention of obesity by stress-induced activation of

brown adipose tissue (Razzoli et al. 2016) or the molecular mechanisms of death and the post-mortem the ischemia of a tissue (Ferreira et al. 2018).

Interestingly, mechanistic models can be used not only to study molecular mechanisms of disease but also to predict the potential consequences that therapeutic interventions can have over the activity of functional modules that account for the disease mechanism (Salavert et al. 2016), thus holding the promise of revolutionizing the future of the practice of medicine. Thus, in a near future systems medicine understood as a discipline that by mathematical modeling of variables from different genomic layers will produce highly accurate relationship between clinical outcomes, such as treatment response, and their genetic and environmental determinants (Kolch and Kholodenko 2013; Wolkenhauer et al. 2014), will play an increasingly important role in the clinical practice.

Beyond systems medicine, this wealth of genomic data produced by the continuous development of NGS technologies is fostering a growing number of applications of Artificial Intelligence (AI) to genomic data (Topol 2019), especially in biomedicine (Wainberg et al. 2018), health care (Miotto et al. 2017) and computational biology (Angermueller et al. 2016; Min et al. 2017) fields. The possibility of using the available genomic Big Data repositories to formulate hypotheses and extrapolate conclusions in complex diseases, but especially in RD, where which the generation of knowledge and the discovery therapies is slow due to an inefficient research approach done in individualized research niches, constitutes an attractive opportunity to demonstrate the power of AI applied to genomic Big Data analysis.

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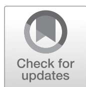
# Chapter 10
# Model Cells and Organisms in Mitochondrial Diseases




**Rhoda Stefanatos, Alberto Sanz, and Daniel J. M. Fernandez-Ayala**

**Abstract** The central role of mitochondria in cell metabolism and physiology is reflected in the diversity of functions that they perform within the cell. Alterations or imbalances in any of these cellular processes can cause mitochondrial dysfunction and disease. In this chapter we review the animal and cellular models used in the research of these diseases. We focus on genes that have been found to be mutated in patients with mitochondrial disease and comment on the potential of these models for use in identification and validation of novel therapies.

## 10.1 Introduction

The mitochondria present within our cells carry out important and diverse functions including oxidative phosphorylation and apoptotic cell death (Nunnari and Suomalainen 2012). Pathologies can arise from inhibition or imbalance in any of the cellular processes for which mitochondria are required. A healthy mitochondrion requires over a thousand proteins encoded by the nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) (Gorman et al. 2016). This means that there are over 1000 mutational targets from which mitochondrial dysfunction can result. Oxidative phosphorylation relies on the formation and stability of 5 multi subunit complexes

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of nuclearly and mitochondrially encoded components (Sanz and Stefanatos 2008). Accordingly, this process is particularly sensitive to mutation and as such, most mitochondrial disease patients carry mutations in genes encoding OXPHOS proteins (Lightowlers et al. 2015).

The current standard of care for mitochondrial disease patients is palliative, with interventions that mainly target specific symptoms (Viscomi et al. 2015; Lightowlers et al. 2015; Gorman et al. 2016). To facilitate the design, discovery and validation of curative treatments, a deeper understanding of disease pathogenesis is required. Predictive models, which recapitulate the clinical and molecular pleiotropy exhibited by mitochondrial disease patients will allow examination of the molecular effects of mitochondrial dysfunction. The mitochondrial research community has accumulated a collection of complementary models with just this aim.

In this chapter, we will attempt to summarise the wide range of models which have been created and used to study the effects of disrupting a variety of mitochondrial proteins, with a focus on those genes which have been found to be mutated in patients (Table 10.1). This will include *in vivo* models such as mice, zebrafish, flies and worms. Finally, we will comment on the potential of these models in investigating the pathophysiology of mitochondrial disease and the development and validation of novel therapies.

*In vitro,* cell culture models have been used to model the pathology of many patient derived mutations, using a variety of techniques. Most studies have used cytoplasmic hybrids (Cybrids). Cybrids allow the assessment of mtDNA alterations in a defined nuclear background. They have been used to study the bioenergetics of specific patient mutations and mtDNA heteroplasmy (Wilkins et al. 2014). Primary cells derived from patient biopsies have also been used to model mitochondrial diseases, however disease pathology is not always recapitulated in culture (Cameron et al. 2004). Finally, with the advent of induced pluripotency, the creation of stem cells from differentiated tissue also known as induced pluripotent stem cells (iPS), researchers can create patient specific iPS cells which can be differentiated into specific cell types, like neurons or muscle cells (Avior et al. 2016). This is particularly important for mitochondrial disease given the tissue specific effects of different mitochondrial syndromes. Despite the obvious limitations of an *in vitro* system, iPS cell models will be useful in validation of potential therapies. However, given the long standing and wide use of cell culture models we will focus this chapter on whole animal models of mitochondrial disease.

## 10.2 Mouse Models of Mitochondrial Disease

The mouse has been well established as the go to model in the study of several human diseases (Peters et al. 2007). Humans and mice share a large degree of genetic and physiological traits which make them an attractive model to study disease pathogenesis (Peters et al. 2007). In the last 20 years, technology for the

**Table 10.1** Models of Mitochondrial disease

| Mitochondrial disease | Gene | Model organism |
|---|---|---|
| CI deficiency, LS | *NDUFS4* | *M. musculus* (Kruse et al. 2008; Quintana et al. 2010, 2012) |
| | *NDUFS6* | *M. musculus* (Ke et al. 2012; Forbes et al. 2013) |
| | *NDUFA5* | *M. musculus* (Forbes et al. 2013) |
| | *NDUFS3* | *D. melanogaster* (Li 2013; Vos et al. 2012) |
| | *NDUFAF1* | *D. melanogaster* (Cho et al. 2012) |
| | *NDUFAF6* | *D. melanogaster* (Zhang et al. 2013) |
| | *MT-ND2* | *D. melanogaster* (Burman et al. 2014) |
| | *NDUFS2* | *C. elegans* (Kayser et al. 2001) |
| | *NDUFV1* | *C. elegans* (Grad and Lemire 2004) |
| CII deficiency | *SDHD* | *M. musculus* (Piruat et al. 2004) |
| | *SDHB* | *D. melanogaster* (Walker et al. 2006) |
| | *SDHC* | *C. elegans* (Ishii et al. 1998) |
| $CoQ_{10}$ deficiency | *COQ2* | *D. melanogaster* (Fernandez-Ayala et al. 2014) |
| | *COQ3* | *D. melanogaster* (Fernandez-Ayala et al. 2014) |
| | *COQ5* | *D. melanogaster* (Fernandez-Ayala et al. 2014) |
| | *COQ7* | *M. musculus* (Wang et al. 2015), Drosophila (Fernandez-Ayala et al. 2014), C. elegans |
| | *COQ8* | *D. melanogaster* (Fernandez-Ayala et al. 2014) |
| | *COQ9* | *M. musculus* (Garcia-Corzo et al. 2013), Drosophila (Fernandez-Ayala et al. 2014) |
| | *COQ10* | *D. melanogaster* (Fernandez-Ayala et al. 2014) |
| | *PDSS1* | *D. melanogaster* (Grant et al. 2010) |
| | *PDSS2* | *M. musculus* (Hallman et al. 2006, Peng et al. 2008) |
| Cyt c deficiency | *CYCS* | *M. musculus* (Li et al. 2000b) |
| CIII deficiency | *BCS1L* | *M. musculus* (Leveen et al. 2011) |
| | *UQCRB* | *D. rerio* (Cho et al. 2013) |
| CIV deficiency, LS | *COX4I2* | *M. musculus* (Huttemann et al. 2012) |
| | *COX10* | *M. musculus* (Diaz et al. 2008, 2012b, 2005) |
| | *COX15* | *M. musculus* (Viscomi et al. 2011) |
| | *COX5A* | *D. rerio* (Baden et al. 2007) |
| | *COX6A1/2* | *D. melanogaster* (Kemppainen et al. 2014) |
| | *COX6B1/2* | *D. melanogaster* (Kemppainen et al. 2014) |
| | *COX7A1/2* | *D. melanogaster* (Kemppainen et al. 2014) |
| | *CEP89* | *D. melanogaster* (van Bon et al. 2013) |
| | *SCO1* | *D. melanogaster* (Porcelli et al. 2010) |
| | *SCO2* | *M. musculus* (Yang et al. 2010), Drosophila (Porcelli et al. 2010) |
| | *SURF1* | *M. musculus* (Agostino et al. 2003; Dell'agnello et al. 2007), *D. melanogaster* (Da-Re et al. 2014), *D. rerio* (Baden et al. 2007) |

(continued)

**Table 10.1** (continued)

| Mitochondrial disease | Gene | Model organism |
|---|---|---|
| CV deficiency | *ATPIF1* | *M. musculus* (Formentini et al. 2014) |
| | *MT-ATP6* | *D. melanogaster* (Celotto et al. 2006; Palladino 2010) |
| French Canadian LS | *LRPPRC* | *M. musculus* (Xu et al. 2012; Ruzzenente et al. 2012) |
| Friedrich's Ataxia | *FXN* | *M. musculus* (Miranda et al. 2002; Martelli et al. 2012; Ristow et al. 2003; Puccio et al. 2001; Cossée et al. 2000) |
| Ethylmalonic encephalopathy (EE) | *ETHE1* | *M. musculus* (Di Meo et al. 2011) |
| | *POLG* | *M. musculus* (Hance et al. 2005; Lewis et al. 2007; Zhang et al. 2000; Bensch et al. 2009; Kasahara et al. 2006; Trifunovic et al. 2005, 2004), *D. melanogaster* (Bratic et al. 2015) |
| | *POLG2* | *M. musculus* (Humble et al. 2013), Drosophila (Baqri et al. 2009) |
| mtDNA depletion | *TWINKLE* | *M. musculus* (Tyynismaa et al. 2005; Milenkovic et al. 2013) |
| | *TFAM* | *M. musculus* (Ekstrand et al. 2007; Li et al. 2000a; Silva et al. 2000; Vernochet et al. 2012; Wang et al. 1999; Wredenberg et al. 2002; Ylikallio et al. 2010) |
| | *ANT1* | *M. musculus* (Graham et al. 1997), *D. melanogaster* (Vartiainen et al. 2014; Zhang et al. 1999) |
| | *TK2* | *M. musculus* (Zhou et al. 2008; Akman et al. 2008) |
| | *TYMP* | *M. musculus* (Haraguchi et al. 2002; Lopez et al. 2009) |
| | *RRM2B* | *M. musculus* (Kimura et al. 2003) |
| | *MPV17* | *M. musculus* (Clozel et al. 1999; Viscomi et al. 2008) |
| | *MFN1* | *M. musculus* (Chen et al. 2003, 2010, 2012) |
| | *MFN2* | *M. musculus* (Chen et al. 2007, 2010, 2012; Detmer et al. 2008; Gall et al. 2012; Sebastian et al. 2012; Papanicolaou et al. 2011; Cartoni et al. 2010), *D. melanogaster* (Dorn et al. 2011), *D. rerio* (Vettori et al. 2011) |
| | *RNASEH1* | *M. musculus* (Lima et al. 2016) |
| mtDNA dynamics | *OPA1* | *M. musculus* (Davies et al. 2007; Alavi et al. 2007, 2009; Moore et al. 2010; Sarzi et al. 2012), *D. melanogaster* (Dorn et al. 2011; Yarosh et al. 2008), *D. rerio* (Rahn et al. 2013) |
| | *DRP1* | *D. melanogaster* (Verstreken et al. 2005) |

genetic modification of mice continues to improve, making the process quicker and cheaper (Van Der Weyden et al. 2011). In that time mouse models corresponding to over 40 nuclearly and mitochondrially encoded mitochondrial proteins have been created. Broadly these can be divided into two categories, those genes involved directly in oxidative phosphorylation (OXPHOS) i.e. subunits and assembly factors of the mitochondrial respiratory complexes, and those proteins required for normal mitochondrial function such as those regulating mitochondrial DNA maintenance.

## *10.2.1 Complex I Deficiency*

### 10.2.1.1 NDUFS4

The first mouse model created which targeted complex I directly was the NADH: Ubiquinone Oxidoreductase Subunit S4 (*Ndufs4*) knockout (KO) mouse created in the Palmiter Lab (Kruse et al. 2008). *Ndufs4* whole body KO mice were created via Cre/loxP excision of exon 2 which resulted in undetectable levels of NDUFS4 protein (Kruse et al. 2008). Phenotypically *Ndufs4*$^{-/-}$ mice started to show signs of illness around 5 weeks of age, with symptoms of increasing lethargy, weight loss, progressive loss of motor ability and reduced survival at around 8 weeks, reminiscent of patients with isolated complex I (CI) deficiency or Leigh syndrome (Kruse et al. 2008; Dahl 1998). Leigh syndrome (LS) is a common infantile mitochondrial disorder which is characterized by progressive neurodegeneration (Dahl 1998). *Ndufs4*$^{-/-}$ mice had reduced levels of assembled CI and a corresponding decrease in CI activity as assayed by oxygen consumption (Kruse et al. 2008). Interestingly, when the same group conditionally removed *Ndufs4* from neurons and glia, they were able to phenocopy whole body KO mice (Quintana et al. 2010). This highlights the importance of the neuronal component in the *Ndufs4*$^{-/-}$ phenotype and suggests it could underlie that of LS associated with CI deficiency. Moreover, neuronal pathology in both models correlated with disease onset and progression in humans (Kruse et al. 2008; Quintana et al. 2010; Rahman et al. 1996). Removal of NDUFS4 globally or specifically from the central nervous system results in a LS-like phenotype which overlaps highly with that of patients suffering from LS, and as such could be useful in the validation of therapies for LS.

The Larsson and Tian groups went on to study the effects of conditional ablation of NDUFS4 in mouse heart, describing a mild phenotype of cardiomyopathy (Karamanlidis et al. 2013; Li et al. 2000a). Given that NDUFS4 levels were undetectable upon ablation, insights into the tissue specific phenotypes observed via conditional knockdown came from a study by Calvaruso and colleagues who measured the residual CI activity in various tissues of the whole-body KO (Calvaruso et al. 2012). The study found that residual CI activity was present in all tissues but that this varied greatly, and in fact that the heart possessed the highest level of residual CI activity (Calvaruso et al. 2012).

Using random transposon insertion Leong and colleagues created another model of NDUFS4 loss, *Ndufs4*$^{fky/fky}$, which in line with what had been observed in previous models displayed reduced CI activity in all tissues, progressive neuropathy and died prematurely at around 7 weeks of age (Leong et al. 2012).

Finally, the Pinkert lab created an NDUFS4 knock in (KI) mouse (*Ndufs4* $^{KI/KI}$) which carries a point mutation which results in the production of a truncated protein (Ingraham et al. 2009). This is a particularly interesting model, as most NDUFS4 mutations in humans lead to expression of truncated versions of the NDUFS4 protein (Budde et al. 2000; Iuso et al. 2006; Van Den Heuvel et al. 1998). Homozygous KI mice were embryonic lethal, while mice heterozygous for the KI allele were

viable and displayed a mild phenotype, characterized by a 30% decrease in CI linked respiration and CI activity, and increased levels of lactate, commonly seen in patients (Ingraham et al. 2009).

This group of studies using several independent models of *NDUFS4* dysfunction firmly establish the importance of this subunit in stability and regulation of respiratory complex I (Ingraham et al. 2009; Karamanlidis et al. 2013; Kruse et al. 2008; Leong et al. 2012; Quintana et al. 2010, 2012; Sterky et al. 2012). They also highlight how residual CI activity can vary greatly tissue to tissue, with implications not only on tissue specific pathology but also on organismal viability. As mutation of *NDUFS4* is associated with LS due to CI deficiency (Ruhoy and Saneto 2014), understanding the effects of its depletion can serve only to aid the development and assessment of targeted therapies.

