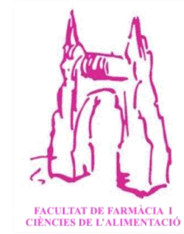

Universitat de Barcelona
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Final Project

COENZYME Q_{10} DEFICIENCIES: A GROUP OF POTENTIALLY TREATABLE MITOCHONDRIAL DISEASES

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Main area: Biochemistry and Molecular Biology
Secondary areas: Physiology and Pathophysiology, Pharmacology and Therapeutics

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ABSTRACT

The main objective of the present project is to critically review the current state of knowledge on a group of mitochondrial disorders known as coenzyme Q_{10} (CoQ) deficiencies. It has been developed thanks to a deep bibliographic research, using an on-line biomedical database in order to search and select the most recent and relevant articles in this field of study.

The project highlights the central role of CoQ in the mitochondrial respiratory chain, but also its involvement in several aspects of cellular metabolism. Given its essential functions, this study also describes how a deficit in this molecule leads to a number of disorders with an unexplained heterogeneous clinical spectrum. In this regard, CoQ deficiencies can occur due to defects of CoQ biosynthesis (primary deficiencies) or due to other causes (secondary forms). In addition, this piece of work emphasizes how biochemical measurements in the patient's tissues or biological fluids and genetic testing play a crucial role in the diagnostic pathway. A reviewed and updated protocol for the biochemical determination of CoQ status in different specimens is proposed. Finally, this project also states that early diagnosis and initiation of specific treatment is of paramount importance, especially as some CoQ-deficient patients respond well to CoQ supplementation.

In conclusion, the study suggests that, despite the advances in the last decades, further elucidation of the biochemistry and genetics of these complex diseases is needed to set the stage for improving the care of patients and the development of novel treatments.

RESUM

Deficiències de coenzim Q_{10}:
un grup de malalties mitocondrials potencialment tractables

El principal objectiu d'aquest treball final de grau és revisar d'una forma crítica el coneixement actual sobre un grup de malalties mitocondrials conegut com a deficiències de coenzim Q_{10} (CoQ). Ha estat redactat gràcies a una minuciosa recerca bibliogràfica, emprant una base de dades biomèdica on-line per tal de cercar i seleccionar els articles més recents i rellevants en aquest camp.

Aquest projecte posa de manifest el rol fonamental del CoQ en la cadena respiratòria mitocondrial, però també la seva participació en un gran nombre de processos del metabolisme cel·lular. Donada la rellevància de les seves funcions, aquest estudi també descriu com el dèficit d'aquesta molècula condueix a un grup de malalties amb una espectre clínic molt heterogeni. En aquest sentit, les deficiències de CoQ poden ser degudes a defectes en la biosíntesi de CoQ (deficiències primàries) o a altres causes (formes secundàries). A més, el treball remarca el paper crucial de les determinacions bioquímiques de CoQ en teixits i fluids biològics, així com el de les proves genètiques, en el procés diagnòstic. També es proposa un protocol actualitzat per a la determinació bioquímica de CoQ en diferents espècimens biològics. Finalment, s'assenyala que el diagnòstic i l'inici del tractament primerencs són de vital importància donat que alguns pacients responen bé a la suplementació amb CoQ.

Com a conclusió, el projecte suggereix que, malgrat els avenços de les últimes dècades, és necessari aprofundir en l'estudi dels aspectes bioquímics i genètics d'aquestes malalties per tal de poder millorar la cura dels pacients i desenvolupar noves teràpies.

