REVIEW

MITOCHONDRIAL DISORDERS OF THE OXPHOS SYSTEM

Erika Fernandez-Vizarra[1] and Massimo Zeviani[2]

[1] Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, University Avenue, Glasgow G12 8QQ, Scotland, UK. Electronic address: Erika.Fernandez-Vizarra@glasgow.ac.uk

[2] Venetian Institute of Molecular Medicine, Via Orus 2, 35128 Padova, Italy; Department of Neurosciences, University of Padova, via Giustiniani 2, 35128 Padova, Italy. Electronic address: massimo.zeviani@unipd.it

ABSTRACT

Mitochondrial disorders are amongst the most frequent inborn errors of metabolism, their primary cause being the dysfunction of the oxidative phosphorylation system (OXPHOS). OXPHOS is composed of the electron transport chain (ETC), formed by four multimeric enzymes and two mobile electron carriers, plus an ATP synthase (also called complex V). The ETC performs the redox reactions involved in cellular respiration while generating the proton motive force used by complex V to synthesize ATP. OXPHOS biogenesis involves multiple steps, starting from the expression of genes encoded in physically separated genomes, such as mitochondrial and nuclear DNA, to the coordinated assembly of multiple components and cofactors building each individual complex and eventually the supercomplexes. The genetic cause underlying around half of the diagnosed mitochondrial disease cases is currently known. Many of these cases result from pathogenic variants in genes encoding structural subunits or additional factors directly involved in the assembly of the ETC complexes. Here we review the historical and most recent findings concerning the clinical phenotypes and the molecular pathological mechanisms underlying this particular group of disorders.

INTRODUCTION

Single or isolated deficiencies in components of the oxidative phosphorylation (OXPHOS) system cause primary mitochondrial disorders, a heterogeneous group of inborn errors of metabolism. The OXPHOS system is located in the mitochondria, eukaryotic organelles of endosymbiotic origin responsible for cellular energy conversion. Four electron transfer chain (ETC) multimeric enzymes, complexes I to IV, plus two mobile electron carriers, coenzyme Q and cytochrome *c*, are responsible for taking reducing equivalents from NADH and $FADH_2$, generated in the upstream catabolic pathways of nutrients or storage compounds (e.g. sugars and fats), and transfer them to O_2, reducing it to water. This process is called cellular respiration. The energy liberated by the electron transfer between the different redox centers is exploited to pump protons across the impermeable mitochondrial inner membrane, in three ETC complexes, complex I (cI) complex III (cIII) and complex IV (cIV) or cytochrome c oxidase (COX), whereas no proton pumping activity is associated with the redox function of complex II (cII). The proton translocation from the matrix to the intermembrane space (IMS), generates an asymmetric distribution of protons across the inner mitochondrial membrane (IMM), which acts as a proton-motive force exploited by the ATP synthase (or complex V) to condensate ADP and inorganic phosphate to generate ATP. The newly synthesized ATP is then translocated to the cytoplasm by the mitochondrial adenine-nucleotide transporter family, to provide chemical energy for virtually all the endergonic biochemical cellular processes. In humans, mitochondria produce, and the cells consume, a daily amount of ATP equivalent to our whole-body weight, around 72 kg on average. Mitochondria are, therefore, central for metabolism and their dysfunctions create not only ATP shortage but also oxidative and metabolic stress, which seems to contribute significantly to the disease phenotypes [1, 2]. Due to their evolutionary origin as an α-

proteobacterium that was engulfed by a primordial non-oxidative cell, nowadays deemed as an archeon organism [3], mitochondria have transferred most of their genes to a newly formed organelle, the nucleus, albeit all the respiring mitochondria have also conserved their own, small genome, mitochondrial DNA (mtDNA). Human mtDNA is a circular double stranded DNA of approximately 16.5 kb in size containing the coding sequence for thirteen polypeptides, all essential components of the OXPHOS system [4]. MtDNA encodes also the RNA elements, two ribosomal RNAs and twenty-two transfer RNAs, necessary for the translation of the thirteen proteins inside the organelle. One of the reasons for the *in situ* translation of the mtDNA genes is that in most eukaryotic species the mitochondrial genetic codes differ from the universal code and therefore the two systems are reciprocally untranslatable. Thus, the translation of mtDNA protein encoding genes relies on the presence of special translators (mt-tRNAs) acting on a peculiar mitochondrial ribosome, whose atomic structure was recently resolved by cryo-EM analysis [5]. This consideration suggests that there must be a compelling evolutionary constraint to maintain this tiny genome as transcriptionally active, albeit for coding only thirteen genes; however, the explanation for this peculiar and fascinating phenomenon is still unclear. The reason that genetic mitochondrial codes vary in different species is not a valid argument, since this modification has occurred in different species, and in some organisms did not occur at all (for instance in plants). Therefore, the reason to maintain a respiring mtDNA and all the costly apparatus to make it expressed has to underpin a more fundamental, biologically essential foundation. For instance, it has been proposed that the protein subunits encoded by mtDNA are too hydrophobic in a way that they could not tolerate the conformational changes occurring during translocation of proteins through the inner membrane and the aqueous environment of the cytoplasm during protein synthesis, so they must be inserted

immediately in the inner mitochondrial membrane [6], with the help of suitable "incorporators" like OXA1 [7]. Other hypotheses have been proposed to explain the maintenance of the mtDNA genes, including protein-encoding and the translational RNA apparatus. Perhaps the most convincing is that the mtDNA must act as a single functional unit, therefore each somatic or inherited mutation in mtDNA has to be probed for bioenergetic proficiency in the context of the whole respiratory chain structures, and a local patrolling provided by the multiple copies of mtDNA distributed within each mitochondria can act as essential checkpoints to probe the bioenergetic efficiency of the system through a local surveillance [8]. All the other components used for mtDNA maintenance and expression, as well as the rest of OXPHOS structural subunits and the factors that take care of their correct assembly, modulate the function of the OXPHOS enzymes and control their turnover, are encoded in the nuclear DNA (Figure 1). All these proteins are translated in the cytoplasmic ribosomes and must be translocated inside the mitochondria using specific and highly sophisticated import machineries [9]. Due to the biogenetic peculiar features of mitochondria, the genetics of mitochondrial disorders is both heterogeneous and unique. In sexuate organisms, mtDNA is transmitted via the maternal gametes and in all respiring eukaryotes there are multiple copies of this genome in each cell. Thus, if the pathological mutation is in the mtDNA, the inheritance mode follows the maternal lineage, and the phenomenon of 'heteroplasmy' usually takes place, where mutated and non-mutated copies co-exist in the same individual [10]. In most of the cases, the clinical disease and the biochemical defect only manifest if the percentage of mutated mtDNA molecules exceeds that of a pathological threshold, which is variable for each kind of mutation [11], but is rarely <50%. The first disease causing mitochondrial mutations were described in the mtDNA itself [12-14] and since then, hundreds of new pathological variants have been and

continue to be discovered in this genome [15]. However, as expected since most of the mitochondrial proteome is encoded in the nucleus, mutations in the nuclear genome causing mitochondrial disease were also discovered [16-18]. In these cases, the inheritance can be X-linked; autosomal dominant or, more commonly, autosomal recessive, but *de novo* sporadic mutations are not uncommon. With the development of next generation sequencing (NGS) techniques of DNA and RNA, there has been a substantial increase in the number of genes with mutations known to cause mitochondrial disease [19-22]. Up to this date, there are mutations described in nuclear genes encoding OXPHOS structural proteins as well as factors involved in basically every step of OXPHOS biogenesis: from mtDNA replication and maintenance, mitochondrial transcription and translation to import, assembly, synthesis and incorporation of redox cofactors, as well as proteins necessary for proper mitochondrial cristae shaping, dynamics and quality control, composition of the lipid milieu or detoxifying pathways, etc. [20]. Mutations that directly affect the assembly of the OXPHOS components can either be found in structural subunits or in assembly factors, being widely defined as proteins necessary for the correct maturation of the complexes, which do not take part in their final structures. The function of these factors can be to either stabilize assembly intermediates, favor the insertion of specific subunits or carry out the biosynthesis and/or incorporation of redox cofactors in the active sites of the enzymes [23]. In the OXPHOS disorders due to mutations in structural subunits and assembly factors, the severity of the biochemical and assembly defects is highly variable and it depends greatly on where during the assembly process the protein is acting and on the nature of the mutation [23, 24].

Here we will review the current knowledge concerning the genetic basis of primary mitochondrial disease associated with pathogenic variants in genes encoding: i) structural

subunits, ii) factors directly responsible for the assembly and maturation of each of the five OXPHOS enzymatic complexes and iii) enzymes responsible for synthesis and function of the mobile electron carriers (coenzyme Q and cytochrome *c*).