#### 10.2.1.2 NDUFS6

NADH: Ubiquinone Oxidoreductase Subunit S6 (*NDUFS6*) is part of the enzymatic core of complex I that has been found to be mutated in patients suffering from isolated CI deficiency (Kirby et al. 2004; Spiegel et al. 2009). Like many patients suffering from CI deficiency, patients carrying mutations in *NDUFS6* display a reduction in CI activity accompanied by acidosis, myopathy and encephalopathy. Several mutations of *NDUFS6* have been described to result in very severe phenotypes, and death within weeks of birth (Spiegel et al. 2009).

There is currently one mouse model of *NDUFS6* loss available, created via gene trap insertional mutagenesis (Forbes et al. 2013). The *Ndufs6*$^{gt/gt}$ mouse has decreased levels of assembled CI and CI activity across all tissues, however like in the case of *Ndufs4*, the degree of reduction varies from tissue to tissue, with the lowest residual activity found in cardiac tissue (Forbes et al. 2013). Interestingly, the authors describe a tissue specific splicing anomaly which may underlie this variability in *Ndufs6* knockdown. *Ndufs6*$^{gt/gt}$ animals develop cardiomyopathy, have reduced ATP synthesis from complex I linked substrates and a deregulation of fatty acid metabolism with sex bias, as males were more severely affected than females. Additionally, both homozygous *Ndufs6*$^{gt/gt}$ and heterozygous *Ndufs6*$^{gt/+}$ mice develop renal disease, with a greater severity in homozygous animals (Forbes et al. 2013).

Although this model displays classic phenotypes of isolated complex I deficiency many of the phenotypes associated with mutations in *NDUFS6* are missing. In contrast to the short survival of affected patients, *Ndufs6*$^{gt/gt}$ can live up to 4 months (Forbes et al. 2013; Spiegel et al. 2009; Kirby et al. 2004).

#### 10.2.1.3 Mt-ND6

*MT-ND6* is a mitochondrially encoded core subunit of CI (Wirth et al. 2016). Mutations and allelic variants of *MT-ND6* have been found in patients suffering from Leber hereditary optic atrophy (LHON), LS and mitochondrial encephalomyopathy lactic acidosis and stroke like episodes (MELAS) (Johns et al. 1992;

Chinnery et al. 2001; Ravn et al. 2001). A severe frameshift mutation of *MT-ND6* (13885insC) was shown to be actively removed from the oocyte in a mouse model of mitochondrial disease (Fan et al. 2008). Two further mouse models were created carrying the same missense mutation (G13997A) isolated from a lung carcinoma cell line, which corresponds to a mutation described in patients (G14600A) (Ishikawa et al. 2008). Homoplasmic mice created in the Hayashi lab had a very mild phenotype consisting of a 20–30% reduction in CI and CIII activity and a modest increase in serum lactate (Hashizume et al. 2012). Long term these mice failed to develop any neurological or ophthalmological pathologies (Hashizume et al. 2012).

The Wallace lab also introduced this mutation into a mouse model but in a different strain (Lin et al. 2012). They observed a clear recapitulation of LHON patient pathology (Lin et al. 2012). In addition to a ubiquitous reduction in CI activity, there were highly elevated levels of ROS in neuronal tissue of affected animals. Thus, the *MT-ND6*$^{P25L}$ model provides an opportunity to investigate the molecular pathogenesis of LHON and test potential therapies (Lin et al. 2012).

### *10.2.2 Complex II Deficiency*

#### 10.2.2.1 SDHD

Succinate Dehydrogenase Complex Subunit D (*SDHD)* has been shown to be mutated in several cases of isolated CII deficiency (Jackson et al. 2014; Alston et al. 2012, 2015). Two separate attempts were made to create a mouse model for the study of this gene (Piruat et al. 2004; Bayley et al. 2009). *Sdhd*$^{-/-}$ mice created in the Barneo lab were embryonic lethal while mice heterozygous for the *Sdhd* KO allele had no observable phenotype, despite a decrease in CII activity (Piruat et al. 2004). As mutations in *Sdhd* have also been shown to be involved in a form of hereditary paraganglioma (Schiavi et al. 2006), the Taschner lab created *Sdhd*$^{-/-}$ mice to assess the role of *Sdhd* as tumour supressor. These mice were also embryonic lethal and aged heterozygous mice did not develop tumours (Bayley et al. 2009). As such these models are not useful for the study of *Sdhd* in isolated CII deficiency or as a tumour suppressor.

### *10.2.3 Complex III Deficiency*

#### 10.2.3.1 BCS1L

BCS1 like Ubiquinol-Cytochrome C Reductase Complex Chaperone (*BCS1L*) has been shown to be involved in the final steps of CIII assembly (Fernandez-Vizarra et al. 2007). Mutations in this gene are associated with cases of LS due to CIII deficiency, GRACILE syndrome (growth restriction, aminoaciduria, cholestasis, iron overload, lactic acidosis and early death), Bjornstad syndrome and mitochondrial

CIII deficiency (Visapaa et al. 2002; Hinson et al. 2007; De Lonlay et al. 2001; Blazquez et al. 2009). The Fellman laboratory created a mouse model of GRACILE syndrome by using gene targeting to introduce the *Bcs1l* 232A > G point mutation, *Bcs1l*$^{g/g}$ (Leveen et al. 2011). The pathology they observed in *Bcs1l*$^{g/g}$ mice displayed remarkable similarities to that observed in patients with fatal GRACILE syndrome (Leveen et al. 2011). This included, growth restriction, progressive liver disease and premature death. Importantly, the model recapitulated the tissue specificity observed in patients with pathology arising only in the kidney and liver. Metabolic comparison of pre-symptomatic and symptomatic mice revealed a deterioration of carbohydrate and fatty acid metabolism leading to a starvation like state which may underlie disease onset (Kotarsky et al. 2012). Further analysis of the molecular pathogenesis underlying the *Bcs1l*$^{g/g}$ phenotype could provide key insights into GRACILE syndrome.

## *10.2.4 Complex IV Deficiency*

### 10.2.4.1 COX4I2

Cytochrome C Oxidase Subunit 4 Isoform 2 (*COX4I2)* is a nuclearly encoded subunit of CIV which has been shown to be expressed in lung and pancreatic acinar cells, with patients carrying mutations in *Cox4I2* suffering from exocrine pancreatic insufficiency (Shteyer et al. 2009; Hüttemann et al. 2001). This subunit and that encoded by Cytochrome C Oxidase Subunit 4 Isoform 1 (*COX4I1)* is thought to play important roles the regulation and assembly of CIV. *Cox4i2* KO mice develop a progressive lung pathology with reduced levels of ATP and CIV activity (Huttemann et al. 2012). However, no pancreatic pathology was observed in these mice, this discrepancy could also result from an absence of *Cox4i2* expression in the pancreas of mice (Huttemann et al. 2012).

### 10.2.4.2 SURF1

*SURF1* is an assembly factor involved in CIV biogenesis (Tiranti et al. 1998; Williams et al. 2001). Patients carrying mutations in *SURF1* suffer from LS due to CIV deficiency (Zhu et al. 1998; Tiranti et al. 1998). Two separate *Surf1* KO mice were created by the Zeviani lab which displayed an expected CIV deficiency but unexpectedly did not have any neuropathology, a hallmark of *SURF1* patients (Agostino et al. 2003; Dell'agnello et al. 2007). Although these models will be useful in understanding the biological role of *SURF1* they do not recapitulate the patient phenotype and therefore may not be suitable for the development and validation of novel therapies.

#### 10.2.4.3 COX10

*COX10* is a CIV assembly protein required for biosynthesis of the heme α prosthetic group required for functional CIV (Antonicka et al. 2003a). Mutations and allelic variants of *COX10* have been identified in patients suffering from isolated CIV deficiency and LS due to CIV deficiency (Antonicka et al. 2003a). A mouse model carrying a conditional allele (*Cox10*$^{cKO}$) was used to create model of mitochondrial myopathy, hepatopathy, neuropathy and encephalopathy (Diaz et al. 2008, 2005, 2012a). Conditional ablation of *Cox10* in the liver of mice resulted in phenotypes which recapitulated clinical features of mitochondrial hepatopathies including enlargement of the liver and steatosis (Diaz et al. 2008). Ablation of COX10 in the peripheral nervous system resulted in a severe neuropathy characterized by demyelination, also observed in several patients carrying *Cox10* mutations (Diaz et al. 2012a). Given the stark recapitulation of pathology seen in patients, these models will be important in understanding tissue specific pathogenesis and in the testing and validation of therapies which directly target the underlying mitochondrial dysfunction.

#### 10.2.4.4 COX15

*COX15* is a CIV assembly protein which plays a key role in the heme biosynthetic pathway (Petruzzella et al. 1998). Patients carrying mutations in this gene suffer from LS due to CIV deficiency and cardioencephalomyopathy (Antonicka et al. 2003b; Oquendo et al. 2004). COX15 is required for mouse embryonic development (Viscomi et al. 2011). Mice lacking COX15 specifically in muscle have acute CIV deficiency accompanied by a severe myopathy from 1 month of age (Viscomi et al. 2011).

#### 10.2.4.5 SCO2

*SCO2* is an assembly factor required for the assembly and function of CIV. Mutations in *SCO2* can result in fatal infant cardioencephalomyopathy due to CIV deficiency (Papadopoulou et al. 1999). The E140K amino acid change, corresponding to the n.1541G > A missense mutation most commonly found in patients, was introduced into a mouse model (Baxevanis et al. 2000). This model had a clear CIV defect but unfortunately did not recapitulate human disease pathology.

### *10.2.5 Complex V Deficiency*

#### 10.2.5.1 ATPIF1

Generation of patient relevant mouse models of CV deficiency will allow testing of therapeutic interventions. There are currently no mouse models of CV deficiency based on patient mutations available. To model CV deficiency, a mouse

overexpressing a modified form of human ATPase Inhibitory Factor 1 (*ATPIF1)* in neurons was created (Formentini et al. 2014). ATPIF1 is thought to play an inhibitory role in the hydrolysis of ATP by ATP synthase (CV) when mitochondrial membrane potential is decreased (Formentini et al. 2012). This mutant form of human ATPIF1 has increased affinity with the beta subunit of ATP synthase, inhibiting CV hydrolysis activity, which resulted in increased oxidative stress, reduced levels of respiration and CIV activation, as well as metabolic reprogramming that prevented cell death (Formentini et al. 2014). Generation of patient relevant mouse models of CV deficiency will allow a deeper investigation of molecular pathogenesis and testing of therapeutic interventions.

### *10.2.6 CoQ Deficiency*

$CoQ_{10}$ plays several roles in electron transport within mitochondria. It facilitates the flow of electrons from CI and CII to CIII, acts as an electron acceptor for several dehydrogenases, such as those involved in NADH shuttling from the cytosol to mitochondria (glycerol-3-phosphate dehydrogenase), in fatty acid beta-oxidation (ETF:QOR) and pyrimidine nucleotide synthesis (DHODH), and functions as a potent lipid soluble antioxidant in the plasma membrane and elsewhere (Lopez-Lluch et al. 2010).

Given the central role that CoQ plays in metabolism, it is unsurprising that mutations involving its biosynthesis lead to severe phenotypes and disorders. Human CoQ deficiency can result in several forms of mitochondrial disease including LS, myopathy, myoglobinuria, ataxia, cerebellar atrophy and severe infantile encephalomyopathy with renal failure (Fernandez-Ayala et al. 2014).

CoQ biosynthesis depends on a highly conserved multi-enzyme complex that involves at least eleven genes (named COQ1 to COQ10 plus PDSS2), although some of them have no catalytic activity (Bentinger et al. 2010). Below, we summarize three mouse models of CoQ deficiency.

*COQ7* encodes a hydroxylase that takes part in the latter stages of CoQ biosynthesis. Tamoxifen-induced knockdown of *Coq7* led to weight loss a few days after drug administration, with reduced respiratory chain electron transfer, mitochondrial respiration and survival (Wang et al. 2015). However ROS production was unchanged (Wang et al. 2015).

*COQ9* encodes a lipid-binding protein that is necessary for incorporation of COQ7 into the Q biosynthetic complex (Lohman et al. 2014) and has been shown to be mutated in a patient suffering with primary $CoQ_{10}$ deficiency (Duncan et al. 2009). This patient mutation was used to create a KI mouse ($Coq9^{X/X}$) model of primary $CoQ_{10}$ deficiency (Garcia-Corzo et al. 2013). Homozygous KI mice developed normally but developed neurological symptoms of paralysis and eventually death within 6 months (Garcia-Corzo et al. 2013). Accordingly, these mice had reduced levels of $CoQ_{10}$ as well as decreased CI, mitochondrial respiration and ATP

synthesis (Garcia-Corzo et al. 2013). As such this KI mouse model is an excellent model of encephalopathy due to primary $CoQ_{10}$ deficiency.

*PDSS2* encodes part of the decaprenyl diphosphate synthase involved in CoQ biosynthesis, which has been found to be mutated in a case of infantile COQ deficiency type 3 (COQ10D3) (Lopez et al. 2006; Salviati et al. 2005). *Pdss2*$^{kd/kd}$ mice carry a missense mutation which results in kidney failure and decreased levels of $CoQ_{10}$ (Peng et al. 2008). This can be rescued by CoQ supplementation (Saiki et al. 2008). Conditional ablation within the liver did not result in pathology despite depletion of $CoQ_{10}$ (Peng et al. 2008). Suggesting that there may tissue specific thresholds for sensitivity to decreases in $CoQ_{10}$.

## *10.2.7 Mitochondrial DNA Maintenance*

### 10.2.7.1 Twinkle

*TWINKLE* is the mtDNA helicase and plays a key role in mtDNA replication and maintenance (Spelbrink et al. 2001; Tyynismaa et al. 2004). Homozygous *Twinkle* KO mice were embryonic lethal but conditional ablation within skeletal and cardiac muscle resulted in depleted levels of mtDNA, OXPHOS defects and a reduced survival of only 19 weeks (Milenkovic et al. 2013). Mutation of *Twinkle* in humans can lead to several pathologies, including autosomal dominant progressive external ophthalmoplegia with mtDNA deletions (adPEOA) and mtDNA depletion syndromes (Spelbrink et al. 2001; Hakonen et al. 2008).

Mice carrying adPEOA associated alleles of *Twinkle* (*Twinkle*$^{A360T}$, *Twinkle*$^{dup353-365}$) recapitulate many aspects the human pathology (Tyynismaa et al. 2005). *Twinkle*$^{A360T}$ and *Twinkle*$^{dup353-365}$ mice progressively accumulated mtDNA deletions, developing mitochondrial myopathy characterised by reduced CIV activity and increased mitochondrial proliferation (Tyynismaa et al. 2005). Interestingly, these "deletor" mice showed selective mitochondrial dysfunction in Purkinje cells, located within the cerebellum, and hippocampal CA2 pyramidal neurons which have been previously shown to be affected in adPEOA and other mtDNA depletion syndromes (Tyynismaa et al. 2005). Importantly, physiological analysis of muscle biopsies from these mice demonstrated that the accumulation of mtDNA deletions was associated with reduced OXPHOS function and induced a local and global starvation response (Tyynismaa et al. 2010). Using the *Twinkle* "deletor" mouse models, Nikkanen and colleagues outline a metabolic response to mitochondrial dysfunction which results in altered 1C metabolism and induction of serine directed glutathione synthesis as a possible mechanism to stabilise dNTP synthesis, which is also present in patients suffering from mitochondrial myopathy (Nikkanen et al. 2016). This validates the *Twinkle* "deletor" mouse as a disease model and provides several targets for therapy of mitochondrial myopathy.

#### 10.2.7.2 *POLG* and *POLG2*

*POLG* encodes the catalytic subunit of the mitochondrial DNA polymerase γ, providing DNA polymerase and 3′-5′ exonuclease activity (Lamantea et al. 2002). Several hundred mutations of *POLG* have been described in patients which can result in several clinical presentations caused by mtDNA instability (Lamantea et al. 2002; Milone and Massie 2010). As such, accurately modelling disease pathogenesis of *POLG* mutation is challenging. Unsurprisingly, mtDNA stability is required during development, *Polg* KO mice are embryonic lethal and display severe mtDNA depletion (Hance et al. 2005).