ABBREVIATIONS

3,4-dHB: 3,4-dihydroxybenzoate

4-HB: 4-hydroxybenzoate

ATP: adenosine triphosphate

BMCs: blood mononuclear cells

CNS: central nervous system

CoA: coenzyme A

CoQ: coenzyme Q_{10}

CoQ_9: coenzyme Q_9

CPEO: chronic progressive external ophthalmoplegia

FGF-21: fibroblast growth factor-21

FPP: farnesyl-pyrophosphate

GDF-15: growth differentiation factor-15

HCM: hypertrophic cardiomyopathy

HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A

HPLC: high-pressure liquid chromatography

HPLC-ED: HPLC coupled with electrochemical detection

ID: intellectual disability

LC-MS/MS: liquid chromatography-tandem mass spectrometry

LHON: Leber hereditary optic neuropathy

MADD: multiple acyl-coenzyme A dehydrogenase deficiency

MD: mitochondrial diseases

MELAS: mitochondrial myopathy, encephalopathy, lactic acidosis with stroke-like episodes

MERRF: myoclonic epilepsy with ragged-red fibers

MNGIE: mitochondrial neurogastrointestinal involvement and encephalopathy

MPS: mucopolysaccharidosis

MRC: mitochondrial respiratory chain

mtDNA: mitochondrial DNA

NARP: neuropathy, ataxia and retinitis pigmentosa

nDNA: nuclear DNA

NGS: next-generation sequencing

OXPHOS: oxidative phosphorylation

PKU: phenylketonuria

PP: pyrophosphate

ROS: reactive oxygen species

SNHL: sensorineural hearing loss

SRNS: steroid-resistant nephrotic syndrome

VA: vanillic acid

WES: whole-exome sequencing

INTEGRATION OF THE DIFFERENT AREAS

Coenzyme Q_{10} (CoQ) deficiency is a biochemical abnormality associated with markedly different clinical presentations. Although the first patients with very low levels of CoQ were described in 1989, the genetic bases of these discoveries remained elusive until 2006. The present project reviews the current state of knowledge on this group of mitochondrial disorders known as CoQ deficiencies. It is a multidisciplinary study that can be related to three different areas: Biochemistry and Molecular Biology, Physiology and Pathophysiology, and Pharmacology and Therapeutics.

First of all, this project has been developed considering the pathophysiology and the clinical manifestations of CoQ deficiencies, with a special focus on the biochemical and molecular mechanisms that play an essential role in the pathogenesis of these diseases. Moreover, it also points out the importance of laboratory analysis in the diagnostic strategy of these disorders, giving emphasis to the fact that biochemical and molecular testing are key elements in this process. Finally, taking into account that some patients respond, sometimes dramatically, to CoQ supplementation and that ubiquinol, the reduced form of CoQ, was recently approved as an orphan drug for primary CoQ deficiencies, this piece of work also reviews the therapeutic use of CoQ in this group of disorders.

The effective integration of the three areas allows the reader of the present study to realise about the vital functions that CoQ plays in human health, from its essential role at a molecular level to the potential therapeutic benefits of its supplementation.

1. INTRODUCTION

Mitochondrial diseases (MD) are a group of genetic disorders that are characterized by dysfunctional mitochondria, with a minimum prevalence of greater than 1 in 5.000 adults (1,2). The concept of MD was introduced in 1962, when a group of investigators in Stockholm described a woman with severe hypermetabolism unrelated to thyroid dysfunction (3). This exemplary piece of translational investigation was based on three sets of data: a) morphological evidence of abnormal mitochondria in muscle, b) biochemical documentation of 'loose coupling' of oxidation and phosphorylation in isolated muscle mitochondria, and c) excellent correlation between biochemical abnormalities and clinical features. This was both the first description of a MD and the first to characterize disease of a subcellular organelle; therefore, this paper introduced not only the concept of mitochondrial medicine but also that of 'organellar medicine' (4,5).

In the pre-molecular era (from 1962 to 1988), MD were defined on the basis of clinical examination, muscle biopsy and biochemical criteria. However, biochemical studies were often inconclusive due to the difficulty of isolating functionally intact mitochondria from human muscle biopsies and to the relatively insensitivity of polarography (the predominant biochemical technique then employed) in detecting partial metabolic blocks. Yet, the application of specific biochemical assays led to the description of increasing numbers of metabolic defects (4).