DISORDERS OF COMPLEX I

Complex I structure and assembly

Complex I (cI) is the NADH:CoQ oxidoreductase enzyme, i.e. the main entry point of electrons into the respiratory chain. It is the largest ETC enzyme, being composed of forty-five subunits in total, but forty-four different proteins, as cI contains two copies of the acyl-carrier protein NDUFAB1 [25]. As shown in Figure 2A, the enzyme consists of two main domains, a hydrophilic arm protruding into the mitochondrial matrix and a hydrophobic arm embedded in the IMM. The hydrophilic arm transfers electrons from NADH to coenzyme Q, through FMN and several Fe-S clusters, whereas the membrane arm is responsible for proton translocation [26, 27]. The two arms are further organized into six functional modules, two (the N- and Q-modules) in the peripheral arm; and four (the ND1-, ND2-, ND4- and ND5-modules) in the membrane arm [28], an arrangement which reflects also the cI modular assembly pathway [29]. The hinge of the L-shaped structure contains a channel harboring the CoQ binding site (therefore called Q-module) [28, 30]. Each of the modules is assembled independently with the assistance of specific assembly factors. All the individual modules, except for the N-module, come together to form the 'pre-cI' or ~830 kDa subcomplex which is stabilized by NDUFAF2. The assembly of cI is completed and the complex activated only once the N-module (the last catalytic module) is incorporated [31-33], as the plug which gets inserted in an electric device to activate an electric current.

Mutations in cI structural subunits

Seven subunits located in the hydrophobic membrane arm, MT-ND1—6 and ND4L, are encoded in the mtDNA (Table 1). They are all 'core' subunits, conserved from bacteria to mammalian mitochondria [34], and a significant number of pathological variants have been found in all of them. As other mtDNA pathological variants, mutations in the *MT-ND* genes are associated with syndromes of different degrees of severity and age of onset, occurring most frequently during late childhood or early adulthood [35-38]. Mutations in the *MT-ND* genes are also the main cause of Leber's hereditary optic neuropathy (LHON), the m.11778G>A variant in *MT-ND4* being the most frequently found in these patients [39]. Other mutations are associated with more severe cases of mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS) (OMIM 540000), Leigh syndrome (OMIM 256000) or other less defined mitochondrial encephalopathies with isolated cI deficiency [35, 37].

Pathological variants have also detected in twenty-four out of the thirty-seven nuclear-encoded cI subunits. Most of these twenty-four subunits are located in the NADH-dehydrogenase and Q-modules of the hydrophilic peripheral arm (Table 1 and Figure 2). Mutations hit both core subunits (NDUFV1, NDUFV2, NDUFS1, NDUFS2, NDUFS3, NDUFS7, NDUFS8) and 'supernumerary' subunits (NDUFS4, NDUFS6, NDUFA2, NDUFA12, NDUFA13, NDUFA1, NDUFA6, NDUFA8, NDUFA9, NDUFA10, NDUFA11, NDUFB3, NDUFB8, NDUFB9, NDUFB10, NDUFB11, NDUFC2), which are not necessary for catalysis but important for the assembly/stability of cI [28]. Mutations in nuclear-encoded cI subunits cause severe encephalopathies, mainly Leigh syndrome, with symptoms starting in early childhood [40]. An interesting exception is that of mutations in *NDUFB11*, which were associated with the X-linked microphthalmia with linear skin defects (MLS) syndrome [41]. This condition is embryonic lethal in males, while affected females, which show a wide spectrum of

abnormalities depending on the degree of X-inactivation expressing the mutant gene, do not display an overt biochemical cI defect, presumably because the cells where the X-chromosome carrying the mutated variant is active are selected out by apoptosis [41]. More recently, patients with NDUFB11 mutations displaying more classical mitochondrial disease syndromes have been described [42, 43].

Mutations in assembly factors/chaperones

Given the size and the structural and functional intricacy of cI, its biogenesis is further complicated by the many assembly factors involved [32]. The genetic basis in many cases of cI deficiency is explained by mutations in nuclear genes encoding these very proteins [44]. As with the deficits originated from mutations in the nuclear-encoded structural subunits, the syndromes associated with cI assembly factors usually present early in childhood. The most typical clinical feature is encephalopathy but also cardiomyopathy and, less frequently, hepatic and renal involvement (Table1) [23]. The different cI chaperones/assembly factors can be classified depending of what module they stabilize or help assemble (Table 1) [33]. Thus, the nature and severity of the assembly defect observed in the cells carrying the deficient alleles will depend on these roles and the impact of the mutant variant on protein function [23]. NDUFAF1 (CIA30), ECSIT, ACAD9 and TMEM126B form the so-called Mitochondrial Complex I Assembly (MCIA) complex [45], with which TMEM186 and COA1 associate [46]. This assembly factor complex is important for the biogenesis of the ND2-module. Pathological variants causing cI deficiency have been described in *NDUFAF1* [47-49], *ACAD9* [50, 51] and *TMEM126B* [52, 53]. NDUFAF3 (C3ORF60) and NDUFAF4 (C6ORF66) work together in the assembly of the Q-module, and mutations in both of them have been associated with different cases of infantile mitochondrial disease [54-59]. Another disease-related cI assembly factor, NDUFAF6 (C8ORF38) [60-64] is also thought to

assist in the assembly of the Q-module and maintain normal levels of the structural subunit MT-ND1 [28, 61, 65]. Both NDUFAF5 (C20ORF7) and NDUFAF7 are modifying enzymes of subunits of the Q-module, catalysing the hydroxylation of NDUFS7 and the dimethylation of NDUFS2, respectively [66, 67]. These are post-translational modifications essential for cI assembly [68, 69]. Mutations in *NDUFAF5* cause severe early onset encephalopathy [68, 70, 71], whereas a heterozygous variant in NDUFAF7 seemed to cause myopia in a Chinese family [72]. A homozygous intronic mutation in the gene encoding an assembly factor of the ND1-module, TIMMDC1 (C1ORF1) [73, 74], was found in several cases of cI deficiency thanks to next-generation RNA sequencing techniques [75]. During the process of modular cI assembly, the ND4-module is stabilized by FOXRED1, ATP5SL/DMAC2 and TMEM70 [33]. Although mutations in *TMEM70* have mainly been associated with complex V deficiency [76, 77], the protein appeared associated with intermediates of the ND4-module when studying the human cI assembly pathway [29]. TMEM70 has now been proposed to act as an assembly factor for both cI and cV [78]. Pathological variants in *FOXRED1* are also the underlying cause of mitochondrial respiratory cI deficiency associated with either Leigh syndrome, encephalocardiomyopathy or ataxia [79-81]. A founder non-sense mutation and, more recently, other missense mutations in TMEM126A, have been pointed out as the cause of autosomal recessive optic atrophy [82-86]. Being that TMEM126A is a paralog of TMEM126B, it was suggested that the former could compensate for the loss of function of the latter [52]. However, recent data point out to a role for TMEM16A as an assembly factor associated with the ND4-module [87, 88]. NDUFAF2 (B17.2L or NDUFA12L) was mutated in patients showing either generic encephalopathic syndromes or Leigh syndrome [89-92]. The study of cI assembly in patient-derived cells carrying mutations in *NDUFAF2* or in genes

encoding structural subunits of the N-module, led to the conclusion that NDUFAF2 binds to a late-stage cI assembly intermediate before the incorporation of the N-module [89, 93].

Mutations in co-factor synthesis/incorporation

NUBPL is the human homolog of *Yarrowia lipolytica* Ind1 [94]. Both NUBPL and Ind1 are essential for cI assembly and well established evidence points out to its role in the specific incorporation of Fe-S clusters into several cI subunits of the peripheral arm [94, 95]. After the identification of NUBPL, originally designated as huIND1, several pathological mutations leading to a characteristic leukoencephalopathy and, in some cases, multisystemic involvement have been described [79, 96-98].

DISORDERS OF COMPLEX II

Complex II structure and assembly

Succinate dehydrogenase (SDH) or complex II (cII) is both an ETC and Krebs cycle enzyme, oxidizing succinate to fumarate and transferring the electrons to coenzyme Q (CoQ). It is composed of four subunits, all encoded in the nuclear genome. The largest hydrophilic subunits, SDHA and SDHB, protrude towards the matrix and contain the redox active groups flavin adenine dinucleotide ($FAD(H_2)$) and three Fe-S clusters, respectively. The small subunits SDHC and SDHD are bound to the inner membrane and contain two CoQ binding sites [99]. The assembly of cII occurs via the independent maturation of SDHA, SDHB and SDHC+SDHD with the assistance of four specific chaperones (SDHAF1-4) necessary for the stabilization and incorporation of the prosthetic groups into each of the structural subunits [33, 100].

Mutations in cII structural subunits

Pathological variants have been found in all four succinate dehydrogenase (SDH or cII) structural subunits, and most of them are causative of hereditary tumors, specifically paragangliomas and pheochromocytomas as well as gastrointestinal cell sarcomas (Table 2). *SDHB* and *SDHD* mutations are the most frequent in these neoplastic disorders [101, 102]. The pathogenetic mechanism is not completely clear but it seems to be related to the accumulation of succinate, which is known to stabilize HIF1-alpha, thus inhibiting its degradation by prolyl-hydroxylase, as a control mechanism activating the hypoxic program of the cell [103]. However, mutations in *SDHA,* encoding the 70 kDa Flavoprotein subunit, have also been found in rare cases of Leigh syndrome, a typical mitochondrial disease phenotype, associated with cII deficiency [16, 104-108]. Other encephalopathic and myopathic syndromes have been more recently associated with pathological variants in *SDHA*, *SDHB* and *SDHD* [108-110]. In addition, cardiomyopathy is also a phenotypic manifestation of cII deficiency associated with mutations in *SDHA* [111, 112] and *SDHD* [113].