Several mouse models have been created to address the pathology underlying *POLG* mutation (Lewis et al. 2007; Bensch et al. 2009; Kasahara et al. 2006; Zhang et al. 2000, 2005). The first *Polg* mouse model created by Zhang et al., used conditional expression of an exonuclease deficient *Polg*, $Polg^{D181A}$ within mouse cardiac muscle (Zhang et al. 2000). These mice developed cardiomyopathy associated with increased levels of mtDNA mutations (Zhang et al. 2000). Further analysis of this model revealed activation of a pro-survival response within the cardiac muscle, reasoned to be a response to activation of cell death pathways in the face of increased mtDNA instability (Zhang et al. 2005). This same mutation was also overexpressed in neurons and pancreatic β cells (Bensch et al. 2009). In neurons, expression of $Polg^{D181A}$ resulted in increased mtDNA instability in the forebrain leading to neurological dysfunction relating to behaviour and mood, in line with what has been observed in patients suffering from progressive external ophthalmoplegia (PEO) with mood disorder (Kasahara et al. 2006). In the pancreas, expression of $Polg^{D181A}$ within islets resulted in early onset diabetes due to β cell death and insulin insufficiency which associated with increased mtDNA mutations (Bensch et al. 2009).

To access the role of mtDNA mutations in aging two Knock IN mice for the $Polg^{D257A}$ mutation, which ablates proof reading of *Polg*, were created (Kujoth et al. 2005; Trifunovic et al. 2004). Although the role of mitochondrial dysfunction in aging is not the focus of this chapter, several relevant phenotypes where described in these mice (Kujoth et al. 2005, Trifunovic et al. 2004) . These mice, often referred to as "mutator" mice, accumulated mtDNA mutations and deletions which led to progressive OXPHOS deficiency (Kujoth et al. 2005; Trifunovic et al. 2005). Importantly, follow up analysis on this model revealed distinct tissue specific pathology due to this KI allele, $Polg^{D257A}$.

Finally, the Y995C mutation associated with PEO was transgenically expressed in mouse cardiac muscle and resulted in increased oxidative stress, reduced levels of mtDNA and cardiomyopathy, as seen with the expression of $Polg^{D181A}$, a proof reading deficient mutant, in mouse heart (Lewis et al. 2007).

*POLG2* encodes the accessory subunit required for Polymerase γ function. Although rarer, mutations of *POLG2* have been described in patients (Longley et al. 2006). Like *Polg*, homozygous loss of *Polg2* results in embryonic lethality with severe mitochondrial defects, underlining the requirement of mtDNA stability for mammalian development (Humble et al. 2013). Importantly half the dose of either *Polg or Polg2* is sufficient for mitochondrial function, development and lifespan.

Given the number of described pathogenic mutations the mouse may not be the most suitable model for assessment of the molecular pathology of each disease causing lesion. This is an example where complementary models such as *Drosophila melanogaster* or *C. elegans* could play a role.

#### 10.2.7.3 TFAM

*Mitochondrial transcription factor A* (*TFAM*) is required for mtDNA transcription and packaging and mitochondrial biogenesis (Stiles et al. 2016). In humans, the singular described mutation of *TFAM* resulted in mtDNA depletion syndrome (Stiles et al. 2016).

Due likely to its role in maintaining mtDNA stability, homozygous loss of *Tfam* is embryonic lethal (Larsson et al. 1998). *Tfam* has been conditionally ablated in skeletal muscle, cardiac muscle, pancreatic β cells, adipose tissue, forebrain and dopaminergic neurons (Wredenberg et al. 2002; Wang et al. 1999; Silva et al. 2000; Sörensen et al. 2001; Ekstrand et al. 2007; Vernochet et al. 2012). In all models, *Tfam* deletion resulted in mtDNA depletion and progressive OXHOS deficiency and except for the adipose tissue, this led to tissue specific pathologies. In the adipose tissue, this dysregulation of mitochondrial biology led to an increased insulin resistance and obesity prevention on a high fat diet.

Given the penetrance of the phenotypes seen in mouse models of *Tfam* deletion it is not surprising that only 2 cases of patients with *TFAM* mutations have been described so far.

#### 10.2.7.4 LRPPRC

*Leucine-rich pentatricopeptide repeat containing protein* (*LRPPRC*) works in complex with other mitochondrial proteins to regulate post transcriptional gene expression of mitochondrial encoded transcripts (Ruzzenente et al. 2012; Sasarman et al. 2010). The French-Canadian variant form of LS (FCLS) is caused by the c.1119C > T mutation of *LRPPRC*, which is characterised by a severe defect in CIV with neuro and hepatopathy (Debray et al. 2011).

Homozygous loss of *Lrpprc* results in developmental delay, CIV deficiency and embryonic lethality (Ruzzenente et al. 2012; Xu et al. 2012). Conditional ablation of *Lrpprc* in the skeletal or cardiac muscle of mice led to reduced lifespan, development of progressive cardiomyopathy and CIV deficiency (Ruzzenente et al. 2012). The sensitivity of CIV function to mutation in *LRPPRC* could be a result of binding of LRPPRC to mRNA of CIV core subunit *COX1* (Xu et al. 2012). However, in a model of *Lrpprc* loss in the mouse heart, a defect in CV assembly was found to underlie the OXPHOS defect (Mourier et al. 2014). Our knowledge of *LRPPRC* function is modest and although those mouse models currently available recapitulate aspects of FCLS, we may need to build new (i.e. neuronal or liver specific

ablation) or utilise complementary models to have a more disease relevant model for analysis of molecular pathology.

#### 10.2.7.5 ANT1

*Adenine nucleotide translocator isoform 1* (*ANT1*) is a member of a family of adenine nucleotides translocators (ANTs) which are responsible for the exchange of ADP and ATP across the inner mitochondrial membrane (Li et al. 1989). Mutations in *ANT1* can be found in patients suffering from several types of mtDNA depletion syndromes with cardiomyopathy and myopathy, as well as adPEO (Kaukonen et al. 2000; Thompson et al. 2016). The Wallace laboratory created a *Ant1* KO mouse (*Ant1$^{PGKneo}$*) which faithfully recapitulated the features of a mitochondrial myopathy and cardiomyopathy (Graham et al. 1997). In line with patients, *Ant1$^{PGKneo}$* mice had myopathy characterised by ragged red fibres (RRFs), COX negative fibres and dysfunctional mitochondria (Graham et al. 1997). These mice also displayed metabolic adaptations, including increased levels of lactate and Krebs cycle intermediates, as well as increased oxidative stress and accumulation of mtDNA deletions (Graham et al. 1997). Importantly and in contrast to patients, *Ant1$^{PGKneo}$* mice do not display any neuronal or ophthalmological pathology which might be due to expression of another *Ant* family member (Lee et al. 2009; Phillips et al. 2010).

Despite the absence of a neuronal or ocular phenotype, the *Ant1$^{PGKneo}$* mouse is a great model of mitochondrial myopathy and as such is an excellent model for molecular investigation of mitochondrial myopathy, as well as validation of targeted therapies.

#### 10.2.7.6 TK2

The mitochondrial thymidine kinase is encoded by *TK2* (Johansson and Karlsson 1997). It catalyses the first step of the nucleotide salvage pathway and has been shown to be mutated in patients suffering from mitochondrial depletion syndrome type 2 (MTDS2) and autosomal recessive PEO (PEOB3) (Boustany et al. 1983; Moraes et al. 1991; Tyynismaa et al. 2012). Homozygous *Tk2$^{-/-}$* mice are viable but develop encephalopathy and die within the first 4 weeks of life (Zhou et al. 2008). This was mirrored in a KI model where a point mutation corresponding to a human pathogenic mutation, H121N, was introduced (Akman et al. 2008). *Tk2$^{KI}$* mice also displayed encephalopathy, early mortality and reduced levels of mtDNA (Akman et al. 2008). Importantly and in contrast to patients, neither mouse model developed myopathy which is a key characteristic of MTDS2. However, a detailed analysis of the *Tk2$^{-/-}$* mouse described an altered transcriptional signature and pre-clinical phenotypes in heart and skeletal muscle (Paredes et al. 2013). Conditional or inducible models of *Tk2* loss specifically in the muscle may allow modelling of this myopathic mtDNA depletion syndrome.

#### 10.2.7.7 TYMP

*TYMP* encodes thymidine phosphorylase which is essential for the nucleotide salvage pathway (Matsukawa et al. 1996). Mitochondrial neuro-gastrointestinal encephalopathy (MNGIE) is a mtDNA deletion syndrome characterised by gastrointestinal blockage, PEO and mitochondrial dysfunction (Hirano et al. 2012). Most MGNIE cases are a result of *TYMP* mutation (Hirano et al. 2012). The phenotype of a mouse model of *TYMP* deficiency (*Tymp* $^{-/-}$, *Up* $^{-/-}$) partially overlapped with that of MNGIE patients (Haraguchi et al. 2002). With increased levels of thymidine and deoxyuridine in several tissues and in the brain, progressive mtDNA depletion (Lopez et al. 2009).

#### 10.2.7.8 RRM2B

*RRM2B* encodes the small subunit of the p53-controlled ribonucleotide reductase (Tanaka et al. 2000). Mutations in *RRM2B* have been associated with mtDNA depletion syndromes, PEO and MNGIE (Bourdon et al. 2007; Kollberg et al. 2009; Tyynismaa et al. 2009).

*Rrm2b* KO mice (*Rrm2b* $^{-/-}$) developed normally, had retarded growth after weaning and died at 14 weeks of severe kidney failure (Kimura et al. 2003). In a comparative analysis of *Rrm2b* $^{-/-}$ mice and patients carrying mutation in *RRM2B*, severe depletion of mtDNA in the absence of a OXPHOS defect was described (Bourdon et al. 2007). In both studies, *Rrm2b* mutation resulted in a decrease in the dNTP pool suggesting that *Rrm2b* is required for replenishing the dNTP pool (Bourdon et al. 2007; Kimura et al. 2003). *Rrm2b* $^{-/-}$ mice mirror the patient phenotype and given the association of *RRM2B* with several mitochondrial diseases this and future models of *Rrm2b* mutation could be used to validate therapies for mtDNA exhaustion resulting from dNTP deficiency.

#### 10.2.7.9 MPV17

*MPV17* encodes a protein of the mitochondrial inner membrane whose function has not yet been delineated. Work in yeast has shown that Sym1 (yeast orthologue of *MPV17*) is required for maintenance of mitochondrial morphology and mtDNA maintenance in stress conditions (Dallabona et al. 2010). In humans, mutation of *MPV17* leads to mtDNA depletion syndrome 6 (MTDPS6) which is an infantile syndrome characterised by liver failure. Unfortunately, *Mpv17* KO mice (*Mpv17* $^{-/-}$) did not develop liver failure despite a reduction in mtDNA levels leading to CI and CIV deficiency (Weiher et al. 1990). Liver failure was induced in *Mpv17* $^{-/-}$ mice fed a ketogenic diet (Bottani et al. 2014). Clues to the pathogenic mechanisms which underlie MTDPS6 may be found by focusing on understanding the function of *MPV17*.

### 10.2.8 Mitochondrial Dynamics

#### 10.2.8.1 MFN1 and MFN2

The mitochondrial GTPases Mitofusin 1 and 2 (*Mfn1/2*) are essential for mouse development (Chen et al. 2003, 2007). However, they are partially redundant with overlapping and distinct roles. Loss of both MFN 1 and 2 in the skeletal muscle results in muscle atrophy, increased levels of lactate and premature death at 6–8 weeks of age (Chen et al. 2010). These mice have severely impaired muscle mitochondria, with a progressive reduction in mtDNA levels, despite a compensatory increase in mitochondrial biogenesis (Chen et al. 2010). Additionally, the remaining copies of mtDNA were found to have an increased number of point mutations and deletions. This work suggests that fusion is required to maintain mitochondrial integrity.

MFN2 has been conditionally deleted in several different tissues including the heart, kidney and liver (Papanicolaou et al. 2011; Gall et al. 2012; Sebastian et al. 2012). In all cases, loss of MFN2 resulted in dysfunction or tissue pathology, but did not mirror phenotypes observed in patients carrying pathological mutations of MFN2. To have a more patient relevant model, mice expressing mutant alleles of MFN2 associated with Type 2A Charcot Marie Tooth Disease (CMT2A), a peripheral polyneuropathy, were created (Detmer et al. 2008; Cartoni et al. 2010). As expected, in both cases animals displayed phenotypes consistent with those of CMT patients such as defective motor skills, axonopathy and incomplete disease penetrance (Detmer et al. 2008; Cartoni et al. 2010). However, the molecular pathogenesis may differ, as density and distribution of mitochondria in axons was not consistent between these models (Detmer et al. 2008, Cartoni et al. 2010). Nevertheless, these models have allowed examination of CMT2A pathogenesis and in future, could be utilised to further understand the interplay between mitochondrial distribution and neuronal function.

#### 10.2.8.2 OPA1

Mutations in Optic atrophy 1 (*OPA1*) are associated with autosomal dominant optic atrophy (adOA) (Delettre et al. 2000). Mouse models homozygous for two mutant alleles which result in truncated forms of Opa1, die during early embryogenesis (Alavi et al. 2007; Davies et al. 2007). Heterozygous animals with around a 50% decrease in Opa1 displayed progressive vision loss as well as neurological symptoms, such as reduced locomotor activity. As seen in the case of MFN2, the phenotypes of these models did not completely overlap. However, further characterisation of both these models uncovered symptoms that were not restricted to retinal ganglion cells (RGCs), the cell type preferentially affected in adOA (Williams et al. 2012).

Mice homozygous for a common adOA deletion (*Opa1*$^{delTTAG}$) died at around E10.5, while heterozygous mice recapitulated phenotypes seen in patients with the syndromic form of adOA (adOA plus) (Sarzi et al. 2012). This included deafness,

encephalomyopathy, cardiomyopathy and defects of the peripheral nervous system (Sarzi et al. 2012). This was in addition to loss of RGCs and vision loss observed in previous mouse models and most adOA patients. At the molecular level these mice displayed reduced CIV activity and incorporation of CIV into stable supercomplexes (Sarzi et al. 2012).

Taken together these models clearly demonstrate the requirement of *OPA1* for normal mammalian development, where complete loss of *OPA1* leads to a fragmented mitochondrial network unable to support life. Analysis of dominant Opa1 alleles highlights the pleotropic effects Opa1 mutations. Mouse models which show RGC specific phenotypes will be useful in further understanding the sensitivity of this tissue to Opa1 loss. The more severe syndromic model of adOA ($Opa1^{delTTAG}$) not only mirrors adOA patients but also several clinical features seen across various forms of systemic mitochondrial disease. Validating this model for use in the identification of potential therapies.

### *10.2.9 Additional Mouse Models*

#### **10.2.9.1 AIFM1**

Apoptosis Inducing Factor, Mitochondria Associated 1 (*AIFM1)* is a flavoprotein located in the mitochondrial intermembrane space, that acts both as a NADH:cytochrome c oxidoreductase in healthy cells and as an inducer of caspase independent apoptosis (Joza et al. 2009). Mutations in this gene can result in combined oxidative phosphorylation deficiency 6 (COXPD6) and Cowchock syndrome (Cowchock et al. 1985; Fischbeck et al. 1986; Ghezzi et al. 2010). A proviral insertion in the *Aifm1* gene resulted in an 80% reduction in the harlequin mouse model (*Hq*) (Klein et al. 2002). Further analysis of this model of neurodegeneration showed an isolated effect on CI function. Mitochondria from retinal and neuronal tissue of *Hq* mice had reduced CI activity, abundance and expression of CI subunits (Vahsen et al. 2004). The relationship between levels of *Aifm1* and CI was further underlined when conditional deletion within skeletal and cardiac muscle resulted in an 80% reduction in CI activity, atrophy of the skeletal muscles and cardiomyopathy (Joza et al. 2005). However, patients who carry mutations in *AIFM1* do not seem to carry complementary defects in CI but instead in respiratory CIII and CIV (Ghezzi et al. 2010). Further analysis of patients and available models will help elucidate the link between these two entities.