The 'big divide' in the history of MD, and the beginning of the molecular age, was the description in 1988 of the first pathogenic mutations in mitochondrial DNA (mtDNA) (4). In the next years, new pathogenic mutations of mtDNA were reported at the rate of about eight per year (6). In the mid-1990s scientists started directing their attention to the nuclear genome, which, after all, encodes about 99% of mitochondrial proteins and about 86% of the respiratory chain subunits. Not too surprisingly, the first 'direct hit' (that is, the first mutations in a gene encoding a respiratory chain subunit) affected complex II, which is entirely encoded by nuclear DNA. The year was 1995, and the patients two siblings with Leigh syndrome who were homozygous for a mutation in the flavoprotein subunit (7,8).

A peculiar kind of 'direct hit' can be considered mutations in genes involved in the biosynthesis of coenzyme Q_{10} (or CoQ, an essential electron carrier in the mitochondrial respiratory chain [MRC]), as they can cause primary CoQ deficiencies, which often result in a severe block of the respiratory chain. The concept that primary CoQ deficiencies were due to mutations in biosynthetic genes was validated in 2006

with the discovery of mutations in *PDSS2* and *COQ2*, and confirmed in 2007 with the report of mutations in *PDSS1* and *COQ2* (4,9–11). These newly recognized disorders are important to consider in the differential diagnosis of infantile encephalomyopathies with nephrosis and in ataxia syndromes because they respond, sometimes dramatically, to oral supplementation of CoQ (8).

In the last 20 years new MD pathogenic mechanisms have been revealed through the identification of numerous nuclear gene mutations. These mitochondrial alterations include defects of mitochondrial protein importation, maintenance of the inner mitochondrial membrane lipid milieu, and even organellar dynamics such as mitochondrial fission, fusion and movement (5).

Although this brief historical review has brought us to the present, it is far from complete. The pathogeneses of known mutations remain largely unexplained, at the same time that new pathogenic mechanisms are emerging (6). In addition, currently there is no effective and specific treatment for vast majority of patients with MD, with few exceptions, such as primary CoQ deficiencies. For these reasons, further elucidation of the biochemistry and genetics of these complex diseases is still needed (2,5).

2. OBJECTIVES

The main objectives of this project are:

1) To briefly describe the pathophysiology and diagnosis of mitochondrial diseases.

2) To review the state-of-the-art knowledge of a group of mitochondrial diseases known as coenzyme Q_{10} (CoQ) deficiencies, including a previous description of the biological functions of CoQ and its biosynthetic pathway.

3) To study the current diagnostic issues of CoQ deficiency syndromes, especially from a biochemical and a molecular point of view.

4) To provide an overview of the role of CoQ therapeutic supplementation in CoQ deficiencies.

3. MATERIALS AND METHODS

In order to learn how to cope with the development of this project, two training courses delivered by the Pharmacy and Food Science CRAI (Learning and Research Resources Centre) Library were attended. Thanks to the first one, not only the structure of an academic work such as this one was learned, but also how to access to a series of information resources (including Google Scholar and specialized databases like Web of Science, Scopus and PubMed), and how to develop and execute an information search strategy. In this regard, the use of Boolean operators and the usefulness of MeSH (Medical Subject Headings) terms when searching PubMed were taught. The second course was focused on citing and managing bibliographical references. On one hand, information and examples of how to cite different documents according to Vancouver rules were provided. On the other hand, detailed information about the use of Mendeley was given. This helpful tool is a reference and citation manager that can also be used to organize the search results and to discover the latest documents published in a specific field.

Taking that in mind, this study has been developed thanks to a wide bibliographic research. The process consisted in identifying, reading, synthesizing and reporting the information from a diversity of sources, mainly journal articles and reviews.

An on-line biomedical database named PubMed (12) was used, which includes citations for biomedical literature from the National Library of Medicine (NLM) database (MEDLINE) plus other life science journals and on-line books. The search strategy and selection criteria followed consisted in searching key terms (*e.g.* 'mitochondrial diseases', 'coenzyme Q_{10} deficiency', 'coenzyme Q_{10} supplementation AND coenzyme Q_{10} deficiency') for articles and reviews published in English in the last 10 years. However, other papers were also selected after identification from the references lists of relevant articles.