Mutations in cII assembly and prosthetic group incorporation

Of the four known cII assembly factors, two are associated to disease in humans: SDHAF1 and SDHAF2. SDHAF1 is a LYR-motif containing protein involved in the insertion of the Fe-S clusters into SDHB [114] and its mutations are the cause of leukoencephalopathy and cII deficiency [115, 116]. Mutations in SDHAF2, with a role in the stabilization and flavinylation of SDHA, are related to the other main group of pathologies associated with SDH defects, i.e., paragangliomas and pheochromocytomas [117-119].

DISORDERS OF COMPLEX III

Complex III structure and assembly

Complex III (cIII) constitutes the central part of the ETC, accepting two electrons from reduced CoQ ($CoQH_2$) and donating them, one by one, to cytochrome c, via a series of catalytic subunits: cytochrome b (MT-CYB in human nomenclature), containing two CoQ binding sites and two heme *b* groups; UQCRFS1, the Rieske Fe-S protein; and CYC1, containing heme *c* as the prosthetic group. The structure is that of a symmetric dimer, it is highly conserved from yeast to mammals and each of the 'monomers' is composed of ten different subunits [120]. In mammals, the N-terminal peptide that is cleaved off during UQCRFS1 maturation is retained and bound in the interface between the UQCRC1 and UQCRC2 subunits [120-122].

The assembly pathway of cIII has been well defined in yeast [123-125] and since the first and last steps are conserved, it has been assumed that in humans it would proceed in a similar way [33]. The cIII assembly begins with the synthesis, membrane insertion and hemylation of cytochrome b, mediated by Cbp3, Cbp6 and Cbp4 (UQCC1-3 in humans) [126-130], followed by the sequential incorporation of the rest of the subunits and early dimerization of the subassembled species [131]. The accumulation of CYC1 together with UQCR10, and most probably UQCRH, in the absence of MT-CYB is peculiar of human cIII, differing from the yeast assembly pathway [132]. The assembly proceeds until the formation of a dimeric pre-$cIII_2$, lacking UQCRFS1, the last incorporated catalytic subunit, and the smallest subunit UQCR11. Three assembly factors, MZM1L (LYRM7), BCS1L and TTC19 are known to be involved in the stabilization, incorporation and metabolism of UQCRFS1 [122, 133-139].

Mutations in cIII structural subunits

As most of the described mutations in OXPHOS structural components, the first ones affecting cIII were identified in the mtDNA gene encoding MT-CYB. Most of these

pathological variants were found in heteroplasmy and mainly associated with late-onset sporadic myopathy and exercise intolerance [140-145]. More rarely, *MT-CYB* mutations can cause histiocytoid cardiomyopathy [146], parkinsonism and MELAS overlap syndrome [147] or multisystem disorders [148-151]. Drastic *MT-CYB* mutations cause combined cI+cIII deficiencies, affecting cIV as well in some cases [132, 144, 152, 153]. This fact will be discussed in further detail in a dedicated section of this review.

Some mutations in nuclear genes encoding cIII structural components have been found in a handful of cases (Table 3). The first ones were described in UQCRB and UQCRQ, the subunits that bind MT-CYB early in the assembly pathway [125, 154] (Figure 2). A homozygous 4-bp deletion in *UQCRB,* causing a frameshift and extended protein product, was present in a girl from a consanguineous family showing hepatopathy and isolated cIII deficiency [155]. In addition, the only missense *UQCRQ* mutation described to date was homozygous in patients of a large consanguineous cohort where all the affected individuals presented with early-onset severe encephalopathy [156]. The pathological substitution p.Arg73Trp in Core 2 (UQCRC2) was found in a Mexican consanguineous family and in an individual of French-Canadian origin [157, 158]. All of these subjects had metabolic decompensation, mainly showing hypoglycemia and lactic acidosis with no direct neurological involvement.

Pathological mutations in the nuclear-encoded catalytical subunits CYC1 and UQCRFS1 have also been identified. In the case of CYC1, two different missense mutations were present in two unrelated individuals showing a similar clinical course with cIII deficiency accompanied by recurrent metabolic crises and insulin-responsive hyperglycemia [159]. Defective UQCRFS1 has been deemed the cause of decreased cIII activity, lactic acidosis, cardiomyopathy and *alopecia totalis* [160].

Mutations in cIII assembly factors

The most frequent cause of cIII deficiency of nuclear origin are mutations in assembly factors [154]. Up to date five of such factors have been associated with mitochondrial disease: BCS1L, TTC19, LYRM7 (MZM1L), UQCC2 and UQCC3. Of all these, mutations in *BCS1L* are by far the most frequent (Table 3). BCS1L is responsible for the translocation of the Rieske Fe-S protein (UQCRFS1) from the matrix to the inner mitochondrial membrane in the process of $cIII_2$ maturation [136-139]. Since the initial description of the first four missense mutations [161], around thirty different pathological variants in *BCS1L* are known to cause a wide range of clinical phenotypes, from the severe growth retardation, aminoaciduria, cholestasis, iron overload, lactacidosis, and early death (GRACILE) syndrome [162] to the mild Björnstad syndrome [163], with everything in between [154, 164]. The origin of this clinical variability is unknown and a clear BCS1L genotype-phenotype relationship has proven difficult to establish [163-165]. LYRM7 (or MZM1L), the human homolog of yeast Mzm1, is involved in the stabilization of UQCRFS1 and Fe-S cluster insertion [133-135, 166]. Mutations in *LYRM7* cause infantile cIII deficiency associated with a characteristic cavitating leukoencephalopathy [167-169]. In addition, compound heterozygous mutations in *LYRM*7 were deemed to cause cIII deficiency in a case of liver failure with metabolic decompensation [170]. Interestingly, these authors proposed the idea that cIII deficiency in the liver impairs the capacity of metabolic adaptation to prolonged fasting, in this case for mutations in *LYRM7*, but also for those in *UQCRB*, *UQCRC2* and *CYC1* (see above). A mitochondrial encephalomyopathic syndrome originated by variants in *TTC19* is the second most frequent cause of cIII deficiency of nuclear origin in humans [154]. TTC19 is a protein only present in metazoans that binds to dimeric cIII ($cIII_2$) once it is fully assembled, i.e. when UQCRFS1 is incorporated. TTC19 maintains cIII enzymatic activity by promoting the proteolysis of the N-terminal fragments of UQCRFS1

produced during its processing due to the import and/or assembly processes [122]. The clinical presentations and age of onset of the affected individuals is also variable and since the description of the first mutations [171], new cases have been reported almost every year [172-181]. All the described mutations are truncating, predicting the partial or total loss of the protein and, accordingly, the protein was absent or markedly reduced in all the samples in which this was assessed [171, 172, 174, 176, 177]. The main clinical presentations are neurological, being either early onset slowly progressive or late onset rapidly progressive conditions [154]. UQCC2 is the human homologue of yeast Cbp6, which forms a complex with Cbp3 (UQCC1) necessary for the initial steps of cIII assembly, i.e., the stabilization of cytochrome b during its synthesis and maturation [127, 128]. Pathological variants in *UQCC2* were found in cases of severe neonatal lactic acidosis and growth retardation with combined cI and cIII deficiency [129, 182]. Cbp4 is an additional factor involved in the early maturation of cytochrome b in yeast [127, 128]. UQCC3 was identified as the human orthologue of Cbp4 and a missense mutation in UQCC3 (C11ORF83) was present in a consanguineous individual displaying isolated cIII deficiency at the biochemical level, and lactic acidosis, hypoglycemia, hypotonia and delayed development as clinical features [130].

DISORDERS OF COMPLEX IV

Complex IV structure and assembly

Cytochrome c oxidase (COX) or complex IV (cIV) is the ETC terminal oxidase, transferring electrons from cytochrome *c* to molecular oxygen. In humans it is composed of 14 subunits but only two, MT-CO1 and MT-CO2, are catalytical. The third subunit encoded in the mtDNA is MT-CO3 and although it does not play a direct catalytic role, it is necessary to maintain cIV

activity levels [183]. The assembly of human cIV has been extensively studied in healthy and pathological cell lines. It seems clear now that the complex grows through the incorporation of modules formed by different subunits and defined by each of the mtDNA-encoded core subunits [33, 184-186]. A myriad of assembly factors is necessary for the synthesis, translocation, stabilization and incorporation of the metal groups in both MT-CO1 and MT-CO2 [187]. Other assembly factors such as PET100, PET117 and MR-1S work on the middle stages of assembly [186] and only one, HIGD2A, is known to promote the incorporation of the MT-CO3 module in the final steps of cIV assembly [188, 189].

Mutations in cIV structural subunits

Numerous mutations in the mtDNA sequences encoding all three cIV subunits (*MT-CO1*, *MT-CO2* and *MT-CO3*) are causative of cytochrome *c* oxidase (COX) deficiency and mitochondrial disease (Table 4). The clinical presentations and degree of severity are also extremely variable in these cases [190]. This variability has been explained by the nature of the mutation and the degree of heteroplasmy [191]. Interestingly, mutations found in *MT-CO3* are frequently truncating and present at high levels of heteroplasmy (> 90%) or even homoplasmy in skeletal muscle, usually the most affected tissue [192-194].