#### **10.2.9.2 FXN**

*FRATAXIN (FXN)* is a mitochondrial protein which has been shown to play roles in several processes, including heme and iron-sulphur cluster biosynthesis and antioxidant defence (Ristow et al. 2000; Shoichet et al. 2002; Martelli et al. 2007).

Friedreich's ataxia (FDRA) is a mitochondrial disease which is caused by expansion of a GAA triplet repeat in an intron of *FXN (*Campuzano et al. 1996*)*. Patients suffer from neurodegeneration, cardiomyopathy and diabetes, presenting with a distinct biochemical signature which includes a decrease in the levels of iron-sulphur cluster containing enzymes and an increase in markers of oxidative damage (Delatycki et al. 2000).

Homozygous *Fxn* null mice are early embryonic lethal, demonstrating the importance of *Fxn* in early embryogenesis (Cossée et al. 2000). Conditional KO of *Fxn* was performed in the heart, brain, liver and pancreatic β cells (Puccio et al. 2001; Ristow et al. 2003; Simon et al. 2004; Martelli et al. 2012). As in patients, tissues specific depletion of *Fxn* in the brain resulted in progressive neurodegeneration, in the heart in cardiomyopathy and in the pancreas, the onset of diabetes (Puccio et al. 2001, Ristow et al. 2003, Simon et al. 2004, Martelli et al. 2012). However, these phenotypes were accelerated and enhanced in mouse models when compared to patients but these mice failed to show any signs of oxidative damage (Puccio et al. 2001, Ristow et al. 2003, Simon et al. 2004, Martelli et al. 2012). Given what is known about other forms of repeat disease such as several forms of spinocerebral ataxia and myotonic dystrophy, the effects of expansion of the GAA triplet on the levels of FXN may be more nuanced than a complete KO of the gene. Accordingly a mouse model which overexpresses the human *FXN* gene with the GAA repeat mirrored the patient phenotype, developing a milder progressive form of ataxia with an accumulation of oxidative damage (Al-Mahdawi et al. 2006).

#### 10.2.9.3 ETHE1

*ETHE1* encodes a detoxification enzyme, present within mitochondria, preventing the accumulation of damaging hydrogen sulphide ($H_2S$) (Tiranti et al. 2004). Ethylmalonic encephalopathy (EE) is a severe childhood mitochondrial disorder characterised by encephalopathy, petechiae and gastrointestinal disturbances. Patients also excrete large quantities of ethylmalonic acid in their urine (Tiranti et al. 2004).

To gain a better understanding of EE pathology Tiranti *et al* created *Ethe1 KO* (*Ethe1*$^{-/-}$) mice and analysed them together with EE patients (Tiranti et al. 2009). They described a striking recapitulation of the patient phenotype, with *Ethe1*$^{-/-}$ mice showing symptoms of EE within the first two weeks of life and succumbing within the first 6 weeks (Tiranti et al. 2009). *Ethe1*$^{-/-}$ mice had arrested growth, pronounced CIV deficiency in muscle, brain and colon, increased levels of lactate, C4 and C5 acylcarnitines and increased levels of ethylmalonic acid and thiosulfate (Tiranti et al. 2009). Conditional ablation in muscle, brain and liver failed to phenocopy *Ethe1*$^{-/-}$ mice (Di Meo et al. 2011). While CIV deficiency was clear in targeted tissues, it was not sufficient to result in increased levels of ethylmalonic acid and thiosulfate in urine, observed in patients and *Ethe1*$^{-/-}$ mice (Di Meo et al. 2011).

The *Ethe1*$^{-/-}$ mouse model provides an excellent opportunity to develop targeted therapies for EE patients which can prevent or negate the accumulation of toxic $H_2S$.

#### 10.2.9.4 RNASEH1

*RNASEH1* encodes a ribonuclease which is required in mitochondria for mtDNA replication (Reyes et al. 2015). *RNASEH1* mutations have been associated with adult-onset encephalopathy with chronic PEO and mtDNA deletions (Reyes et al. 2015). *RNaseH1* null mice are embryonic lethal due to depletion of mtDNA resulting in cell death (Cerritelli et al. 2003). Conditional and inducible deletion of *RNaseH1* specifically in the liver also resulted in reduced levels of mtDNA, with clear defects in mitochondrial morphology. These mice also displayed progressive liver degeneration (Lima et al. 2016). These models have allowed the delineation and conformation of *RNASEH1* function. The liver specific models could in future allow modelling of liver dysfunction (hepatopathy) due to mtDNA depletion.

## 10.3 *Drosophila* Models of Mitochondrial Disease

The fruit fly, *Drosophila melanogaster,* has long been used in the study of several human pathologies (Vidal and Cagan 2006; Chan and Bonini 2000; Stefanatos and Vidal 2011). Despite its' relative simplicity, the fly genome shows remarkable conservation with that of humans, with the advantage of reduced genetic and functional redundancy. As a model organism to study mitochondrial disease, *Drosophila* present many advantages. There is a high degree of conservation of mitochondrial proteins and many patient phenotypes can be assessed in a whole animal model (Jacobs et al. 2004). The major advantage of the fly is the plasticity that the genetic tools available in *Drosophila* confer (Jennings 2011). While several mutations in mitochondrial proteins have been isolated in *Drosophila*, the true power of this model organism lies in the ability to utilise methods such as RNA interference (RNAi) in combination with the binary GAL4/UAS system for modulation of gene expression as well as the several methods of transgenesis available (Brand and Perrimon 1993; Lee and Luo 2001; St Johnston 2002; Dietzl et al. 2007; Perkins et al. 2015). Together these tools allow tissue specific or global, depletion or deletion of any gene of interest. Over the last 10 years the *Drosophila* research community has established several models of mitochondrial dysfunction. While we must keep in mind the differences between mammalian and insect physiology, the fly possesses similar if not the same cellular and physiological functions with a high degree of conservation. Importantly, flies with mutations in mitochondrial genes recapitulate several aspects of mitochondrial disease (Toivonen et al. 2001; Vartiainen et al. 2014).

### 10.3.1 Complex I

Li and colleagues have utilized P-element mediated excision of *CG12079/ND-30,* the fly orthologue of CI subunit *Ndufs3,* to create novel mutant alleles (Li 2013). They find that *CG12079/ND-30* mutants have severely reduced CI activity and aberrant assembly (Li 2013). Most developing larvae do not make it to adulthood but characterization of rare escapers revealed increased oxidative stress as well as activation of compensatory increases in mitochondrial biogenesis (Li 2013). These flies also display seizure sensitivity and defective climbing ability, phenotypes which can be extrapolated to mitochondrial disease patients (Jacobs et al. 2004). RNA interference (RNAi) mediated depletion of *Ndufs3* results in decreased ATP levels and membrane potential (Vos et al. 2012). Although this model was not further analysed it demonstrates the power of the availability of RNAi constructs against most of the genome to induce mitochondrial dysfunction (Dietzl et al. 2007, Perkins et al. 2015).

Mutants have also been isolated for two CI assembly factors *Ndufaf1* (*CG7598*) and *Ndufaf6* (*CG15738*) (Cho et al. 2012; Zhang et al. 2013). In the case of *Ndufaf1*, deletion resulted in developmental arrest with defects in mitochondrial structure and a loss of CI holoenzyme (Cho et al. 2012). RNAi mediated depletion of *Ndufaf1* (*CG7598*) also resulted in loss of CI holoenzyme and defects in mitochondrial structure (Cho et al. 2012). Two separate mutations of *Ndufaf6* isolated from an unbiased forward genetic screen resulted in decreased CI activity and increased ROS levels (Zhang et al. 2013).

Burman and colleagues took advantage of a *mt-ND2* mutant created using a mitochondrially targeted restriction enzyme (Burman et al. 2014). In humans, mutations in *MT-ND2* are associated with Leber Optic Atrophy (LHON) and CI deficiency (Johns and Berman 1991; Schwartz and Vissing 2002; Pulkes et al. 2005). $ND2^{del1}$ flies, which carry a small deletion in *ND2*, show an increase in heat, mechanical stress, hypoxic and hypercarbic induced paralysis as well as a reduced lifespan (Burman et al. 2014). Although there were no signs of muscle pathology, aged mutants displayed progressive neurodegeneration (Burman et al. 2014). Biochemically $ND2^{del1}$ flies have an isolated CI respiration defect, reduced levels of assembled CI, reduced membrane potential and ATP production. This model recapitulates many aspects of CI deficiency and provided insights into the role of *mt-ND2*. Importantly this model has also been recently used to parallel the therapeutic effects of rapamycin described in a mouse model of CI deficiency further validating its use in the investigation of mitochondrial disease pathogenesis (Johnson et al. 2013; Wang et al. 2016).

### 10.3.2 Complex II

*SDHB* is one of four subunits of CII. An insertional mutant for *Sdhb* (*CG3283)* in flies displayed a CII specific respiratory defect accompanied by a reduction in CII activity and an increase in mitochondrial ROS (Walker et al. 2006). Mutation

of *Sdhaf3* (*CG14898*) resulted in reduced CII activity, increased sensitivity to oxidative stress and motility defects (Na et al. 2014). Loss of *Sdhaf4* (*CG7224*) also resulted in a severe decrease in CII activity and increased sensitivity to oxidative stress as well as neurodegeneration and reduced survival (Van Vranken et al. 2014).

### *10.3.3 Coenzyme Q*

In flies, the first model of CoQ deficiency was isolated in the Gould lab using a mosaic screen to identify modifiers of neuroblast size (Grant et al. 2010). This technique in fruit flies allows analysis of wild type and mutant cells within the same tissue, as well as allowing the study of mutations that are lethal, at any stage in development (Lee and Luo 2001). They identified *qless* (*CG31005*), the *Drosophila* orthologue of *PDSS1*, involved in the first step of COQ biosynthesis (Grant et al. 2010). Investigation of *qless* in the developing CNS revealed a growth defect in *qless* mutant clones which were smaller than wild type clones (Grant et al. 2010). *qless* mutant clones undergo caspase dependant apoptosis and express the marker of mitochondrial stress, Hsp60A. These phenotypes are rescued by dietary supplementation with CoQ10 (Grant et al. 2010). As well as being the first to study the effects of *PDSS1* loss in the fly, Grant and colleagues highlight how the power of mosaic analysis for the investigation of gene function.

*COQ2* catalyses one of the final reactions in CoQ biosynthesis (Forsgren et al. 2004). Mutants of *Coq2* (*CG9613*) created using P-element mediated excision display developmental arrest and decreased levels of ATP (Liu et al. 2011). Animals heterozygous for mutant *CoQ2* had increased lifespan and inhibited insulin signalling which has been shown to have life extending effects (Liu et al. 2011). This model does not present a viable model of mitochondrial disease; however, mosaic analysis of this mutation may provide more insights into gene function.

Finally using RNAi technology, Fernandez-Ayala and colleagues have systematically depleted enzymes required for CoQ biosynthesis including, *Pdss1* (*CG31005*), *Coq2* (*CG9613*), *Coq3* (*CG9249*), *Coq5* (*CG2453*), *Coq6* (*CG2453*), *Coq7* (*CG14437*), *Coq8* (*CG32649*), *Coq9* (*CG30493*) and *Coq10* (*CG9410*) ubiquitously. Apart from *Coq6*, depletion of these enzymes resulted in development lethality (Fernandez-Ayala et al. 2014). *Coq6* knockdown flies had severe CoQ deficiency and the presence 5-dimethoxy ubiquinol (DMQ) a late stage CoQ biosynthesis intermediate was detected in COQ3, 6, and 9 knockdown animals (Fernandez-Ayala et al. 2014).

Mutation or RNAi mediated depletion of biosynthetic CoQ enzymes in flies results in primary CoQ deficiency. As such these models will allow study of the role of CoQ in development and mitochondrial biology in health and disease.

### 10.3.4 Complex III

The Zeviani Laboratory described mutations in tetratricopeptide repeat protein *TTC19* which resulted in severe CIII deficiency accompanied by progressive encephalopathy (Ghezzi et al. 2011). A fly KO line for *Ttc19* (*CG15173*) created by transposon insertion displayed a severe deficiency in CIII activity, reduced locomotor activity and an increased sensitivity to mechanical stress (Ghezzi et al. 2011). The parallels between the patient phenotype and that seen in the KO flies underlines the high degree of conservation of genes involved in mitochondrial respiration. Importantly, using RNAi technology to knockdown *Ttc19* down by 70% did not result in the same phenotypes observed in KO flies, highlighting that for some disease genes more than one model may be required (Ghezzi et al. 2011).

### 10.3.5 Complex IV

The first model of CIV deficiency described in *Drosophila* was the *levy*[1] mutation in the orthologue of *COX6A1*, *CG11015* (Liu et al. 2007). Initially identified in a screen of temperature sensitive paralytic mutants, *levy*[1] flies have a severe reduction in CIV activity, less ATP, increased sensitivity to mechanical stress and neurodegeneration as early as seven days old (Liu et al. 2007). Even though mutations in *Cox6a1* have not been described in patients we have included this model as the severity of the phenotype and clear neurodegeneration recapitulates the main features of CIV deficiency in humans. This is very important given that knockdown of *Surf1*, that is often found mutated in patients with LS due to CIV deficiency, does not result in neurodegeneration in flies or mice (Da-Re et al. 2014; Dell'agnello et al. 2007).

Ubiquitous depletion of several nuclearly encoded subunits of CIV via RNAi, including *Cox6b1,6c, and 7a,* results in developmental lethality (Kemppainen et al. 2014). Tissue specific depletion in the CNS resulted in a variety of phenotypes including no effect i.e. indistinguishable from wild type and severe neurodegeneration depending the targeted subunit (Kemppainen et al. 2014). Again, highlighting that for some disease genes RNAi knockdown models may not be appropriate.

The *SCO1* and *SCO2* genes, represented by one member in flies (*CG8885*), are required for the biogenesis of the core subunit of CIV, *MT-CO2*. Patients carrying mutations in *SCO1* suffer from CIV deficiency (Valnot et al. 2000). In flies, null mutations of *CG8885* result in developmental lethality while milder alleles result in female sterility (Porcelli et al. 2010). All mutants display a reduction in CIV activity with a reduction in locomotor activity in mutant adults (Porcelli et al. 2010). Given these robust phenotypes flies carrying patient relevant mutations in this gene may provide a more tractable system to study disease pathogenesis and therapeutic validation.

Finally, van Bon and colleagues studied the role of *Cep89* thought to modulate CIV activity *in vitro* in cell culture and *in vivo* in *Drosophila,* after isolating a mutation in a patient suffering from CIV deficiency (Van Bon et al. 2013). Ubiquitous RNAi depletion of the *Drosophila* orthologue of *Cep89* (*CG8214*), resulted in reduced CIV activity and developmental lethality (Van Bon et al. 2013). Tissue specific loss of *Cep89* in either the muscle or CNS also resulted in lethality with a few escapers who presented with severe motor defects. Closer inspection of viable knockdown flies revealed serious neuronal aberrations and learning defects. This study highlights how use of complementary models can allow molecular and pathological examination which can elucidate the role of novel mitochondrial disease genes identified in patients.

### *10.3.6 Complex V*

Mutations of the mitochondrially encoded CV subunit *MT-ATP6* have been shown to cause maternally inherited LS (MILS), Neuropathy, ataxia, retinitis pigmentosa (NARP) and familial bilateral striatal necrosis (FBSN). An endogenous missense mutation of *mt-atp6* was isolated from *Drosophila ANT1* mutants, $sesb^{1}$ (Celotto et al. 2006; Palladino 2010). *mt-atp6* mutant flies ($ATP6^{1}$) had abnormal mitochondrial morphology, no detectable CV activity but respiration was not affected. $ATP6^{1}$ flies had a reduced survival, progressive neuromuscular pathology and locomotor deficiency, mirroring the main features observed in MILS patients (Celotto et al. 2006, Palladino 2010).