Finally, bibliographical references were recorded following the NLM citation style, using the Mendeley reference manager (both web and desktop 1.19.4 versions).

4. RESULTS AND DISCUSSION

4.1. Mitochondrial diseases: mechanisms and diagnosis

Mitochondria are cellular organelles found in all nucleated human cells. They have a crucial function which consists in generating energy in the form of ATP (adenosine triphosphate) via oxidative phosphorylation (OXPHOS), using predominantly carbohydrates and fatty acids as fuel. The OXPHOS system is located in the inner membrane and it consists of five multimeric protein complexes (Figure 4.1.). The first four complexes (I-IV) form the respiratory chain, and complex V is an ATP synthase (2). OXPHOS is a complex process involving the transfer of electrons along the MRC through a series of oxidation and reduction reactions that ultimately results in the consumption of oxygen at complex IV. Essential to this electron transport are two electron carriers, CoQ and cytochrome *c*, which shuttle between the complexes. A consequence of this electron transport is the extrusion of protons into the intermembrane space at complexes I, III and IV. The proton gradient generated is then dissipated through complex V, resulting in the condensation of adenosine diphosphate and inorganic phosphate to form ATP (13).

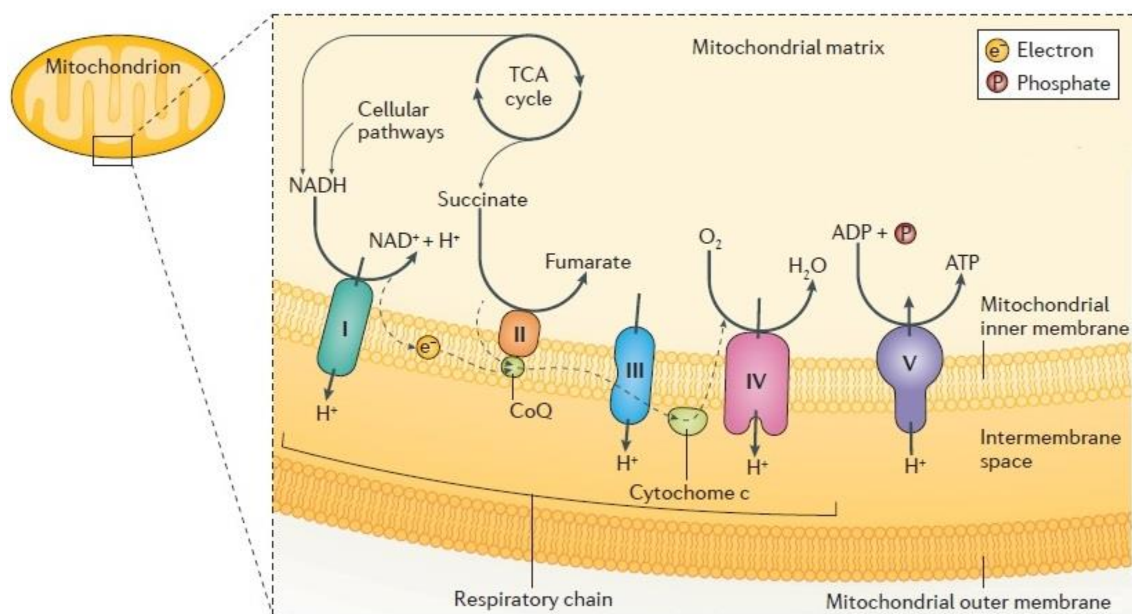

Figure 4.1. Schematic representation of mitochondrial respiratory chain and oxidative phosphorylation system. Adapted from Ref. (1).