COX6B1 was the first nuclear-encoded cIV subunit in which a pathological variant was identified in association with infantile encephalomyopathy [195, 196]. Prior to 2008, it was believed that these kind of mutations were incompatible with life, even if they would affect 'supernumerary' subunits, because they were not being found in a number of mutational screenings [197-200]. Currently, with the development of next generation sequencing techniques, several cIV subunits have now been added to the catalogue of disease-causing genes. One of the peculiar features of cIV is that some nuclear-encoded subunits are tissue and development-specific, being some isoforms expressed in specific cell types or

developmental stages instead of the ubiquitous ones [201]. Pathological mutations have been identified in some of these isoforms, being *COX4I2* mutations the first example, which were found in two consanguineous families with exocrine pancreatic insufficiency, anemia and hyperostosis of calvarium [202]. In most cell types COX4I2 expression is induced in hypoxia [203, 204], but it is constitutively expressed in pulmonary and pancreatic acinar cells [202, 205]. In addition, mutations in the gene encoding the ubiquitous isoform, *COX4I1*, have also been associated with poor growth and Fanconi anemia [206], and with Leigh-like syndrome [207]. COX5A forms an early subassembly complex with COX4 [186]. A pathological variant in *COX5A* found in two siblings from a consanguineous family was associated with reduced cIV levels and pulmonary arterial hypertension, lactic acidemia and failure to thrive [208]. Missense variants in *COX6A2*, encoding the muscle-specific isoform of COX6A, were discovered as the cause of myopathy in two unrelated Japanese patients [209]. Interestingly enough, mutations in the X-chromosome encoding *COX7B* are another cause of microphthalmia with linear skin defects (MLS) syndrome [210]. Loss of COX8A due to a splice-site mutation in COX8A, was associated with Leigh syndrome and epilepsy [211]. NDUFA4 was firstly considered to be a cI structural subunit [212], but more recently it was proven to belong to cIV [186, 213-215]. As such, mutations in the gene encoding NDUFA4 (or COXFA4) produce isolated cIV deficiency associated with a Leigh syndrome neurological phenotype [216].

Mutations in cIV assembly factors

There are more assembly factors known to have a role in the biogenesis of cIV than actual structural subunits [187] and most of them have been discovered because they are the human orthologues of already characterized yeast proteins [217]. However, in the last few years several proteins involved in cIV biogenesis specific to metazoa have been described,

mostly by exome sequencing of affected individuals as well as other unbiased approaches. This is also reflected by the fact that the majority of the cases of mitochondrial cIV deficiency of nuclear origin are caused by mutations in genes encoding proteins mostly involved in the stabilization of cIV subunits/assembly intermediates and co-factor synthesis and insertion [23, 190]. The most prominent of these is SURF1 (Table 4), the functional absence of which causes Leigh syndrome [23, 218, 219] and, much more rarely, Charcot-Marie-Tooth disease [220]. Even though its involvement in the stability, maturation and/or assembly of the core MT-CO1 subunit is clear, the exact molecular role of SURF1 in the process remains unknown [187]. TACO1 is an MT-CO1 translational activator, binding to the *MT-CO1* mRNA and promoting the synthesis of the protein [221, 222]. Defects in TACO1 have been confirmed to produce cIV deficiency and Leigh syndrome [221, 223]. COX14 (C12ORF62) and COA3 (CCDC56 or MITRAC12) interact with the nascent MT-CO1 peptide in the early stages of cIV assembly [224-226]. A disease-causing homozygous mutation in *COX14* was found in three siblings from a consanguineous family presenting with fatal neonatal cIV deficiency, dysmorphism, multiorgan failure and severe lactic acidosis [227]. Mutations in *COA3* were shown to cause peripheral neuropathy, exercise intolerance, obesity and short stature, a mild phenotype considering the severe COX deficiency found in the patient muscle biopsy [228]. COX20 is involved in the stabilization of MT-CO2 early after its synthesis [229, 230] and when defective causes child-onset neurological phenotypes characterized by cerebellar ataxia [22, 229, 231].

PET100 and PET117 are the human homologues of two *S. cerevisiae* assembly factors involved in the intermediate steps of cIV assembly, stabilizing the MT-CO2 module [186]. A particular pathological variant in *PET100* affecting the starting codon was identified as the cause of Leigh Syndrome in a group of patients of Lebanese origin [232, 233]. In addition, a

different *PET100* truncating mutation was determined as the cause of cIV deficiency and fatal infantile lactic acidosis [234]. A homozygous *PET117* mutation was present in two sisters displaying neurodevelopmental regression and bulbar lesions associated with cIV deficiency [235]. COA5 (C2ORF64) is the human ortholog of yeast Pet191, a protein associated with the mitochondrial inner membrane, facing the matrix, involved in an unknown early step of cIV biogenesis [236, 237]. A mutation in *COA5* was described in two siblings born from consanguineous parents showing fatal neonatal cardiomyopathy [237]. COA7 is a respiratory chain biogenetic factor located in the intermembrane space (IMS) where it interacts with the human ortholog of MIA40 (CHCHD4), the main component of the disulfide-relay system necessary for IMS protein import [238, 239]. Mutations in *COA7* cause complex IV deficiency in nearly all the cases and a neurological phenotype characterized by cavitating leukodystrophy of the brain with spinal cord hypotrophy and spinocerebellar ataxia with peripheral neuropathy [240, 241]. Pathological variants in *COA8* (previously known as *APOPT1*) have been found in eight subjects belonging to seven different families [242-244]. All the described mutations are nonsense and associated with a characteristic brain MRI pattern of leukoencephalopathy and a relatively mild neurological phenotype, which is usually aggravated after infectious disease but tends to stabilize with time [242-244]. The specific function of COA8 is still unclear and currently under investigation. However, it is known that oxidative stress prevents its proteasomal degradation and promotes its import inside mitochondria, suggesting its involvement in a stress-response mechanism to enhance cIV biogenesis and/or protect cIV from oxidative damage [245, 246].

Mutations in co-factor synthesis/incorporation

MT-CO1 contains cytochrome *a* and *a3* as co-factors necessary for its catalytic activity. The products of two disease genes, *COX10* and *COX15*, are involved in heme A synthesis and

essential for cIV activity. COX10 catalyzes the farnesylation of a vinyl group at position C2 of heme B to obtain heme O. Mutations in *COX10* cause Leigh Syndrome and also a different fatal early onset neurological syndrome [247-249]. COX15 catalyzes the subsequent step of synthesis of heme A from heme O. The clinical presentations of patients carrying pathological mutations in *COX15* are variable, presenting either with hypertrophic cardiomyopathy [250, 251] or Leigh syndrome with either rapid or slow disease progression [252, 253]. Copper delivery to the active sites of MT-CO1 and MT-CO2 involves various factors essential for cytochrome c oxidase activity [24, 33]. The biogenesis of the Cu_A center in MT-CO2 involves, among others, SCO1, SCO2 and COA6, the malfunction of which cause mitochondrial disease [254, 255]. *SCO1* mutations have been found in patients showing cIV deficiency and fatal outcomes. The variable clinical manifestations include neonatal hepatopathy [256], encephalopathy with hepatopathy and cardiomyopathy [257], pure encephalopathy [258] or an exclusively metabolic syndrome with fatal lactic acidosis but no encephalopathy, hepatopathy, hypotonia, or cardiac involvement [259]. Mutations in *SCO2* are often found in cases of cIV deficiency and have been associated with severe phenotypes of cardiac hypertrophy principally, frequently with variable degrees of neurological involvement [260-267]. A recurrent p.Glu140Lys mutation in the *SCO2* product has been described in several patients, suggesting the presence of a founder effect or a mutational hotspot [261]. COA6 mutations were more recently identified as the cause of fatal infantile cardioencephalomyopathy [268, 269].

DISORDERS OF COMPLEX V

Complex V structure and assembly

The complete structure of the dimeric and monomeric mammalian mitochondrial F1Fo-ATP synthase, ATPase or complex V (cV) has been just recently resolved by Cryo-EM [270, 271]. The enzyme contains two main domains, the F1 domain protruding to the matrix, where the condensation of ADP+Pi occurs; and the Fo membrane domain where the rotational movement, necessary for energy transduction and induced by the translocation of H^+ from the intermembrane space to the matrix, is generated [272]. Both domains are connected by the peripheral stalk (PS). The assembly pathway of human cV is known, at least for the order of subunit incorporation [273-276]. It starts with the assembly of the three alpha and three beta subunits of the F1 domain, to which the rest of the subunits bind subsequently. The c-ring composed of eight units is assembled in the IMM. When these two precursors get together, the subunits of the PS join followed by the remaining subunits of the membrane domain, including MT-ATP6 and MT-ATP8 [33]. Surprisingly, only three assembly factors have been described so far and just two of them have a well-defined function. Yeast Atp11 binds and stabilizes subunit beta [277], while Atp12 does the same with subunit alpha [278]. Both these factors have human orthologues (ATPAF1 and 2) carrying out the same function in the assembly of the F1 domain of cV [279].