### *10.3.7 Associated Mitochondrial Proteins*

Excluding those genes which are directly implicated in OXPHOS, knockout and knockdown models of several associated mitochondrial proteins have been created in *Drosophila*. In humans, mutation of *ANT1*, the ADP/ATP transporter, results in Progressive External Ophthalmoplegia with Mitochondrial DNA deletions (adPEO) and mitochondrial DNA depletion syndrome (Kaukonen et al. 2000; Thompson et al. 2016). Flies carrying a point mutation in *sesB* (*CG16944*), the *Drosophila ANT1* orthologue, are developmentally delayed, sensitive to mechanical stress and have compromised respiration (Zhang et al. 1999; Vartiainen et al. 2014). Analysis at the transcriptional and metabolic level revealed a glycolytic shift and increased mitochondrial stress (Vartiainen et al. 2014). Importantly, this phenotype overlapped with that of the fly model of mitochondrial disease, $tko^{25t}$, orthologous to human *MRPS12* (Royden 1987; Toivonen et al. 2001; Vartiainen et al. 2014). $tko^{25t}$ flies are developmentally delayed, sensitive to mechanical stress, have a combined OXPHOS defect and display metabolic remodelling (Fernandez-Ayala et al. 2010;

Toivonen et al. 2001). This strongly suggests that some mitochondrial diseases could have common therapeutic targets.

Mutation of the DNA polymerase *POLG2* (*CG33650*) in developing larvae leads to depletion of mtDNA, increased mitochondrial biogenesis and altered mitochondrial neuronal transport (Baqri et al. 2009). This study provided key insights into how altered mitochondrial trafficking in neurons populated with mtDNA deficient mitochondria may underlie pathogenesis of mtDNA depletion syndromes. The Larsson lab have recently used genetic engineering to create flies with an exonuclease (DmPOLγ$^{exo-}$) deficient form of *POLG* (*CG8987*) (Bratic et al. 2015). They found that DmPOLγ$^{exo-}$ flies accumulated mtDNA mutations, although several fold less than the equivalent "deletor" mouse model despite a clear developmental arrest at the larval stage (Bratic et al. 2015). Using this model, the authors could assess the transmission of these deletions and the existence of a mtDNA mutation threshold after which pathogenic effects were observed.

The fluid state of the mitochondrial network is regulated by the action of Dynamin related protein 1 (*DRP1*), which promotes mitochondrial fission, and Optic Atrophy 1 (*OPA1*) and Mitofusin 1 and 2 (*MFN1/2*) which promote mitochondrial fusion. Homozygous mutation of *Drp1* (CG3210), *Opa1*(*CG8479*) and *Marf* (*CG3869*) are developmentally lethal (Verstreken et al. 2005; Dorn et al. 2011; Debattisti and Scorrano 2013). Examination of *Drp1* mutant larvae uncovered a role for *DRP1* in maintaining cellular distribution of neuronal mitochondria and mitochondrial function as ATP levels were also depleted in *Drp1* mutants (Verstreken et al. 2005). UAS/GAL4 mediated depletion of *Opa1* or *Marf*, the single orthologue of MFN1 and 2, within cardiac muscle resulted in altered mitochondrial morphology and cardiomyopathy (Dorn et al. 2011). Confirming the functional redundancy between the human and fly genes, co-expression of MFN1 or MFN2 could rescue these phenotypes in *Marf* knockdown flies (Dorn et al. 2011). *Opa1* heterozygous flies have a reduced lifespan, increased oxidative stress, altered mitochondrial morphology and reduced activity of CII and III. Mosaic analysis of *Opa1* mutations demonstrated the requirement of Opa1 in the developing eye, where altered mitochondrial morphology and increased ROS levels resulted in a miss-patterned eye due to ectopic cell death (Yarosh et al. 2008).

## 10.4 Zebrafish Models of Mitochondrial Disease

*Danio rerio*, commonly referred to as the zebrafish, is a popular vertebrate model system used extensively in developmental studies (Eisen 1996; Fishman 1999). As tools for reverse genetic approaches in zebrafish have improved, their use to study the function of human disease genes has increased (Eisen 1996, Fishman 1999). There are several advantages to working with zebrafish including the availability of genetic tools for gene silencing and transgenesis, as well as high degree of genetic conservation (Wienholds et al. 2003; Soroldoni et al. 2009; Nasevicius and Ekker 2000).

To study mitochondrial disease using zebrafish, several tools and assays have been developed over the last ten years that allow real time visualization of mitochondrial networks and the assessment of physiological effects of mitochondrial dysfunction (Broughton et al. 2001; Kim et al. 2008; Plucinska et al. 2012). Below we highlight those cases where genes described to be mutated in patients have been modelled in zebrafish.

The first zebrafish model of mitochondrial dysfunction was described by Baden and colleagues (Baden et al. 2007). Using morpholinos against CIV subunit, COX5A (*cox5aa* and *cox5ab*) and CIV assembly factor, SURF1 (*surf1*), they could reduce CIV activity by around 50% (Baden et al. 2007). They also found a concomitant decrease in the levels of the mitochondrially encoded core subunit MT-CO1 (*mt-co1*). In all cases, morpholinos against *cox5aa*, *cox5ab*, or *surf1* resulted in severe developmental phenotypes and ectopic apoptosis (Baden et al. 2007). Mutant embryos displayed delayed and malformed development of the nervous system, gut and heart. As such these embryos die in embryogenesis. Although this model reveals details around the effects of CIV deficiency during development which may be informative for infantile syndromes associated with CIV deficiency there are many patient features that cannot be extrapolated here. However, given the strong developmental effects which have been so carefully characterised here, this model could be combined with the developmental screens for small molecules which alleviate the phenotypes described.

More recently zebrafish were used to study the role of CIII subunit Ubiquinol-Cytochrome C Reductase Binding Protein (UQCRB) *in vivo* (Cho et al. 2013). UQCRB has been shown to be mutated in a patient suffering from CIII deficiency (Haut et al. 2003). The focus of the study was the role of CIII in angiogenesis, which was impaired in embryos treated with a small molecule inhibitor of *uqcrb*, terpestacin or injected with morpholinos against *uqcrb* (Cho et al. 2013). Importantly, there were also clear developmental effects of *uqcrb* knockdown suggesting that this model could also be used for developmental screening of effective therapies.

Developmental defects were also observed when two regulators of mitochondrial dynamics, *MFN2* (*mfn2*) and *OPA1* (*opa1*), were depleted independently using specific morpholinos (Vettori et al. 2011; Rahn et al. 2013). *mfn2* knockdown resulted in decreased survival, with only 60% of injected embryos surviving (Vettori et al. 2011). Surviving animals had neuromuscular defects resulting in motility defects, reminiscent of patients carrying *MFN2* mutations which cause CMT2A (Vettori et al. 2011). As such this is a useful model for the investigation of CMT2A while the characterization of the developmental defects will allow easy screening of effective small molecules. Depletion of *opa1* also resulted in developmental defects with larvae dying at 7 days post fertilisation (dpf). *opa1* knockdown larvae had a fragmented mitochondrial network, reduced respiration and compensatory upregulation of mitochondrial biogenesis and transcription (Rahn et al. 2013). Although not directly extrapolatable to human patients with *OPA1* mutations, this like those zebrafish models described above could be used to identify active therapeutic small molecules.

## 10.5 *Caenorhabditis elegans* Models of Mitochondrial Disease

The transparent nematode *C. elegans* has been used extensively over the last 50 years to study diverse and complex biological processes (Kenyon 1988). Its short life cycle, defined cellular lineage and array of tools for genetic manipulation have made it a powerful force in the study of gene function (Kenyon 1988). The simplicity of its nervous system coupled with conservation of neuronal subtypes and communication make it an attractive model to study neurological disorders. Given the severe effects that mitochondrial dysfunction has on the nervous system of patients with mitochondrial disease, using *C .elegans* to study mutations identified in patients will allow investigation of the neuropathology of several mitochondrial disorders.

Several classical genetic mutants which correspond to mitochondrial genes found mutated in patients have been isolated in *C. elegans* (Kayser et al. 2001; Kayser et al. 2004; Ishii et al. 1998; Grad and Lemire 2004; Tsang et al. 2001). Mutants of the *NDUFS2* orthologue, *gas-1* have a reduce lifespan, retarded growth, increased sensitivity to temperature, oxidative stress and hyperoxia, as well as a reduced CI dependant respiration (Kayser et al. 2001). Mutation of *mev-1*, the orthologue of *SDHC* result in increased oxygen sensitivity and accelerated ageing. Analysis of the CII function demonstrated the requirement of this subunit for the participation of CII in electron transport (Ishii et al. 1998). Grad and Lemire created 3 models expressing distinct patient mutations of *nuo-1*, the orthologue of *NDUFV1* (Grad and Lemire 2004). In all three cases, mutations of *nuo-1* resulted in decreased lifespan, premature ageing, compromised respiration and lactic acidosis (Grad and Lemire 2004). *clk-1*, *orthologue* of *COQ7*, mutants are unable to produce $CoQ_9$, accumulate the intermediate $DMQ_9$ and display defects in CoQ dependant activities of CI and CIII, as has been described in other animal models of *COQ7* loss (Kayser et al. 2004; Fernandez-Ayala et al. 2014).

These studies clearly demonstrate that mutation of genes found in patients with mitochondrial disease can induce many of same phenotypes in worms, validating their use as a model for the study of molecular pathogenesis and the effectiveness of available therapies.

## 10.6 Conclusion

As can be appreciated from the studies summarised, the extent of mitochondrial disease models currently available is impressive. Each with its advantages and limitations. The last 25 years of mitochondrial research has elucidated many important aspects of these organelles functions in health and disease. Despite this progress patients remain without treatments. As we move forward the focus remains on understanding the molecular pathology underlying mitochondrial disease but increasingly it will be on the development and validation of therapies. These aims both rely on a foundation of disease relevant and genetically defined models. Models

of mitochondrial disease in flies, worms and fish display complex and pleiotropic phenotypes of mitochondrial syndromes which can be extrapolated to human patients. Although the mouse is an excellent model for human disease, the use of complementary models suitable for high throughput screening, where patient specific mutations can be introduced using state of the art genome editing will accelerate progress in both these areas.

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# Chapter 11
# Therapies Approaches in Mitochondrial Diseases

**Valentina Emmanuele, Catarina M. Quinzii, and Michio Hirano**

**Abstract** Therapies for mitochondrial diseases has been largely limited to supportive and symptomatic therapies; however, in the last decade, advances in understanding the causes and pathomechanisms of these diverse disorders have enabled development of novel treatment strategies. Here, we highlight current use of dietary supplements and exercise therapy as well as emerging treatments in preclinical and clinical trial stages of development. Broad-spectrum therapies that may be applied multiple diseases include: activation of mitochondrial biogenesis, regulation of mitophagy and mitochondrial dynamics, bypass of mitochondrial biochemical defects, mitochondrial replacement therapy, and hypoxia. Tailored disease-specific therapies in development include: scavenging of toxic compounds, deoxynucleoside therapy, cell replacement therapies, viral-mediated gene-delivery, shifting heteroplasmy of mitochondrial DNA pathogenic variants, and stabilization of mitochondrial transfer RNAs.

## 11.1 Introduction

Mitochondrial disorders (MDs) represent a heterogeneous group of inborn errors of the oxidative phosphorylation (OXPHOS) system in mitochondria, where most of the cell's ATP is generated. This metabolic pathway is under the dual genetic control of the mitochondrial and nuclear genomes.

The genetic complexity only partially accounts for the clinical heterogeneity of these disorders. Onset varies widely from childhood to adulthood, even among members of the same family. Virtually every organ system can be affected by mitochondrial dysfunction (in particular brain, skeletal and cardiac muscle) and as a consequence, MDs are often multisystemic (DiMauro et al. 2013).

Taken together, MDs have an estimated prevalence greater than 1:5000 individuals (Schaefer et al. 2008). The 10 most common pathogenic mitochondrial DNA

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(mtDNA) mutations alone have an estimated incidence of 1 in 200 infants (Elliott et al. 2008). Given their prevalence and their progressive nature, often worsening over many decades, these disorders cause substantial morbidity among both pediatric and adult populations.

Currently, only supportive care is available for the vast majority of patients with MDs (Pfeffer et al. 2012; Kerr 2013). However, extraordinary progress has been made in recent years in understanding the pathogenesis of these disorders (Hirano et al. 2018). Based on this knowledge, therapeutic strategies have been proposed and experimental evidence is increasing in *in vitro* and *in vivo* studies.

Numerous challenges albeit hamper the translation of these therapies from bench to bedside. The main challenges are caused by the genetic, biochemical, and phenotypic variability of MDs. This heterogeneity, for instance, makes it difficult to collect a sufficient number of patients to conduct reliable natural history studies, clinical trials, and to identify universally validated outcome measures.

In this chapter, we will discuss separately current therapeutic approaches, including supportive therapy, the results of previous clinical trials, and emerging therapies that have shown promising results at preclinical and clinical level.[1]

## 11.2 Current Treatments in Clinical Practice

### *11.2.1 Supportive Treatments*

Supportive treatments are often the only available option in the management of mitochondrial patients when a specific therapy is lacking. For the vast majority of patients, therapy is limited to either preventing or treating the complications of MDs. Nevertheless, supportive measures are extremely important and can significantly improve quality of life and survival in this group of patients. Symptomatic approaches often require a multidisciplinary team for early recognition and treatment of complications/manifestations.

Epileptic seizures commonly occur in MDs, such as, for example, Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS), Myoclonic epilepsy with ragged red fibers (MERRF) and Leigh syndrome. Most available anticonvulsants can be used, except sodium valproate that can cause carnitine deficiency and may precipitate hepatic failure in Alpers syndrome (Rahman 2012). Myoclonic epilepsy in MERRF can be treated effectively with levetiracetam, clonazepam, or zonisamide (DiMauro and Hirano 1993). Dystonia, often present in Leigh syndrome, can benefit of anti-dystonia oral medications (e.g. anticholinergic, neuroleptics) or botulinum toxin injection.

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[1] This chapter is based upon our published review article (Hirano et al. 2018) available via Open Access (https://portlandpress.com/essaysbiochem/article/62/3/467/78638/Emerging-therapies-for-mitochondrial-diseases) CCBY license ELVJWX.

Heart is often affected in MDs. Conduction defects are commonly present in patients with Kearns-Sayre syndrome (KSS), but also in Leber hereditary optic neuropathy (LHON), and m.3243A>G mutation. Timely placement of a pacemaker can be lifesaving in KSS patients. Cardiomyopathy can also occur and it is estimated to affect 20–40% of children with MDs (Scaglia et al. 2004). Hypertrophic cardiomyopathy is the most common manifestation, being present in 50% of mitochondrial patients with cardiac involvement (Finsterer and Kothari 2014). MDs presenting cardiomyopathy can be due to defects in respiratory chain complexes subunits and assembly factors, mitochondrial tRNAs, rRNAs, ribosomal proteins, translation factors, mtDNA maintenance, $CoQ_{10}$ synthesis, or defect of lipid milieau, like Barth syndrome (El-Hattab and Scaglia 2016). Cardiac involvement should be closely monitored with by a cardiologist and pharmacologically or surgically treated when necessary.

Endocrine dysfunction can be present in MDs, and hormone replacement can be necessary (insulin, thyroxine, growth hormone). In the case of diabetes mellitus, for instance in patients with MELAS and Maternally Inherited Diabetes-Deafness (MIDD) syndrome, diet and low doses of insulin/oral hypoglycemic drugs are usually sufficient to maintain the euglycemic state. Metformin should be avoided because it can cause lactic acidosis (Murphy et al. 2008).

Gastrointestinal (GI) problems are also common in patients with mitochondrial disorders and include dysphagia, weight loss, constipation, pseudo-obstruction, nausea, failure to thrive. Among syndromic MDs, Mitochondrial neurogastrointestinal encephalopathy (MNGIE) is the one with prevalent GI involvement (Garone et al. 2011). Adequate nutrition can be achieved with hypercaloric nutritional supplements but may require PEG (percutaneous or parenteral nutrition endoscopic gastrostomy) feeding in severe cases (Finsterer and Frank 2017).