Mutations in cV structural subunits

The coding sequences of two Fo subunits cV are overlapping in the human mtDNA [280], and pathological variants in both of them are the cause of sporadic and maternally inherited mitochondrial disease (Table 5). Mutations in *MT-ATP6* have been found in cases of mitochondrial disease with different clinical phenotypes, the most frequent presentations being Neuropathy, Ataxia and Retinitis Pigmentosa (NARP) syndrome [281] and Maternally Inherited Leigh Syndrome (MILS) [282, 283]. Most of the times NARP and MILS are associated with mutations in position m.8993 changing from T to C or G [284-286]. The T>G

transition is usually more severe, and the severity of the disease (NARP is an adult-onset slowly progressive form, whereas MILS is an early onset, highly disabling, often fatal condition) depends rather tightly by the degree of heteroplasmy found in stable tissues (e.g. muscle) [286, 287]. The MT-ATP6 protein is unique as it forms a channel that crosses obliquely (by approximately 30°) the IMM. A Glu58 residue attracts the proton coming from the IMS, which is then expelled into the matrix with the aid of Arg159. The 8993T>G most frequent NARP mutation causes the change Leu156Arg, therefore disturbing the release of the proton from the channel. The proton flow promotes the rotation of the cylinder formed by the c subunits of Fo [272]. MT-ATP6 is one of the subunits incorporated last in the assembly pathway, and when defective there is a prominent accumulation of a very advanced intermediate denominated cV* [273, 288-290]. Other less frequently observed syndromes associated with mutations in *MT-ATP6* are Mitochondrial Myopathy, Lactic Acidosis, and Sideroblastic anemia (MLASA) [291], adult-onset ataxia and polyneuropathy [292-294], bilateral striatal necrosis [295, 296] and motor neuron syndrome [297]. *MT-ATP8* mutations are much rarer than those in *MT-ATP6* but appeared in a case of valproate induced reversible brain atrophy [298] and in homoplasmic state in a patient with apical hypertrophic cardiomyopathy and neuropathy [299]. Cardiomyopathic syndromes were also the hallmark of mutations found in the MT-ATP6/ATP8 overlapping region [300], as well as ataxia, peripheral neuropathy, diabetes mellitus, and hypergonadotropic hypogonadism [301] or early-onset ataxia, psychomotor delay and microcephaly [302].

Until now, only three out of the fifteen nuclear-encoded cV subunits have been associated with mitochondrial disease. A homozygous pathological variant in *ATP5F1E*, encoding subunit epsilon of the ATP synthase, was found in a woman showing neonatal-onset lactic acidosis, 3-methylglutaconic aciduria, mild mental retardation, hypertrophic

cardiomyopathy and peripheral neuropathy [76, 303]. Two Dutch siblings from unrelated parents presenting with cV deficiency and fatal infantile encephalopathy carried a heterozygous pathological mutation in *ATP5F1A*, encoding cV subunit alpha of the F_1 domain [304]. A homozygous mutation in the same gene was associated with combined OXPHOS deficiency and early death [305]. Mutations in *ATP5F1D*, encoding subunit delta, were identified in two unrelated patients one showing metabolic decompensation starting in the neonatal period, and the other childhood-onset acute encephalopathy [306].

Mutations in cV assembly factors

A homozygous missense mutation in ATPAF2 (ATP12) was found in an infant with cV deficiency and severe atrophic encephalopathy and elevated lactate in body fluids [307]. The molecular role of TMEM70 in cV biogenesis is still not clear, although its mutations are the most common cause of ATP synthase deficiency in humans [308]. Its proposed function is to facilitate the incorporation of subunit c into the rotor structure in the mitochondrial inner membrane [309]. However, it has been suggested to be also involved in the assembly of cI (see above and [29, 78]). The majority of the patients with *TMEM70* mutations present a characteristic phenotype of neonatal-onset severe muscular hypotonia, hypertrophic cardiomyopathy, facial dysmorphism, profound lactic acidosis and 3-methylglutaconic aciduria, although the severity and clinical outcomes are variable [23]. The first described 317-2A>G splice-site variant was determined to be a founder mutation in a population of Roma descent, but throughout the years the ethnic groups in which *TMEM70* mutations have been found has expanded, as well as the clinical spectrum associated with them [76, 77, 310-312].

COMBINED RESPIRATORY CHAIN DEFICIENCIES DUE TO DEFECTS IN ONE STRUCTURAL COMPONENT—ROLE OF SUPERCOMPLEXES IN MITOCHONDRIAL DISEASE

Nowadays the existence of higher-order associations of the respiratory chain enzymes forming supercomplexes (SCs) is undeniable, especially after resolving the structure of the mammalian 'respirasome' [313-317]. The respirasome is defined as the association of complexes I, III_2 and IV and envisioned in principle as a functional unit capable of transferring electrons from NADH to O_2 [318]. Apart from the respirasomes, other prominent respiratory SCs found in human cells and tissues, when disrupting the mitochondrial membranes with mild detergents, are $I+III_2$ and III_2+IV [319]. The respirasome organization has been proposed to be functionally advantageous making electron transfer from cI to cIV through $cIII_2$ more efficient, thus functionally dividing the coenzyme Q (CoQ) present in the mitochondrial inner membrane in two non-interchangeable pools, while increasing respiratory function and decreasing the formation of deleterious reactive oxygen species (ROS) [320-323]. However, the phenomenon of 'substrate channeling' between the complexes and the non-diffusion of CoQ from the respirasome structures is deemed as highly unlikely once the SCs structure was obtained by cryo-EM, just by looking at the actual reciprocal position of the active centers [324]. Nevertheless, a recent paper provides evidence in yeast that suggests a role of SCs III_2+IV_{1-2} in enhancing electron transport by decreasing the diffusion distance of cytochrome c [325]. In addition, there are functional demonstrations arguing against the advantage for electron transfer through cI over cII or in favor of the functional segmentation of the CoQ-pool into two separated electron transfer routes [326-328].

Understanding the structure and assembly of the SCs is very relevant for the field of mitochondrial disease mainly to explain the cases of combined respiratory chain deficiency

due to complexes functional and structural interdependency, but also to determine the pathophysiological mechanisms of respiratory chain functional adaptation if the SCs were finally shown to be important for this role. As described before in the suitable sections, strong defects in the biogenesis of cIII cause combined defects due to a secondary loss of cI, affecting also cIV in some cases [129, 132, 144, 151-153, 156, 182, 329]. Impairment in the early stages of cIV assembly also leads to decreased cI levels [330, 331]. However, when the respiratory chain defect is originated from mutations in structural cI components or ancillary proteins, the biochemical manifestation is almost invariably isolated cI deficiency [23, 24].

In human cell mitochondria solubilized with the mild detergent digitonin, the near totality (90-95%) of cI appears associated inside the SCs, whereas approximately 50% of $cIII_2$ is inside SCs and 90% of cIV is in the 'free' monomeric form [319]. Mitochondria from other mammalian species appear to contain higher amounts of 'free' cI [318, 332, 333]. The fact that the SCs co-exist with the 'free' versions of the respiratory complexes gave rise to the idea that each of the enzymes could assemble independently and then dynamically join and separate in response to varying metabolic settings, according to the so called 'plasticity model' [318, 320, 334]. However, there is growing evidence indicating that subunits belonging to different complexes can associate before their assembly is completed. This was firstly observed in patient-derived human cell lines with defects in the late stages of cI or $cIII_2$ assembly, where the 'pre-complexes' were found associated in SCs [24]. Further evidence has been obtained through detailed studies of cell lines carrying null-mutations in structural subunits or biogenetic factors in which a cIV subassembly was shown to be associated with complexes I and III_2 [189, 335]. In addition, these and previous studies convincingly indicate the existence of alternative maturation routes for $cIII_2$ and cIV

depending on whether they associate into SCs or not [189, 335, 336]. This is exemplified by the fact that the incorporation of UQCRFS1 into the 'free' $cIII_2$ is dependent on BCS1L function, whereas HIGD1A plays the same role in the $cIII_2$ that is associated in the SCs [189]. In the case of human cI, its final and complete assembly, i.e. N-module incorporation, only occurs efficiently in the context of the SCs [132, 336]. This may be due to safely activate cI, which alone would be a highly reactive enzyme, producing undesired redox reactive species in the absence of the downstream electron acceptors of the ETC. The dependent assembly of cI on one hand, and independent biogenesis of $cIII_2$ and cIV on the other, would explain why strong biogenetical defects in the latter enzymes induce secondary defects in cI assembly, but only very rarely the other way around [337, 338].

The idea that SC formation could be a regulated process prompted the quest to find factors that would promote respirasome assembly and potentially affect mitochondrial function when mutated. The first and only candidate protein so far is COX7A2L, renamed SuperComplex Assembly Factor 1 (SCAFI or SCAF1) [320]. However, the commonly used laboratory mouse C57Bl/6 strains naturally carry a deletion that inactivates the protein and these animals do not show any mitochondrial disease-like phenotypes [320, 339]. In addition, whether SCAF1-deficient mitochondria show biochemical and/or respiratory activity alterations remains controversial [320, 323, 339-342]. So far, no pathological variants in *COX7A2L* have been identified in humans.

PRIMARY COENZYME Q DEFICIENCIES

Coenzyme Q (CoQ) is an essential lipidic component of the mitochondrial respiratory chain responsible for the transfer of electrons to $cIII_2$. It receives electrons from cI and cII as well as from other metabolic pathways that converge in the CoQ pool [343]. It is composed of a

benzoquinone head and a polyisoprenoid tail, which in humans has 10 isoprene units (CoQ_{10}) [344]. The CoQ_{10} biosynthetic pathway involves a series of activities for the synthesis of both the quinone head from the 4-hydroxybenzoic acid (4HB) and the isoprenoid tail from isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Most of the enzymes performing these steps have been identified and are believed to interact physically, forming a CoQ biosynthetic complex [344-346].

Mutations in the genes encoding ten of CoQ synthesis enzymes are known to cause primary CoQ deficiency. The clinical phenotypes are multisystemic and variable, similarly to other mitochondrial disorders [346]. However, many of these patients suffer from corticosteroid-resistant nephrotic syndrome, which is characteristic of primary CoQ deficiency [347]. CoQ supplementation can be used to treat these conditions, although it is not an efficient therapy in all cases and diagnosis should be done early to avoid irreversible damage to the brain or kidneys [346].

Most of the patients carrying mutations in *COQ2* show early-onset nephropathy in some cases accompanied by neurological or other organ involvement [348-354]. A homozygous mutation in *PDSS1* was found in two siblings from a consanguineous Moroccan family with CoQ_{10} deficiency and a multisystem disorder [351]. PDSS2 forms a heterotetramer with PDSS1 and pathological variants in *PDSS2* also cause combined respiratory and CoQ deficiencies associated with the typical renal phenotype together with encephalopathy [350, 355-357]. Mutations in *COQ8A* (*ADCK3*) are causative of autosomal recessive spinocerebellar ataxia [358-362]. Defects in COQ9 are severe and manifest as neonatal Leigh-like syndrome or multisystem disorders [363-366]. Pathological variants in *COQ6* have been associated with the CoQ-deficient nephrotic syndrome and sensorineural hearing loss [367-369]. A polymorphism in *COQ8B* (*ADCK4*) was proposed as a modifier of the COQ6-

associated renal phenotype [369, 370], whereas in other cases the mutations found in *COQ8B* are considered the cause of primary CoQ deficiency and steroid-resistant nephrotic syndrome [371-373]. COQ4 was found mutated in a number of patients with a wide range of clinical symptoms with different severity and age of onset, although the majority of the described patients showed encephalomyopathy [374-377]. An encephalopathic infant also with hearing impairment, failure to thrive and peripheral neuropathy as well as eye, renal and cardiac involvement carried a homozygous missense variant in *COQ7* [22, 378]. *COQ5* was added to the list of primary CoQ deficiency disease genes when biallelic duplications, affecting the 3'-UTR sequence, were found in three siblings from non-consanguineous parents presenting cerebellar ataxia and encephalopathy [379].

CYTOCHROME C BIOGENESIS DEFECTS (HCCS)

The covalent attachment of the heme group to apo-cytochrome *c* is performed by the cytochrome *c*-type lyase, which in humans is denominated HCCS and is also needed for the maturation of the cIII subunit CYC1 [380, 381]. The *HCCS* gene is located in the X-chromosome and pathological variants are yet another cause of microphthalmia with linear skin defects (MLS) syndrome [382, 383]. The pathological mechanism is thought to be the activation of a non-canonical apoptosis pathway during embryonic development, which is deemed as the reason of developmental dysmorphism, a feature that is not typical in mitochondrial disorders [41, 384].

FINAL REMARKS

During the past ten years, there has been a significant improvement in the genetic diagnostic capacity thanks to the development and decreasing costs of the Next Generation

Sequencing technologies. This has increased spectacularly our knowledge regarding the genetic basis of mitochondrial disease in general, and in the number of altered genes encoding structural subunits and assembly factors in particular.

The study of pathological samples derived from individuals carrying mutations that directly affect the assembly of the mitochondrial ETC complexes has been invaluable to unravel the pathways and functions involving the defective proteins as well as the molecular pathological mechanisms underlying these diseases. The careful analysis at the cellular, mitochondrial and molecular levels of the growing number of identified cases will also potentially help us understand the genotype-phenotype correlations, which is still a challenge in the field of mitochondrial disorders.

We expect to find more and more pathological variants in genes encoding OXPHOS structural proteins and assembly factors. However, some of these elements are strictly conserved throughout evolution and so fundamental for life that it is probable that none or only mild mutations will ever be described. There is a hierarchy in the type of mutations that can be found in the assembly factors, which probably relates to the biogenetical essentiality of their function or whether they might be part of secondary stress-response mechanisms. For example, the mutations found in factors like COA8 or TTC19 are exclusively non-sense mutations. MT-CO1 metal centers are incorporated early during the process of cIV assembly, while the subunit is still in the form of an individual complex bound to its several assembly factors [217, 385]. Yeast cells devoid of downstream cIV biogenetic factors, accumulate a hemylated Cox1 subunit resulting in a pro-oxidant intermediate that creates a signal for its own proteolysis, preventing further oxidative damage [386, 387]. COA8-null cells are more sensitive to oxidative stress and produce more ROS when stimulated [242, 246] and COA8 levels are increased in response to oxidative stress [245]. We hypothesized a

role for COA8 in responding to the oxidative stress created by a partially assembled MT-CO1 in order to protect this intermediate, precluding its degradation and stimulating the continuation of the assembly pathway, rather than being directly involved in the pathway itself. Another example of this kind of factors is TTC19, which is dispensable for the completion of $cIII_2$ assembly and activation, but necessary to maintain the structural and functional integrity of the enzyme. We determined that this is achieved by a proteolytic mechanism involving the N-terminal peptide of UQCRFS1 which could regulate $cIII_2$ turnover in response to stress or metabolic stimuli [122, 388]. The mishandling of oxidative or metabolic stress could play a role in the pathogenesis of the disease observed in these patients. On the other hand, in an essential process such as the actual translocation of UQCRFS1 for the activation of $cIII_2$ mediated by BCS1L, only missense mutations that just reduce the activity of the protein have been described in *BCS1L*, thus suggesting that BCS1L, as many other components of the biogenetic machinery of the ETC are essential genes, and only hypomorphic alleles are tolerated for extra-uterine life.

Despite the enormous advances in genetic screening and knowledge of the mitochondrial proteome, genetic diagnosis of mitochondrial disorders is still limited to about half of the suspected cases. In some instances, of course, the clinical diagnosis may be wrong, or include features that may phenocopy a mitochondrial disease. In other cases, mutations of genes encoding proteins localized outside mitochondria can be a source of ambiguity (this is the case for instance for MSTO1, a cytoplasmic protein whose mutations lead to a fragmentation of mitochondria, for *TYMP*, whose mutations cause MNGIE, which encodes pyrimidine-nucleotide phosphorylase, or for the gene encoding ribonucleotide reductase, leading to severe mtDNA depletion). In other cases proteins that are not included in the current dedicated databases may be responsible of a mitochondrial disease (as was the case

of TTC19) or the function and supposed localization of these proteins may have not been demonstrated to be in mitochondria (this was the case, for instance, for MPV17). However, we must also hypothesize that the clinical phenotype may diverge from the canonical signs and symptoms we are used to attribute to mitochondrial disorders (such in the case, for instance, of mutations in PITRM1), the whole mitochondrial proteome is still not completely defined especially for proteins quantitatively scarce, and the possibility of digenic or oligogenic inheritance must always be considered in controversial cases. The more information will be accumulated on the variance of the mitochondrial proteome in human populations, the more precise and focused the genetic diagnosis will be. But we also have to admit that the biology of mitochondria is still partly unknown, the impact of mitochondrial pathways on a number of metabolic function is a territory that needs to be explored in full, and the possible connections of mitochondrial functions with previously unsuspected, yet essential responses, such as inflammation, immunity, cell degeneration, are just about to leak some information for future research. Thus, although we can certainly be proud of the impressive amount of information gained in fundamental and translational biomedicine concerning the role of mitochondria in cells, tissues and organs, a wide landscape stands in front of the new generations of investigators keen to take the challenge to further explore and understand the complexity of this essential component of our life machinery.

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TABLES

Table 1: Disease genes encoding structural subunits and assembly factors associated with mitochondrial cI deficiency.

MUTATED GENE	MAIN CLINICAL FEATURES	OMIM # [389]	REFERENCES
STRUCTURAL SUBUNITS OF THE PERIPHERAL ARM N-MODULE			
NDUFA2	Mitochondrial complex I deficiency. Leigh syndrome. Cavitating leukoencephalopathy.	602137 618235	[390-392]
NDUFA6	Early onset mitochondrial complex I deficiency. Mitochondrial encephalopathy.	602138 618253	[393]
NDUFA12	Mitochondrial complex I deficiency. Leigh syndrome.	614530 618244	[394]
NDUFS1	Mitochondrial complex I deficiency. Leigh syndrome. Cavitating leukoencephalopathy.	157655 618226	[395-398]
NDUFS4	Mitochondrial complex I deficiency. Combined complex I + complex III deficiency. Leigh syndrome.	602694 252010	[337, 399-404]
NDUFS6	Mitochondrial complex I deficiency. Fatal neonatal onset lactic acidosis. Mitochondrial encephalopathy. Leigh syndrome.	603848 618232	[405-407]
NDUFV1	Mitochondrial complex I deficiency. Mitochondrial encephalopathy. Cerebellar ataxia. Leigh syndrome.	161015 618225	[395, 408-411]
NDUFV2	Mitochondrial complex I deficiency. Hypertrophic cardiomyopathy and encephalopathy. Leigh syndrome.	600532 618229	[412-414]
ASSEMBLY FACTORS OF THE PERIPHERAL ARM N-MODULE			
NDUFAF2	Fatal mitochondrial complex I deficiency. Mitochondrial encephalopathy. Leigh syndrome	609653 618233	[89-92]
STRUCTURAL SUBUNITS OF THE PERIPHERAL ARM Q-MODULE			
NDUFS2	Mitochondrial complex I deficiency.	602985	[415, 416]