Other supportive therapies include electrolyte replacement, renal dialysis or transplantation in patients with renal involvement, uncommon but described in patients with mitochondrial DNA or nuclear DNA mutations (O'Toole 2014); blood transfusion in case of anemia in Pearson syndrome or other forms of anemia; respiratory support for restrictive lung disease; and psychological support for patients and their families.

Non-pharmacologic approaches, include the use of hearing aids or cochlear implants for patients with hearing loss (Sinnathuray et al. 2003), and eyelid surgery for ptosis, as a mean to improve not only the vision but also the psychological well-being and social interaction of the patient.

### *11.2.2 Pharmacological Approaches*

Multiple vitamins and cofactors are often used in patients with mitochondrial disorders, although these therapies are not yet standardized or definitively proven to be effective. The dietary supplements are used with different purposes, such as: (1) increase the respiratory chain flux (Coenzyme$Q_{10}$ [$CoQ_{10}$], riboflavin), (2) serve as

antioxidants (e.g. $CoQ_{10,}$ idebenone, alpha-lipoic acid, vitamins C and E), and/or act as cofactors (e.g. riboflavin, thiamine), or (3) function as mitochondrial substrates (L-carnitine). Accumulation of reactive oxygen species (ROS) as a toxic byproduct of a mitochondrial respiratory chain dysfunction may lead to cellular damage contributing to the pathogenesis of MDs. Moreover, a transgenic murine model overexpressing a catalase targeted to mitochondria extended life span (Schriner et al. 2005). Based on this rational, antioxidants have been frequently used in the treatment of mitochondrial patients. $CoQ_{10}$ is the most commonly utilized and many clinical trials have been investigating its efficacy and that of its analogues, like idebenone and EPI-743, in different MDs (see Sect. 11.3). Other antioxidants like vitamins C and E might also be beneficial in patients with MDs. An example is an analogue of vitamin E, trolox ornithylamide hydrochloride, when applied to fibroblasts from patients with Leigh syndrome reduced ROS levels and an increased activities of mitochondrial complexes I and IV, and citrate synthase (Blanchet et al. 2015). The efficacy of antioxidants in patients with MDs nonetheless remains controversial. A Cochrane review of mitochondrial therapies has found little evidence supporting the use of any vitamin or cofactor (Pfeffer et al. 2012). However, benefits of various agents (riboflavin, alpha-lipoic acid, etc) have been anecdotally reported. Consensus recommendations from the Mitochondrial Medicine Society recently reported (Parikh et al. 2015) aimed to standardize treatment options for mitochondrial patients. According to these recommendations, patients with primary mitochondrial disorders, and not only $CoQ_{10}$ deficiency, should be offered $CoQ_{10}$ in its reduced form (ubiquinol), and plasma or leukocyte levels should be monitored to assess adherence to treatment.

In addition, alpha-lipoic acid (ALA) and riboflavin are frequently offered to mitochondrial patients. L-carnitine should be administered when deficient. Folinic acid should be given to mitochondrial patients when deficient and the central nervous system is involved. Moreover, supplements should be given starting with one supplement at the time, avoiding "cocktails" initially.

Effective treatment of acute stroke-like episodes or their prevention has not been established. Open-label studies suggest that treatment of acute mitochondrial stroke-like episodes with intravenous (IV) arginine hydrochloride, a precursor of nitric oxide, is beneficial for patient with the m.3243 A>G mutation in *MTTL1* (Koenig et al. 2016). IV arginine administration should be considered in acute stroke-like episodes associated with other primary mitochondrial disorders. Open-label studies also suggest that daily oral arginine to prevent strokes should be considered in MELAS patients with the m.3243 A>G mutation (Parikh et al. 2015).

Lastly, some drugs should be avoided in patients with mitochondrial dysfunction, or used with caution: valproic acid, statins, metformin, high-dose acetaminophen, and specific antibiotics (i.e. aminoglycosides, linezolid, tetracycline, azithromycin, erythromycin).

A survey on the use of dietary supplements was recently conducted by the North American Mitochondrial Disease Consortium (NAMDC) (Karaa et al. 2016). The survey pointed out how the majority of patients takes four or more dietary supplements, despite the recent recommendation of the Mitochondrial Medicine Society.

Even though no or minor side effects were reported, and patients noted overall some improvements, the economic burden to the families was considerable; 90% of patients purchased the supplements out-of-pocket. The authors conclude that this burden and the potential side effects are not justifiable, considering the lack of evidence for using these "cocktails". Importantly, this survey reveal an increase interest towards patients' perception of their care and quality of life; this approach is fundamental to determine reliable outcome measures to use in future well-conducted clinical trials.

### *11.2.3 Exercise*

Exercise has been proven beneficial in some patients with MDs (Voet et al. 2013; Tarnopolsky 2014). In particular, aerobic endurance training can increase mitochondrial mass, by stimulating mitochondrial biogenesis, and increase muscle mitochondrial enzyme activities and muscle strength. Endurance training has been proven beneficial and safe in trials of patients with mitochondrial DNA mutations (Taivassalo et al. 2001, 2006). A combination of progressive endurance with or without resistance exercise should be recommended to mitochondrial patients (Parikh et al. 2015).

## 11.3 Clinical Trials

In 2012 a systematic review by the Cochrane collaboration evaluated 1335 studies comparing pharmacological and non-pharmacological treatments for MDs (Pfeffer et al. 2012). Only 12 studies were selected for inclusion in the review, the most common reason for exclusion being lack of randomization/blinding and presence of methodological biases. The primary outcome measures included any change in muscle strength or neurological features. Secondary outcome measures included quality life evaluation, biochemical biomarkers (e.g. lactic acidosis), and negative outcomes. The 12 studies investigated the effects of $CoQ_{10}$, dichloroacetate (DCA), creatinine, dimethylglycine, whey-based cysteine and combination therapy of creatine, α-lipoic acid and $CoQ_{10}$. Dramatic effects were not observed in any of these studies, and one trial assessing the effects of DCA in MELAS patients had to be terminated because of toxicity (NCT00068913). Several clinical trials are currently underway or have been recently completed, but the results were not published for most of them and are still unclear. The majority of the studies focused on patients with MELAS and LHON, which could be studied in relatively large cohorts. Many other trials analyzed less homogeneous cohort of patients, including, for instance, patients with similar phenotype (i.e. mitochondrial myopathy) but different genetic background. A summary of the studies is reported in Table 11.1. We will briefly discuss some examples.

**Table 11.1** Recent clinical trials in mitochondrial disorders

| Treatment | Disease | Design | Mechanism | Status | Outcome | Trial number |
|---|---|---|---|---|---|---|
| **ACTIVE STUDIES** | | | | | | |
| EPI-743 | MDs | Phase 2, Emergency use protocol in acutely ill patients (90 days endo-of-life care) | Antioxidant | Active/not recruiting | na | NCT01370447 |
| EPI-743 | Children (2–11 years of age) with MDs or metabolic diseases | Phase 2, randomized, double blind, placebo-controlled, crossover | Antioxidant | Active/not recruiting | na | NCT01642056 |
| MTP-131 (Elamipretide) ophthalmic | LHON | Phase 2, prospective, randomized, double blind, vehicle-controlled | Cardiolipin stabilization | Active/not recruiting | na | NCT02693119 |
| DCA (dichloroacetate) | PDH deficiency | Phase 3, randomized, placebo-controlled, crossover | Lowering lactate levels | Recruiting | na | NCT02616484 |
| Resistance exercise | Barth syndrome | Phase 2, open label | Increase glycolitic type 1 muscle fibers | Recruiting | na | NCT01629459 |
| scAAV2-P1ND4v2 | LHON m.11778G>A | Phase 1, open label, dose-escalating | Gene therapy | Recruiting | na | NCT02161380 |
| rAAV2/2-ND4 (GS010) | LHON | Phase1/2 safety, open label, dose escalating | Gene therapy | Active/not recruiting | na | NCT02064569 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Allogenic HSCT | MNGIE | Phase 1 safety study | Production of TP | Recruiting | na | NCT02427178 |
| **COMPLETED STUDIES** | | | | | | |
| KH176 | MELAS, MIDD, mitochondrial myopathies, MDs | Phase 2, randomized, double blind, placebo-controlled, crossover | Antioxidant | Completed | Twice daily oral 100mg KH176 was well-tolerated and appeared safe. Primary outcome (gait measures) did not reach statistical significance, but possible positive effects on alterness and mood were noted. | NCT02909400 |
| RTA 408 (omaveloxolone) | Mitochondrial myopathy | Phase 2 randomized, double blind, placebo-controlled, dose-escalating | Antioxidant, NRF2 activator, NFkB inhibitor | Completed | Oral omaveloxone up to 160mg daily was well-tolerated and appeared safe. Primary (peak cycling exercise workload) and secondary (6-minute walk test) did not achieve statistical significance, but improvements in exploratory endpoints (lowering heart rate and lactate production during submaximal exercise) were reported. | NCT02255422 |
| MTP-131 (Elamipretide) | Mitochondrial myopathy | Phase 2, randomized, double-blind, placebo-controlled, crossover | Cardiolipin stabilization | Completed | na | NCT02805790 |
| MTP-131 (Elamipretide) | MDs | Phase 2, open label | Cardiolipin stabilization | Completed | na | NCT02976038 |
| CoQ10 | Children with MDs with mtDNA mutations or specific OXPHOS complexes defects | Phase 3, randomized, double blind | OXPHOS/ROS | Completed | na | NCT00432744 |

(continued)

**Table 11.1** (continued)

| Treatment | Disease | Design | Mechanism | Status | Outcome | Trial number |
|---|---|---|---|---|---|---|
| Idebenone | LHON | Phase 2, randomized, double blind, placebo-controlled | Antioxidant | Completed | Primary endopoint did not reach statistical significance; secondary outcomes significantly differ in a subgroup ot patients with discordant acuity at baseline | NCT00747487 |
| Idebenone | MELAS | Phase 2, randomized, double blind, placebo-controlled, dose-finding | Antioxidant | Completed | Primary endopoint did not reach statistical significance | NCT00887562 |
| EPI-743 | Leigh syndrome | Phase 2b, randomized, double blind, placebo-controlled | Antioxidant | Completed | na | NCT01721733 |
| EPI-743 | Pearson syndrome | Phase 2, open label | Antioxidant | Terminated | Results from other studies did not support continuation of this trial | NCT02104336 |
| KH176 | MELAS, LHON, Leigh, and other MDs | Phase 1, randomized, double blind, placebo-controlled, crossover | Antioxidant | Completed | Well tolerated with promising pharmacokinetic profile | NCT02544217 |
| Bezafibrate | Mitochondrial myopathy (m.3243A>G) | Phase 2, open label | Mitochondrial biogenesis | Completed | na | NCT02398201 |
| Curcumin | LHON | Phase 3, randomized, double blind, placebo-controlled | Antioxidant | Completed | na | NCT00528151 |

| | | | | | | |
|---|---|---|---|---|---|---|
| MTP-131 | Mitochondrial myopathy | Phase 1/2 randomized, double-blind, placebo-controlled, dose-escalating | Cardiolipin stabilization | Completed | na | NCT02367014 |
| RP103 (Cysteamine bitartrate delayed-release) | Childhood MDs including Leigh syndrome | Phase 2 open-label, dose-escalating | Cystine-depleting agent | Completed | Primary endopoint did not reach statistical significance; high percentage of serious Adverse Events (30.56%) | NCT02023866 |
| RP103 (Cysteamine bitartrate delayed-release) | Childhood MDs including Leigh syndrome | Phase 2, long term open-label extension study | Cystine-depleting agent | Completed | na | NCT02473445 |
| Medium chain triglycerides | MELAS | Phase 1, open label | Shift heteroplasmy | Completed | na | NCT01252979 |
| L-Arginine | MELAS | Phase 2, open label | NO precursor | Completed | Improvement in aerobic capacity and muscle metabolism | NCT01603446 |
| L-Arginine (IV) | MELAS | Phase 3, open label | NO precursor | Completed | Improvement of strok-like symptoms | JMA-IIA00023 |
| L-Arginine (PO) | MELAS | Phase 3, open-label | NO precursor | Completed | Improved endothelial dysfunction | JMA-IIA00025 |
| Arginine and citrulline | MELAS | Phase 1, open-label | NO precursor | Completed | na | NCT01339494 |
| Lipoic acid | Mitochondrial myopathy | Pilot compassionate use study | – | Completed | na | NCT00004770 |
| RG2133 (2′,3′,5′-tri-o-acetyluridine) | MDs | Phase 1, open label, dose escalating | – | Completed | na | NCT00060515 |
| SPP-004 (5-Ala and SFC) | MDs, mainly to cranial nerve symptoms | Phase 2, randomized, placebo-controlled | – | Completed | na | JMA-IIA00200 |

(continued)

**Table 11.1** (continued)

| Treatment | Disease | Design | Mechanism | Status | Outcome | Trial number |
|---|---|---|---|---|---|---|
| Taurine | MELAS | Phase 2/3 open-label | Taurine modification | Completed | na | UMIN000011908 |
| Pyruvate | MELAS | Phase 2, randomized, placebo-controlled | NAD donor | Unknown | na | JMA-IIA00093 |
| DCA (dichloroacetate) | MELAS | Phase 2, randomized, double blind, crossover | Lowering lactate levels | Terminated | Terminated because of peripheral nerve toxicity | NCT00068913 |
| Cyclosporine | LHON acute phase | Phase 2, open label | Inhibition of mitochondrial PTP | Unknown | na | NCT02176733 |
| rAAV2-ND4 | LHON | Open label | Gene therapy | Completed | Improvement of visual acuity and enlargement of visual field | NCT01267422 |

*MDs* mitochondrial disorders, *MELAS* mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes, *MIDD* maternally inherited diabetes-deafness syndrome, *LHON* Leber hereditary optic neuropathy, *PDH* pyruvate dehydrogenase deficiency, *HSCT* hematopoietic stem cell transplantation, *MNGIE* mitochondrial neurogastrointestinal encephalomyopathy, *IV* intra-venous, *PO* oral, *PTP* permeability transition pore, *na* not available

**MELAS Syndrome**

MELAS is one of the most frequent maternally inherited mitochondrial disorders. The pathogenesis of this disorder is not completely understood and results from different factor. Energy failure due to faulty mitochondria is a common feature of MDs as well as the overproduction of ROS. Different approaches have been studied in order to reduce the oxidative stress in MDs and in particular in MELAS patients. Idebenone is a short-tail ubiquinone synthetic analog, which is more water-soluble compared to $CoQ_{10}$ and act as antioxidant. Several studies have been conducted to assess the efficacy and safety of idebenone in Friedreich Ataxia (FA), and proven that idebenone is in fact well tolerated and may stabilize neurological features in this disorder (Mariotti et al. 2003; Di Prospero et al. 2007a, b; Meier et al. 2012; Lynch et al. 2010). A phase 2a, randomized, double blind, placebo-control, dose-finding study in patients with MELAS syndrome has recently been completed and showed that the primary endopoint did not reach statistical significance (NCT00887562). KH176 is a small molecule derived from Vitamin E and is a potent ROS scavenger. After the completion of a dose escalating clinical trial with KH176 in healthy individual that has demonstrated good tolerability and a promising pharmacokinetic profile (NCT02544217), a randomized trial studied 18 adults with the m.3243A>G mutation (NCT02909400). Twice daily oral 100mg KH176 was well-tolerated and appeared safe. Primary outcome (gait measures) did not reach statistical significance, but possible positive effects on alterness and mood were noted (Janssen et al. 2019). Moreover, bezafibrate is an activator of the transcription factor peroxisomal proliferator receptors (PPARs), that in turn when activated promote transcription of mitochondrial genes. Its efficacy is being evaluated in patients with m.3243A>G mutation (NCT02398201) and evidence of myopathy, but results are not available at the moment. In addition to energy failure and ROS accumulation, there has been growing evidence that nitric oxide (NO) deficiency play a central role in the pathogenesis of the stroke-like episodes (El-Hattab et al. 2016). Arginine is the substrate of nitric oxide synthase, which produces NO, therefore arginine is a promising treatment for MELAS patients. Multiple open-label trials have been conducted (NCT01603446, JMA-IIA00023, and JMA-IIA00025) and have shown the efficacy of chronic oral administration (Koga et al. 2006, 2007; Rodan et al. 2015) and acute intravenous administration (Koga et al. 2005, 2006) of arginine in patients with MELAS syndrome, although a placebo-controlled randomized clinical trial has not yet been conducted. Furthermore, preliminary evidence has been provided for the effect of citrulline in stroke-like episodes in MELAS patients (El-Hattab et al. 2016) and a Phase 1, open-label study has been conducted (NCT01339494), although data are not available yet. Other molecules investigated in clinical trials for patients with MELAS include pyruvate (JMA-IIA00093), taurine (UMIN000011908), and supplemental medium chain triglycerides (NCT01252979); however, those results have never been reported.

**Leber Hereditary Optic Neuropathy (LHON)**

LHON is a mitochondrial disorder characterized by painless, subacute visual loss affecting the central visual field in one eye, followed by similar symptoms in the

other eye typically with 2 or 3 months of delay. Three mtDNA mutations are commonly associated with LHON: m.3460G>A in MT-ND1, m.11778G>A in MT-ND4, or m.14484T>C in MT-ND6 (Yu-Wai-Man and Chinnery 1993). Many therapeutic approaches have been tested in patients with LHON, the majority of which are focused on the use of antioxidants. In particular, Idebenone has been evaluated in a clinical trial of LHON and, even if primary endpoints did not reach statistical significance, a possible beneficial effect has been shown in a subgroup of patients with discordant visual acuity at baseline (Klopstock et al. 2011) (NCT00747487). Its use has been recently approved for the treatment of this disease in Europe. MTP-131 (elamipretide) is a small molecule targeting inner mitochondrial membrane that has been demonstrated to correct excessive ROS and increase ATP synthesis in preclinical studies (Szeto 2014); this molecule has entered clinical trials for LHON (NCT02693119), as well as for mitochondrial myopathy and Barth Syndrome (NCT02367014, NCT02976038, NCT02805790). Although the preliminary results of phase I and II studies of this compound for mitochondrial myopathy were promising, the phase III study failed to reach primary endpoints. Further assessments of elamipretide for Barth syndrome are ongoing (NCT03098797). Curcumin, a derivate of the spice turmeric (*Curcuma longa*), has also displayed antioxidant properties and a trial has been completed in patients with LHON, but no results are available (NCT00528151). Lastly, AAV-mediated allotopic ND4 gene therapy has been attempted by three groups in patients with LHON (NCT02161380; NCT02064569; NCT02652767; NCT02652780). Curiously, the three teams have reported improvement in best corrected visual acuity in both treated and sham/untreated eyes in some subjects. These results were interpreted as possible transfer of the AAV-ND4 across the optic chiasm (Wan et al. 2016; Feuer et al. 2016; Vignal et al. 2018; Yang et al. 2016).

**MNGIE**

MNGIE (mithochondrial neuro-gastro-intestinal encephalomyopathy) is an autosomal recessive mitochondrial disorder characterized by severe gastrointestinal dysmotility, cachexia, progressive external ophthalmoplegia, myopathy, and peripheral demyelinating neuropathy (Hirano 1993). It is caused by autosomal recessive mutations in *TYMP* gene, encoding thymidine phosphorylase (TP). TP is a cytosolic enzyme that catalizes the first step of thymidine and deoxyuridine catabolism. When the enzyme is deficient, thymidine and deoxyuridine accumulates and become toxic, leading to mtDNA instability. Two trials at Columbia University, USA, are currently recruiting to define natural history of the disease (NCT01694953) and to assess the safety of hematopoetic stem cell transplant (HSCT), as a mean to replace TP enzyme, for MNGIE patients (NCT01694953) (see also Sect. 11.4). Orthoptic liver transplantation has been performed on three MNGIE patients with early promising results and possible greater safety than HSCT) (D'Angelo et al. 2017; De Giorgio et al. 2016).

**Primary Mitochondrial Myopathies**

Primary mitochondrial myopathies, defined as genetically confirmed disorders of oxidative phosphorylation affecting predominantly skeletal muscle (Mancuso et al.

2017), have emerged as targets for novel potential therapies (Madsen et al. 2020; Karaa et al. 2020). Oral omaveloxone, a semi-synthetic oleanoic triterpenoid activator of Nrf2, up to 160mg daily was well-tolerated and appeared safe. Primary (peak cycling exercise workload) and secondary (6-minute walk test) did not achieve statistical significance, but improvements in exploratory endpoints (lowering heart rate and lactate production during submaximal exercise) were reported (Madsen et al. 2020). A phase 3 trial of MTP-131 (elampretide), a small peptide stabilizer of cardiolipin, has been completed but publication of results are pending (Karaa et al. 2020).

**Heterogeneous Cohort of Patients with MDs**
Many of the therapeutic interventions acting on common pathogenic pathways of MDs have been tested in non-homogenous cohort of patients or in different disorders. One example is EPI-743, a para-benzoquinone analog modified to exert a higher antioxidant effect compared with $CoQ_{10}$ and idebenone (Enns et al. 2012). This molecule is supposed to enhance the biosynthesis of glutathione (GSH), which is an important cellular antioxidant. Two open label studies conducted independently in North America and in Italy showed promising results in a cohort of patients with various mitochondrial diseases and with Leigh syndrome, respectively (Blankenberg et al. 2012; Martinelli et al. 2012). As a result, a randomized clinical trial has started in patients with Leigh syndrome (ClinicalTrials.gov NCT01721733) as well as in patients with FA (NCT01728064), and in acutely ill patients (90 days of end-of-life care) (NCT01370447). A clinical trial on Pearson Syndrome has been terminated because results of other studies have not supported continuation (NCT02104336). EPI-743 has also been studied in an open-label trial of patients with LHON with favorable outcome (Sadun et al. 2012).

## 11.4 Emerging Therapies

In the past few years, many potential treatments have been proposed for mitochondrial disorders. These approaches act on different mechanisms and can be broadly divided in "non-tailored strategies", acting on common pathways thus in theory relevant to different MDs, and "disease-tailored" strategies (Viscomi 2016). Examples of these strategies are summarized in Table 11.2. Components of the first group are, for instance, strategies aiming at: (1) activation of mitochondrial biogenesis; (2) regulation of mitophagy and mitochondrial dynamics; (3) bypass of OXPHOS defects; (4) mitochondrial replacement therapy (MRT). Part of the second group includes: (5) scavenging of specific toxic compounds; (6) supplementation of nucleosides; (7) cell replacement therapies; (8) gene therapy; (9) shifting heteroplasmy; and (10) stabilizing mutant tRNAs. Some of these approaches have been proven effective only in preclinical models while others have already been successfully applied in anecdotic patients with MDs (Table 11.2).

**Table 11.2** Examples of experimental therapies in mitochondrial disorders

| Strategy | Method | Model | References |
|---|---|---|---|
| **NON-TAILORED** | | | |
| Activation of mitochondrial biogenesis | Nicotinamide riboside and PARP1 | *Sco2* knockout/knockin mouse, Deletor mouse model | Khan et al. (2014) and Cerutti et al. (2014) |
| | AICAR | Patients fibroblasts and mouse models of COX deficiency | Viscomi et al. (2011) |
| | Bezafibrate | Patients fibroblasts, cybrids, mouse models of COX deficiency, Deletor mouse model | Bastin et al. (2008), Noe et al. (2013), Hofer et al. (2014), Yatsuga and Suomalainen (2012), and Dillon et al. (2012) |
| | Resveratrol | *In vitro* models, Drosophila models, human fibroblasts | Lopes Costa et al. (2014), Mizuguchi et al. (2017), and De Paepe et al. (2014) |
| | Retinoic acid | Cybrids (m.3243A>G) | Chae et al. (2013) |
| | Endurance exercise | Mouse models of COX deficiency, mtDNA mutator mice, patients with MDs | Safdar et al. (2011), Jeppesen et al. (2009), Zeviani (2008), and Rowe et al. (2012) |
| Regulating mitophagy and mitochondrial dynamics | Rapamycin | *in vitro* models and *Ndufs4*$^{-/-}$ mouse model of Leigh syndrome; *Tk2*$^{H126N/H126N}$ mouse model | Johnson et al. (2013) and Siegmund et al. (2017) |
| | P110 | *In vitro* models | Qi et al. (2013) |
| Bypassing OXPHOS blocks | Ndi1 (Complex I defect) and AOX (Complex III and IV defects)¡ | *In vitro* models and Drosophila models | Perales-Clemente et al. (2008), Sanz et al. (2010), Dassa et al. (2009), and Fernandez-Ayala et al. (2009) |
| Mitochondrial replacement therapy | Oocyte nuclear genetic material transfer | Non-human primates; healthy subjects and patients with MDs | Tachibana et al. (2009, 2013), Craven et al. (2010), Paull et al. (2013), Kang et al. (2016), and Zhang et al. (2017) |
| **DISEASE-TAILORED** | | | |
| Scavenging of specific toxic compounds | N-acetyl cysteine and metronidazole | *Ethe1*$^{-/-}$ mouse model of EE; patients with EE | Viscomi et al. (2010) |
| | Hemodialysis | Patients with MNGIE | Spinazzola et al. (2002) |

(continued)

**Table 11.2** (continued)

| Strategy | Method | Model | References |
|---|---|---|---|
| Supplementation of nucleotides/ nucleosides | Deoxycytidine and deoxythymidine monophosphates; deoxycytidine and deoxythymidine | *Tk2*$^{H126N/H126N}$ mouse model; patients with TK2 deficiency | Garone et al. 2014 and López-Gómez et al. (2017) |
| | Deoxycytidine or tetrahydrouridine | Thymidine-induced mtDNA depleted cells and *Tymp/Upp1* knockout murine model of MNGIE | Camara et al. (2014) |
| | Deoxyguanosine | dGK deficient human fibroblasts | Camara et al. (2014) |
| Cell replacement therapies | Platelets or erythrocyte-encapsulated thymidine phsphorylase | Patients with MNGIE | Lara et al. (2006) and Bax et al. (2013) |
| | Allogenic HSCT | Patients with MNGIE | Hirano et al. (2006) and Halter et al. (2015) |
| | Liver transplantation | One patient with EE | Dionisi-Vici et al. (2016) |
| Gene therapy | AAV-mediated gene therapy | *Ant1*$^{-/-}$ mouse model; harlequin mouse; mouse model of EE; mouse model of MNGIE; mpv17 knockout mouse model | Flierl et al. (2005), Bouaita et al. (2012), Di Meo et al. (2012), Torres-Torronteras et al. (2014), and Bottani et al. (2014) |
| | Allotopic expression of mtDNA encoded proteins | Human fibroblasts (mutations in ND1, ND4, and ATP6 genes) and a mouse model of LHON | Bonnet et al. (2007, 2008), Kaltimbacher et al. (2006), and Ellouze et al. (2008) |
| | CRIPR/Cas9 | iPS cell model of $CoQ_{10}$ deficiency (c.483G > C in *COQ4* gene) | Romero-Moyà et al. (2017) |

(continued)

**Table 11.2** (continued)

| Strategy | Method | Model | References |
|---|---|---|---|
| Shifting heteroplasmy | Mitochondrial-targeted restriction endonucleases | Cybrids (m.8993T>C) Artificial mammalian oocytes and the NZB/BALB heteroplasmic mouse model | Srivastava and Moraes (2001), Tanaka et al. (2002), Alexeyev et al. (2008), Bayona-Bafaluy et al. (2005), Bacman et al. (2007, 2010, 2012), and Reddy et al. (2015) |
| | mZFNs | Cybrids (m.8993T>C, mtDNA common deletion) | Minczuk et al. (2008) and Gammage et al. (2014) |
| | TALENs | Cybrids (mtDNA common deletion, m. 14459G>A) Artificial mammalian oocytes and the NZB/BALB heteroplasmic mouse model | Bacman et al. (2013) and Reddy et al. (2015) |
| Stabilizing mutant tRNAs | Overexpressing cognate and non-cognate aminoacyl mt-tRNA synthetase | *In vitro* yeast and human cell line models | De Luca et al. (2006, 2009), Li and Guan (2010), Rorbach et al. (2008), Perli et al. (2014), and Horning-Do et al. (2014) |

*MDs* mitochondrial disorders, *EE* ethylmalonic encephalopathy, *MNGIE* mitochondrial neurogastrointestinal encephalomyopathy, *HSCT* hematopoietic stem cell transplantation, *iPS* induced pluripotent stem, *ZFNs* zinc finger endonucleases, *TALENs* transcription activator-like effectors nucleases, *CRISPR* clustered regularly interspaced palindromic repeat

### *11.4.1 Activation of Mitochondrial Biogenesis*

Energy failure is a hallmark of mitochondrial diseases and various therapeutic interventions have been used to stimulate mitochondrial biogenesis. Although these interventions do not fix the underlying cause of the disease, increasing the mitochondrial mass might increase energy production, thus ameliorating the phenotype. There is increasing evidence in *in vitro* studies and animal models that increased mitochondrial biogenesis might be beneficial in many mitochondrial diseases. Interestingly, a recent observation suggested that increased mitochondrial content influences incomplete penetrance in LHON patients and that mitochondrial biogenesis could be used as a therapeutic strategy in this group of patients (Giordano et al. 2014). The biological pathway that controls mitochondrial biogenesis is complex and relies mostly on the peroxisome proliferator-activated receptor gamma (PPAR γ) coactivator 1α (PGC1α). PGC1α interacts with several transcription factors, including nuclear respiratory factors 1 and 2 (NRF1 and NRF2) and the

peroxisome proliferator-activated receptors α, β, and γ. Once activated, NRFs increases the transcription of OXPHOS genes and PPARs increase the expression of genes related to fatty acid oxidation (FAO) (Scarpulla 2008). Besides, PGC1α activity is increased by deacetylation and phosphorylation. Importantly, two enzymes responsible for these modifications, deacetylation by Sirt1 and phosphorylation by AMPK, can be modulated by drugs (Puigserver and Spiegelman 2003) and have been tested in preclinical models. Different agents used with this purpose are listed below.

Sirt1 is a nuclear deacetylase that utilizes $NAD^+$ to deacetylate residues of acetyl-lysine of proteins. Notably, Sirt1 is activated by increased cellular levels of $NAD^+$. This increase can be achieved by providing NAD precursor, such as nicotinamide riboside, or inhibiting NAD consuming enzymes, such as poly(ADP) ribosylpolymerase1 (PARP1). These approaches have been tested in animal models of mitochondrial myopathies with beneficial effects (Cerutti et al. 2014; Khan et al. 2014).

5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), an adenosine monophosphate analog, is an agonist of AMPK that has been used to increase the respiratory chain complex activities in three mouse models of COX deficiency ($Surf^{-/-}$, $Sco2^{KOKI}$, and ACTA-$Cox15^{-/-}$) with striking improvement of motor performances only in the Sco2 model (Viscomi et al. 2011).

Bezafibrate, a pan-PPAR activator, was tested in fibroblasts of patients with different MDs and was able to stimulate PGC1α and improve the mitochondrial respiratory chain defects (Bastin et al. 2008). These findings were subsequently confirmed by *in vivo* studies in mouse models of COX-deficiency (Noe et al. 2013; Hofer et al. 2014). However, studies on other mouse models did not show induction of mitochondrial biogenesis or increased mitochondrial respiratory chain enzyme activities (Viscomi et al. 2011; Yatsuga and Suomalainen 2012; Dillon et al. 2012). PPAR-δ agonists have also been proposed as potential therapeutic agents for primary mitochondrial myopathies.

Resveratrol has also been described as an activator of mitochondrial biogenesis in animal models and in human fibroblasts (Lopes Costa et al. 2014; Mizuguchi et al. 2017), although its mechanism of action is still unknown and it did not appear to increase OXPHOS activities in another study on human fibroblasts (De Paepe et al. 2014).