	Encephalomyopathy. Hypertrophic cardiomyopathy. Severe neonatal lactic acidosis.	618228	
NDUFS3	Mitochondrial complex I deficiency. Leigh syndrome. Encephalomyopathy and lactic acidosis.	603846 618230	[417-419]
NDUFS7	Mitochondrial complex I deficiency. Leigh syndrome.	601825 618224	[420-422]
NDUFS8	Mitochondrial complex I deficiency. Leigh syndrome. Multisystem disorder.	602141 618222	[418, 423, 424]
ASSEMBLY FACTORS OF THE PERIPHERAL ARM Q-MODULE			
NDUFAF3	Mitochondrial complex I deficiency. Severe neonatal lactic acidosis. Encephalopathy. Leigh syndrome.	612911 618240	[55, 56, 58]
NDUFAF4	Mitochondrial complex I deficiency. Encephalomyopathy. Cardiomyopathy. Severe neonatal lactic acidosis. Leigh syndrome.	611776 618237	[54, 57, 59]
NDUFAF5	Mitochondrial complex I deficiency. Leigh syndrome.	612360 618238	[68, 70, 71]
NDUFAF6	Mitochondrial complex I deficiency. Leigh syndrome. Acadian-variant Fanconi syndrome.	612392 618239 618913	[60, 62-64, 425]
NDUFAF7	Pathological myopia	615898	[72]
NUBPL	Mitochondrial complex I deficiency. Leukoencephalopathy. Encephalomyopathy.	613621 618242	[79, 96-98]
STRUCTURAL SUBUNITS OF THE MEMBRANE ARM ND1-MODULE			
MT-ND1	Leber's hereditary optic atrophy (LHON): major allele m.3460G>A. Mitochondrial complex I deficiency. Mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS) syndrome. Myopathy.	516000	[36, 426-431]
NDUFA8	Mitochondrial complex I deficiency. Developmental delay, microcephaly, and epilepsy.	603359	[432, 433]

	Failure to thrive and language difficulties.		
NDUFA13	Mitochondrial complex I deficiency. Encephalopathy with sensorial deficiencies. Leigh syndrome.	609435 618249	[434, 435]
NDUFA11	Mitochondrial complex I deficiency. Fatal infantile metabolic acidosis. Encephalopathy. Cardiomyopathy. Myopathy.	612638 618236	[436, 437]
ASSEMBLY FACTORS OF THE MEMBRANE ARM ND1-MODULE			
TIMMDC1	Mitochondrial complex I deficiency. Early onset severe neurological dysfunction. Leigh syndrome.	615534 618251	[75]
STRUCTURAL SUBUNITS OF THE MEMBRANE ARM ND2-MODULE			
MT-ND2	LHON. Mitochondrial complex I deficiency. Leigh syndrome.	516001	[438-442]
MT-ND3	Mitochondrial complex I deficiency. Infantile encephalopathy. Leigh syndrome. LHON and dystonia. Adult onset encephalopathy.	516002	[443-450]
MT-ND6	LHON: major allele m.14484T>C. Leigh syndrome. MELAS syndrome.	516006	[451-458]
NDUFA1	Mitochondrial complex I deficiency. X-linked Leigh syndrome. Milder form with symptoms only during intercurrent infections.	300078 301020	[459-461]
NDUFA10	Mitochondrial complex I deficiency. Leigh syndrome. Intrauterine growth retardation, lactic acidosis and pulmonary hypertension.	603835 618243	[42, 462, 463]
NDUFA9	Mitochondrial complex I deficiency. Leigh syndrome.	603834 618247	[464, 465]
NDUFC2	Mitochondrial complex I deficiency. Leigh syndrome.	603845	[466]
ASSEMBLY FACTORS OF THE MEMBRANE ARM ND2-MODULE			
NDUFAF1	Mitochondrial complex I deficiency. Cardioencephalopathy. Hypertrophic cardiomyopathy.	606934 618234	[47, 48]
ACAD9	Mitochondrial complex I deficiency.	611103	[50, 51, 467-471]

	Early-onset hypertrophic cardiomyopathy. Exercise intolerance. Mild deficiency of beta oxidation. May be responsive to riboflavin.	611126	
TMEM126B	Mitochondrial complex I deficiency. Infantile or childhood onset myopathy.	615533 618250	[52, 53]
STRUCTURAL SUBUNITS OF THE MEMBRANE ARM ND4-MODULE			
MT-ND4	LHON: major allele m.11778G>A. LHON and Dystonia. Mitochondrial complex I deficiency. Encephalopathy.	516003	[13, 472-475]
NDUFB10	Mitochondrial complex I deficiency. Neonatal pulmonary hypertension, cardiomyopathy and lactic acidosis.	603843 619003	[476, 477]
NDUFB11	Microphtalmia with linear skin defects (MLS) syndrome with multiple congenital anomalies. Usually embryonic lethal in males. Mitochondrial complex I deficiency. Cardiomyopathy and lactic acidosis.	300403 300952 301021	[41-43]
ASSEMBLY FACTORS OF THE MEMBRANE ARM ND4-MODULE			
FOXRED1	Mitochondrial complex I deficiency. Leigh syndrome with or without cardiomyopathy. Ataxia, epilepsy and psychomotor developmental delay.	613622 618241	[79-81]
TMEM70	Complex V deficiency. Neonatal encephalocardiomyopathy. Occasionally facial dysmorphisms and complex I deficiency.	612418 614052	[76, 77, 310-312]
TMEM126A	Autosomal recessive optic atrophy (OPA7)	612988 612989	[82-86]
STRUCTURAL SUBUNITS OF THE MEMBRANE ARM ND5-MODULE			
MT-ND5	LHON. MELAS syndrome. Mitochondrial complex I deficiency. Leigh syndrome with Wolff-Parkinson-White syndrome and/or optic atrophy.	516005	[36, 478-482]
NDUFB3	Mitochondrial complex I deficiency. Infantile encephalomyopathy.	603839 618246	[418, 483]
NDUFB8	Mitochondrial complex I deficiency.	602140 618252	[484]

	Early onset encephalocardiomyopathy.		
NDUFB9	Early-onset mitochondrial complex I deficiency	601445 618245	[485]

Table 2: Disease genes encoding structural subunits and assembly factors associated with mitochondrial cII deficiency.

MUTATED GENE	MAIN CLINICAL FEATURES	OMIM # [389]	REFERENCES
CII STRUCTURAL SUBUNITS			
SDHA	Mitochondrial complex II deficiency. Leigh syndrome. Dilated cardiomyopathy. Paragangliomas.	600857 614165	[16, 104-108, 486, 487]
SDHB	Gastrointestinal stromal tumors. Paragangliomas. Pheochromocytomas. Mitochondrial complex II deficiency. Leukodystrophy.	185470 115310	[109, 488-491]
SDHC	Gastrointestinal stromal tumors. Paragangliomas.	602413 605373	[118, 492-494]
SDHD	Gastrointestinal stromal tumors. Paragangliomas. Pheochromocytomas. Mitochondrial complex II deficiency. Encephalomyopathy. Prenatal hypertrophic cardiomyopathy.	602690	[110, 113, 489, 491, 495-498]
CII ASSEMBLY FACTORS			
SDHAF1	Mitochondrial complex II deficiency. Leukoencephalopathy.	612848	[115, 116]
SDHAF2	Paragangliomas. Pheochromocytomas.	613019 601650	[117-119]

Table 3: Disease genes encoding structural subunits and assembly factors associated with mitochondrial cIII deficiency.

MUTATED GENE	MAIN CLINICAL FEATURES	OMIM # [389]	REFERENCES

EARLY ASSEMBLY CIII STRUCTURAL SUBUNITS			
MT-CYB	Mitochondrial complex III deficiency. Combined respiratory chain deficiency. LHON. Sporadic exercise intolerance. Myopathy. Histiocytoid cardiomyopathy. Parkinsonism/MELAS overlap syndrome. Multisystem disorders.	516020	[140-151, 194, 329, 438, 499, 500]
UQCRB	Mitochondrial complex III deficiency. Hepatopathy.	191330 615158	[155]
UQCRQ	Mitochondrial complex III deficiency. Early onset encephalopathy.	612080 615159	[156]
MT-CYB ASSEMBLY FACTORS			
UQCC2	Mitochondrial complex III deficiency. Combined respiratory chain deficiency (cI+cIII) Lactic acidosis and dysmorphic features. Growth retardation, respiratory distress and seizures.	614461 615824	[129, 182]
UQCC3	Mitochondrial complex III deficiency. Feeding difficulties, hypoglycemia, and severe lactic acidosis.	616097 616111	[130]
INTERMEDIATE ASSEMBLY CIII STRUCTURAL SUBUNITS			
CYC1	Mitochondrial complex III deficiency. Recurrent metabolic crises and insulin-responsive hyperglycemia.	123980 615453	[159]
UQCRC2	Mitochondrial complex III deficiency. Metabolic decompensation, hypoglycemia and lactic acidosis.	191329 615160	[157, 158]
LATE ASSEMBLY CIII STRUCTURAL SUBUNITS			
UQCRFS1	Mitochondrial complex III deficiency. Lactic acidosis, cardiomyopathy and *alopecia totalis.*		[160]
LATE ASSEMBLY CIII ASSEMBLY FACTORS			
BCS1L	GRACILE syndrome. Mitochondrial complex III deficiency. Björnstad syndrome. Encephalopathy either isolated or combined with visceral disease.	603647 124000	[137, 161-164, 501-509]

	Proximal tubulopathy and/or hepatopathy.		
LYRM7	Mitochondrial complex III deficiency. Lactic acidosis and early onset leukoencephalopathy. Metabolic decompensation and liver failure.	615831 615838	[167-170]
TTC19	Mitochondrial complex III deficiency. Progressive encephalopathy: early onset and slowly progressive or late onset and rapidly progressive. Spinocerebellar ataxia. Leigh syndrome. Stroke-like episodes. Psychiatric symptoms.	613814 615157	[171-176, 177 Conboy, 2018 #2207, 178, 179, 181]

Table 4: Disease genes encoding structural subunits and assembly factors associated with mitochondrial cIV deficiency.