Retinoic acid has been used to stimulate the retinoid X receptor-alfa (RXRalfa) in cybrid containing the m.3243A>G mutation, ameliorating the respiratory chain defect (Chae et al. 2013).

Endurance training has also been used as an activator of mitochondrial biogenesis and has been reported to be beneficial and safe in the mtDNA *mutator* mice (Safdar et al. 2011), and in patients with MDs (Jeppesen et al. 2009; Zeviani 2008). Endurance training seems able to regulate not only PGC1α but also PGC1β, AMPK, and the hypoxia inducible factors (HIFs) (Rowe et al. 2012).

### *11.4.2 Regulating Mitophagy and Mitochondrial Dynamics*

Mitophagy is the selective elimination of dysfunctional mitochondria, a physiological process fundamental for maintaining normal mitochondrial function (Kim et al. 2007; Ashrafi and Schwarz 2013). This process is under the regulation of various pathways. One way of targeting mitophagy is via mTOR inhibition, which can be achieved by rapamycin. This approach has been investigated in a mouse model of Leigh syndrome (Ndufs4$^{-/-}$) (Johnson et al. 2013) and in a knock-in mouse model of mtDNA depletion syndrome, (Siegmund et al. 2017) and appeared to ameliorate the clinical phenotype and life-span of the treated mice, even though the biochemical defect was not rescued.

The balance between mitochondrial fusion and fission also contributes to the maintenance of mitochondrial function and can theoretically be regulated by specific modulators. An inhibitor of the mitochondrial fission protein dynamin-related protein1 (DRP1), for example the selective inhibitor P110, could potentially decrease pathological hyper-fragmentation observed in some MDs (Qi et al. 2013).

### *11.4.3 Bypassing OXPHOS Blocks*

The use of single-peptide enzymes derived from yeast or low eukaryotes to bypass mitochondrial respiratory chain defects has been tested in *in vitro* and in vivo models. In particular, Ndi1 substitutes complex I in yeast and transfers electron to CoQ, without pumping protons across the membrane. AOX is present in lower eukaryotes and bypasses complex III and IV transferring electrons from CoQ. Expression of these enzymes has been used to bypass complex I deficiency (Perales-Clemente et al. 2008; Sanz et al. 2010) and Complex III-IV deficiencies in human cells and drosophila (Dassa et al. 2009; Fernandez-Ayala et al. 2009), but not in mammals *in vivo*.

### *11.4.4 Mitochondrial Replacement Therapy*

Mitochondrial DNA mutations are transmitted maternally and can cause fatal or severe disorders in children (Schon et al. 2012). Moreover, these mutations are relatively common with an estimated 12,423 women at risk for transmitting mtDNA pathogenic mutations and 778 affected children per year in the United States (Gorman et al. 2015). Prenatal and preimplantation diagnoses are currently the only options available to women carrying mtDNA mutations who want to give birth to healthy children genetically related to them (Richardson et al. 2015). These techniques can accurately predict the risk of the embryo of carrying a high mtDNA mutation load. However, they cannot be applied to women with homoplasmic or

nearly-homoplasmic mutations. Mitochondrial replacement therapy is a promising new reproductive technique that prevents the transmission of mtDNA mutations. It consists in combining nuclear DNA (nDNA) from a woman with a mtDNA mutation with the mitochondria of a healthy donor. This can be achieved either transferring nuclear genetic material between the oocyte of the woman carrying the mutation and the oocyte of a healthy donor or between embryos (pronuclear transfer). These techniques have been first performed in non-human primates (Tachibana et al. 2009), and then in human embryos and oocytes of healthy individuals (Craven et al. 2010; Paull et al. 2013; Tachibana et al. 2013) and women with mtDNA mutations (Kang et al. 2016) with success. Optimization of these techniques is *ongoing* and long-term efficacy and safety still under examination. A reversion to the original mtDNA after MRT, for example, has been recently reported from different groups (Yamada et al. 2016; Hyslop et al. 2016; Kang et al. 2016) pointing towards the necessity of additional studies to evaluate the compatibility of donor mtDNA haplogroups. Moreover, there is a fervent debate on the ethical issues concerning manipulating oocytes. Nonetheless, 2 years after the approval of the UK parliament, the UK Human Fertilisation and Embryology Authority (HFEA) authorized the use of mitochondrial replacement on a case-by-case basis in 2016. The authorities have not yet approved mitochondrial replacement techniques in the United States, even if there is evidence that women carrying mtDNA mutations and oocyte donors would support the development of these procedures (Engelstad et al. 2016). The first successful mitochondrial replacement therapy via oocyte chromosomal spindle transfer has been reported; a woman carrier of the m.8993T>G mutation with a prior history of four miscarriages and 2 children who died of Leigh syndrome gave birth to a healthy boy with neonatal mtDNA mutation loads 2.36–9.23% in tested tissues (Zhang et al. 2017).

### *11.4.5 Scavenging of Specific Toxic Compounds*

Ethylmalonic encephalopathy (EE) is a devastating disorder of infancy due to ETHE1 mutations. ETHE1 encodes a mitochondrial sulfur dioxygenase (SDO) involved in the elimination of $H_2S$. Accumulation of $H_2S$, produced by the catabolism of amino acids and by the anaerobic flora of the intestine, is toxic and leads to inhibition of COX activity and to endothelial damage. N-acetyl cysteine is a precursor of glutathione and can be used to buffer intracellular $H_2S$. Metronidazole is an intestinal antibiotic active against anaerobic bacteria that produce $H_2S$.The use of metronidazole and N-acetyl cysteine in a mouse model of ethylmalonic encephalopathy (*Ethe1*$^{-/-}$) prolonged the lifespan and ameliorated the clinical phenotype of this model. Moreover, the administration of these compounds in a cohort of patients with EE was able to improve some of the clinical features of the disease (Viscomi et al. 2010). This treatment has not been tested in clinical trials yet.

In MNGIE, hemodialysis has been used to remove these toxins but was not effective in decreasing thymidine or deoxyuridine levels (Spinazzola et al. 2002).

### 11.4.6 Supplementation of Nucleotides/Nucleosides

Supplementation of deoxyribonucleotides and deoxyribonucleosides has been exploited in *in vitro* and *in vivo* models of mitochondrial deoxynucleotide triphosphate (dNTP) pool unbalance. Mitochondrial dNTP pool unbalance causes mtDNA instability and consequent mtDNA depletion, multiple deletions, and point mutations. Different enzymes are involved in the maintenance of dNTP pools, such as thymidine kinase 2 (TK2), deoxyguanosine kinase (dGK), and thymidine phosphorylase (TP).

Thymidine kinase 2 (TK2) is a mitochondrial matrix protein that phosphorylates thymidine and deoxycytidine nycleosides to generate deoxythymidine and deoxycytidine monophosphate (dTMP, dCMP), which are then converted to dNTPs, fundamental for mtDNA synthesis. Recessive mutations in *TK2* gene cause dNTP pool unbalance and mtDNA instability. The consequent clinical phenotypes range from a severe infantile neuromuscular form to adult-onset chronic progressive external ophthalmoplegia. Promising results were obtained in a Tk2 knockin mouse model ($Tk2^{H126N/\ H126N}$) with oral administration of deoxycytidine and deoxythymidine monophosphates and subsequently deoxycytidine and deoxythymidine; both treatments increased mtDNA levels and mitochondrial respiratory chain enzyme activities, and prolonged the lifespan of the homozygous mutant mice (Garone et al. 2014; Lopez-Gomez et al. 2017). In 2019, a compassionate use (expanded access) study of 16 patients demonstrated safety and improved survival in early onset TK2 deficiency patient (onset <2 years-old) as well as well as motor functions in all forms of TK2 deficiency relative to the natural history studies (Dominguez-Gonzalez et al. 2019).

Depletion of mtDNA has been corrected in vivo in a Tymp knockout mouse model of MNGIE disease by administrating deoxycytidine or tetrahydrouridine (Camara et al. 2014). In the same study, the addition of deoxycytidine and tetrahydrouridine to a cell model of MNGIE disease (dThd-induced mtDNA depleted fibroblasts) was also able to prevent mtDNA depletion. mtDNA depletion was also corrected in dGK deficient human fibroblasts by adding deoxyguanosine (Camara et al. 2014).

### 11.4.7 Cell Replacement Therapies

Cell replacement has been explored in different mitochondrial disorders as a method to deliver a specific protein when deficient. For instance, platelets (Lara et al. 2006) or erythrocyte-encapsulated thymidine phsphorylase (Bax et al. 2013) have been transfused in patients with MNGIE disease with temporary reduction of thymidine and deoxyuridine levels, but currently there is no evidence of the sustained effect of these approaches.

Allogenic hematopoietic stem cell transplantation (HSCT) has shown potential long-term effects in treating MNGIE patients (Hirano et al. 2006) being able to restore TP function and improve biochemical and clinical manifestation of the disease. This procedure albeit has been associated with high mortality. A retrospective study (Halter et al. 2015) evaluated the experience of HSCT in MNGIE patients and underlined the effectiveness of this treatment and the importance of balancing risks and benefits of this procedure. A consensus statement for future transplants in MNGIE patients has been published (Halter et al. 2011) and HSCT should be recommended in selected patients with optimized transplant conditions (Halter et al. 2015).

Liver transplantation has been recently performed in an infant with EE due to ETHE1 mutations (Dionisi-Vici et al. 2016). The patient showed progressive improvement of the neurological function and normalization of the biochemical abnormalities. Liver transplantation can replace the deficient enzyme and clear the toxic compounds that accumulate in this disorder, constituting a feasible therapeutic option in patients with EE.

### *11.4.8 Gene Therapy*

**Nuclear DNA Defects**

Adeno-associated viruses (AAV) are ideal candidates as viral vectors for gene therapy, given their low risk of insertional mutagenesis, and are currently the most widely used. AAV-mediated gene therapy has been performed in different mouse models of nuclear-encoded MDs. The first animal model was a $Ant1^{-/-}$ treated with muscle injection of AAV2 (Flierl et al. 2005). AAV2 vector targeted to retina was used to express AIF in the eye of the Harlequin mouse and restore complex I deficiency (Bouaita et al. 2012). A liver specific AAV2,8 serotype was used in a mouse model of EE and was able to dramatically improve the clinical course and the biochemical abnormalities of mutant mice (Di Meo et al. 2012). This study demonstrated that restoring ETHE1 activity selectively in the liver was sufficient to correct the enzymatic defect and led to the hypothesis that liver transplant could be used in patients with EE. Similarly, the same hepatotropic vector was used in a mouse model of MNGIE disease and was proven successful (Torres-Torronteras et al. 2014), suggesting that liver transplantation could be an option also in MNGIE patients (Boschetti et al. 2014). The first transplanted patient was described in 2016 (De Giorgio et al. 2016). The biochemical abnormalities rapidly normalized after the transplant, and clinical conditions remained stable after 400 days. AAV2,8 vector was also used to express the wild-type MPV17 protein in a mpv17 knockout mouse model of mtDNA depletion and hepato-cerebral syndrome (Bottani et al. 2014). The vector was able to rescue the mtDNA depletion and prevent liver steatosis induced by ketogenic diet in this model.

**mtDNA Defects**
Even more challenging is delivering gene therapy into mitochondria. One attempted approach is to allotopically express recombinant mtDNA encoded proteins containing a mitochondrial targeting sequence (MTS) in the nucleus. This approach has been tried in fibroblasts carrying mutations in ND1, ND4, and ATP6 genes (Bonnet et al. 2007, 2008; Kaltimbacher et al. 2006) and in an animal model of LHON (Ellouze et al. 2008). Despite the controversial preclinical results, clinical trials have started in patients with LHON (NCT01267422, NCT02064569 NCT02161380) and one recently completed clinical trials has shown promising results (Wan et al. 2016) (See Sect. 11.3).

### *11.4.9 Shifting mtDNA Mutation Heteroplasmy*

Pathogenic mtDNA mutations are usually heteroplasmic, requiring a minimum critical mutation load to cause mitochondrial dysfunction. Shifting heteroplasmic levels in order to reduce the amount of mutated DNA below this threshold, therefore, has been used as a therapeutic approach. This can be achieved with different techniques: mitochondrial-targeted restriction endonucleases (Srivastava and Moraes 2001), zinc finger endonucleases (ZFNs) (Minczuk et al. 2008), transcription activator-like effectors nucleases (TALENs) (Bacman et al. 2013), and CRISPR (clustered regularly interspaced palindromic repeat)/Cas9. Restriction endonuclease *Sma*I has been used in cybrids carrying the m.8399T>G mutation and was able to reduce the mutation load and increase ATP levels (Tanaka et al. 2002; Alexeyev et al. 2008). Restriction endonucleases have been exploited also in heteroplasmic mouse models, using AAV vectors, with promising results (Bayona-Bafaluy et al. 2005; Bacman et al. 2007, 2010, 2012). The main limitation of this approach is that requires the generation of a suitable restriction site by the mtDNA mutation. The introduction of ZFNs and TALENs overcomes this limitation. ZFNs are engineered mitochondrially targeted heterodimeric zinc finger nucleases conjugated to the restriction enzyme *Fok*I. Each zinc finger domain recognizes three nucleotides, so arranging zinc finger modules appropriately allow for recognition of virtually any DNA sequence. Expression of mtZFNs in cybrids was able to reduce the mutant mtDNA and restore mitochondrial function (Gammage et al. 2014). TALENs also work as heterodimers, requiring two monomers to bind close DNA sequence in order to allow the *Fok*I nuclease to dimerize and cleave DNA, as for the mtZFNs. Reengineered TALENs targeted to different mtDNA point mutations and deletion (MitoTALENs) were able to permanently reduce the mutation load in patient-derived cells (Bacman et al. 2013). The CRISPR/Cas9 system, another endonuclease-based system, has been reported to be more effective than TALENs and has been demonstrated to rescue mitochondrial and skeletal muscle impairment in an iPS cell model of $CoQ_{10}$ deficiency due to a mutation in the *COQ4* gene (Doudna and Charpentier 2014; Romero-Moya et al. 2017). A possible limitation to the use of ZNFs and MitoTALENs in clinical practice is that AAV vectors usually are able to

fit smaller constructs. Moreover, the risk of a rapid reduction in mtDNA copy numbers of inducing a potential mtDNA depletion syndrome remains a limitation for the potential clinical application of these approaches. Lastly, mitochondria-targeted restriction endonucleases and TALENs have also been used in the selective elimination of mtDNA mutations in the germline of the heteroplasmic mouse model and artificial mammalian oocytes as a potential approach for preventing transmission of mtDNA mutations (Reddy et al. 2015).

### *11.4.10 Stabilizing Mutant tRNAs*

The majority of the mtDNA mutations are localized to tRNA genes. It is not surprising therefore that several therapies have been targeting mt tRNAs. In particular, tRNA synthetases are enzymes that catalyze the attachment of amino acids to their cognate tRNA during protein synthesis. Many studies indicated that overexpressing cognate and non-cognate aminoacyl mt-tRNA synthetase can stabilize mt-tRNAs and attenuate the detrimental effect of the mutation in yeast and human cell lines (De Luca et al. 2006, 2009; Li and Guan 2010; Rorbach et al. 2008; Perli et al. 2014; Hornig-Do et al. 2014).

## 11.5 Conclusions

Remarkable progress has been made in the past years in mitochondrial medicine. Many potential therapeutic approaches for MDs have been proposed and are now at different stages of development. Translating preclinical studies to bedside remains challenging and well-controlled trials of high quality are necessary to define the efficacy of potential therapies already in use and novel drugs (Pfeffer et al. 2013). Based on the knowledge acquired with the previous studies, these future trials may overcome the challenges posed by this heterogeneous group of disorders in the context of multicenter collaborations, by selecting numerous subgroups of homogeneous patients and by selecting outcome measures that are objective and relevant to patient care and quality of life. Undoubtedly, there is a need for evidenced-based guidelines in the treatment of mitochondrial patients and the development of more effective therapies is an exciting perspective for the near future.

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