MUTATED GENE	MAIN CLINICAL FEATURES	OMIM # [389]	REFERENCES
STRUCTURAL SUBUNITS OF THE EARLY MODULE			
COX4I1	Mitochondrial cIV deficiency. Fanconi anemia. Leigh-like síndrome.	123864	[206, 207]
COX4I2	Exocrine pancreatic insufficiency, anemia and hyperostosis of calvarium.	607976 612714	[202]
COX5A	Mitochondrial cIV deficiency. Pulmonary arterial hypertension, lactic acidemia and failure to thrive	603773	[208]
STRUCTURAL SUBUNITS OF THE MT-CO1 MODULE			
MT-CO1	Mitochondrial cIV deficiency. Sideroblastic anemia. Neurological syndromes. Myopathy and rhabdomyolysis. Cardiomyoencephalopathy.	516030	[199, 510-514]
ASSEMBLY FACTORS OF THE MT-CO1 MODULE			
TACO1	Mitochondrial complex IV deficiency. Leigh syndrome.	612958	[221, 223]
SURF1	Mitochondrial complex IV deficiency. Leigh syndrome.	185620	[218-220, 515-520]

	Charcot-Marie-Tooth syndrome.		
COA3	Mitochondrial complex IV deficiency. Exercise intolerance and neuropathy.	614775	[228]
COX14	Mitochondrial complex IV deficiency. Severe congenital lactic acidosis and dysmorphic features.	614478	[227]
COX10	Mitochondrial complex IV deficiency. Leigh syndrome. Encephalopathy with proximal tubulopathy. Hypertrophic cardiomyopathy, sensorineural hearing loss and metabolic acidosis.	602125	[247-249]
COX15	Mitochondrial complex IV deficiency. Fatal infantile hypertrophic cardiomyopathy. Leigh syndrome.	603646 615119	[250-253]
STRUCTURAL SUBNITS OF THE MT-CO2 MODULE			
MT-CO2	Mitochondrial complex IV deficiency. Encephalomyopathy. Myopathy and lactic acidosis. Multisystem disorder.	516040	[521-524]
COX7B	Microphtalmia with linear skin defects (MLS) syndrome with multiple congenital anomalies. Usually lethal in males.	300885 300887	[210]
COX8A	Mitochondrial complex IV deficiency. Leigh-like syndrome and epilepsy.	123870	[211]
ASSEMBLY FACTORS OF THE MT-CO2 MODULE			
COX20	Mitochondrial complex IV deficiency. Growth retardation, hypotonia, and cerebellar ataxia.	614698	[229]
PET100	Mitochondrial complex IV deficiency. Leigh syndrome. Fatal infantile lactic acidosis.	614779	[232-234]
PET117	Mitochondrial complex IV deficiency. Neurodevelopmental regression.	614771	[235]
SCO1	Mitochondrial complex IV deficiency. Neonatal hepatopathy and severe ketoacidosis. Encephalopathy and lactic acidosis. Cardiomyopathy and hepatomegaly.	603644	[256-258]
SCO2	Mitochondrial complex IV deficiency. Fatal infantile cardioencephalomyopathy.	604272 604377	[260, 261, 263, 266, 267, 525, 526]

	Charcot-Marie-Tooth syndrome. Cerebellar ataxia and progressive peripheral axonal neuropathy.		
COA6	Mitochondrial complex IV deficiency. Combined respiratory chain deficiency. Fatal infantile cardioencephalomyopathy.	614772 616501	[268, 269]
STRUCTURAL SUBNITS OF THE MT-CO3 MODULE			
MT-CO3	Mitochondrial complex IV deficiency. Myoglobinuria. Encephalomyopathy and lactic acidosis. LHON.	516050	[192-194, 527, 528]
MT-ATP6/MT-CO3 junction	Seizures and lactic acidosis.	516050	[529]
COX6B1	Mitochondrial complex IV deficiency. Encephalomyopathy and lactic acidosis. Hypertrophic cardiomyopathy.	124089	[195, 196]
COX6A2	Mitochondrial complex IV deficiency. Myopathy.	602009	[209]
FINAL ASSEMBLY SUBUNITS			
COXFA4	Mitochondrial complex IV deficiency. Leigh syndrome.	603833	[216]
CIV ASSEMBLY FACTORS OF UNDEFINED FUNCTION			
COA5	Mitochondrial complex IV deficiency. Fatal infantile hypertrophic cardiomyopathy.	613920 616500	[237]
COA7	Mitochondrial complex IV deficiency. Cavitating leukodystrophy and spinocerebellar ataxia with axonal neuropathy.	615623 618387	[240, 241]
COA8	Mitochondrial complex IV deficiency. Leukoencephalopathy.	616003	[242-244]

Table 5: Disease genes encoding structural subunits and assembly factors associated with mitochondrial cV deficiency.

MUTATED GENE	**MAIN CLINICAL FEATURES**	**OMIM # [389]**	**REFERENCES**
STRUCTURAL SUBUNITS OF THE Fo DOMAIN			
MT-ATP6	Mitochondrial complex V deficiency. Neuropathy, Ataxia and Retinitis Pigmentosa (NARP) syndrome.	516060 500015	[281, 282, 285, 287, 291-295, 296de Coo,

	Leigh syndrome. Major alleles: m.8993T>G or C Adult-onset ataxia and polyneuropathy. Bilateral striatal necrosis. Motor neuron syndrome. Mitochondrial myopathy, lactic acidosis and sideroblastic anemia (MLASA).		1996 #2652, 297, 530-532]
MT-ATP8	Mitochondrial complex V deficiency. Valproate induced reversible brain atrophy. Hypertrophic cardiomyopathy.	516070	[298, 299]
MT-ATP6/8 overlap region	Mitochondrial complex V deficiency. Infantile hypertrophic cardiomyopathy.	516060 516070	[300]
STRUCTURAL SUBUNITS OF THE F1 DOMAIN			
ATP5F1A	Mitochondrial complex V deficiency. Combined OXPHOS deficiency. Fatal infantile encephalopathy.	164360 615228 616045	[304, 305]
ATP5F1D	Mitochondrial complex V deficiency. Metabolic decompensation with lactic acidosis, hypoglycemia, hyperammonemia, and 3-methylglutaconic aciduria. Encephalopathy.	603150 618120	[306]
ATP5F1E	Mitochondrial complex V deficiency. Neonatal-onset lactic acidosis, 3-methylglutaconic aciduria, mild mental retardation, hypertrophic cardiomyopathy and peripheral neuropathy.	606153 614053	[303]
CV ASSEMBLY FACTORS			
ATPAF2	Mitochondrial complex V deficiency. Encephalopathy, lactic acidosis and 3-methylglutaconic aciduria.	608918 604273	[307]
TMEM70	Mitochondrial complex V deficiency. Neonatal encephalocardiomyopathy. Occasionally facial dysmorphisms and complex I deficiency.	612418 614052	[76, 77, 310-312]

FIGURE LEGENDS

Figure 1: The biogenesis of the OXPHOS system relies on structural components encoded both in the mitochondrial genome (mtDNA) and in the nucleus. All the assembly factors are encoded in the nuclear genome, synthesized in the cytoplasm and imported inside the mitochondria, where they coordinate the assembly of the subunits coming from inside and outside the organelle. Mutations in the genes encoding a large number of these elements have been determined as the cause of mitochondrial disease. The figure depicts the organization of the human respiratory chain in which the practical totality of complex I (cI) is associated in supercomplexes (SC) I_1III_2 and the respirasomes $I_1III_2IV_1$. The structures shown were generated with PyMOL and correspond to: the human respirasome (PDB: 5XTH) [317], ovine (SC) I_1III_2 (PDB: 6QBX) [533], porcine cII (PDB: 1ZOY) [99], human $cIII_2$ (PDB: 5XTE) [317], human cIV (PDB: 5Z62) [215], human cytochrome *c* (PDB: 2N9J) [534] and bovine cV (PDB: 6ZQN) [270]. Created with BioRender.com.

Figure 2: Localization of the disease-associated structural subunits of (A) cI, (B) $cIII_2$ and (C) cIV. MtDNA-encoded subunits, all of them found mutated in mitochondrial disease, are depicted in magenta. In A and B, nuclear-encoded core subunits are indicated in red, whereas supernumerary subunits are indicated in pink. The topology of cI and the division into functional and assembly modules (mod.) is indicated in A (see main text for details). In C, subunits with tissue-specific isoforms involved in mitochondrial disease are indicated in pink, the rest of the nuclear-encoded disease-linked subunits are colored in red. The subunits for which no mutations have been found are indicated in cyan. IMS: intermembrane space; IM: inner membrane. The images were created with PyMOL using

the human cryo-EM structures for cI (PDB: 5XTD), $cIII_2$ (PDB: 5XTE) [317] and cIV (PDB: 5Z62) [215].