Likewise, mitochondria are unique in that they have their own DNA pool (mtDNA), distinct from that of nuclear DNA (nDNA). mtDNA is maternally inherited and it has independent evolutionary origins from nDNA that date back to the time when

mitochondria were separate organisms before forming a symbiotic relationship with eukaryotes (14). mtDNA is a molecule of double-stranded DNA that encodes thirteen structural peptide subunits of the OXPHOS system and twenty-four RNA molecules that are required for intra-mitochondrial protein synthesis. As opposed to nDNA, mtDNA has a circular structure and lacks an intron-exon structure. In addition, the mitochondrial genome is polyploidy, with multiple copies of mtDNA within each cell, and the total amount can vary depending on the cell type. This results in complex transmission of mutations and genotypic-phenotypic variation in the presentation of MD. In fact, many patients with a MD have a mixture of mutated and wild-type mtDNA, a situation known as heteroplasmy. This explains why disease does not occur in everyone with mtDNA mutations; in certain mutations, a threshold level of mutated mtDNA is required before function is compromised and clinical consequences become apparent. This threshold may vary between individuals, tissue types and specific mtDNA mutations (1,13).

MD represent a clinically, biochemically and genetically heterogeneous group of diseases in which the process of ATP production is disrupted. As approximately 90% of ATP arises from mitochondria, cells with high-energy requirements such as neurons, skeletal and cardiac muscle are particularly vulnerable to this limited ATP supply and feature prominently in the various mitochondrial phenotypes (13,14). MD can be caused by mutations in either mtDNA or nuclear genes that directly or indirectly interfere with the MRC function. To date, mitochondrial proteomic analysis reveal that, in addition to the thirteen proteins encoded by the mitochondrial genome, around 1.500 proteins are linked to various mitochondrial functions and so far almost 290 genes have been implicated in the development of human disease (2,15). On one hand, pathogenetic mutations in mtDNA can affect the structural subunits of the MRC or the mitochondrial protein synthesis machinery; in this sense, hundreds of different point mutations and large-scale mtDNA rearrangements have been shown to cause disease (1). On the other hand, the nDNA mutations may be structural (that is, coding for respiratory complexes) or non-structural, affecting mtDNA replication and repair, metabolism and mitochondrial integrity (13).

The clinical manifestations of MD are very heterogeneous, and the presentation usually depends on generalised or tissue-specific decrease in ATP production. Some of these disorders affect a single organ (*e.g.* the eye in Leber hereditary optic neuropathy [LHON]), but many involve multiple organ systems. Virtually any organ may be impaired but, as previously described, the organs with the highest energy demands are most frequently involved, including brain, muscle, heart and liver. Besides, MD may

manifest at any age since birth until late-adulthood, with acute manifestation or as a chronic progressive disease (16). Paediatric onset disease is associated with more severe multi-systemic involvement, relentless progression and poorer prognosis (2).

Many classic syndromes have been described over the last few decades (Table 4.1.). However, there is often considerable clinical variability and many affected individuals do not fit into one particular category (16). Moreover, many symptoms associated with MD (such as deafness, diabetes, myopathy, gastrointestinal symptoms and others) are also common on their own in the population (2).

Clinical syndromes with neonatal and childhood onset
✓ Alpers-Huttenlocher syndrome ✓ Ataxia neuropathy spectrum ✓ Childhood myocerebrohepatopathy spectrum ✓ Congenital lactic acidosis ✓ Leigh syndrome ✓ 3-methylglutaconic aciduria with deafness, encephalopathy and Leigh-like syndrome ✓ Myoclonic epilepsy myopathy sensory ataxia ✓ Pearson syndrome ✓ Sengers syndrome
Clinical syndromes associated with adolescence and adulthood
✓ Chronic progressive external ophthalmoplegia (CPEO) ✓ Kearns-Sayre syndrome ✓ Leber hereditary optic neuropathy (LHON) ✓ Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) syndrome ✓ Mitochondrial neurogastrointestinal encephalopathy (MNGIE) syndrome ✓ Myoclonic epilepsy with ragged-red fibres (MERRF) ✓ Neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP)

Table 4.1. Clinical syndromes of mitochondrial disorders. Adapted from Ref. (1).

In general, many MD do not have pathognomonic features that point towards a particular genetic diagnosis (2). The diagnosis is further complicated by an often tenuous relationship between the genotype and the observed clinical phenotype. For example, some mtDNA mutations can give rise to several different clinical syndromes:

the m.3243A>G mutation (in *MT-TL1*) can cause CPEO, MELAS syndrome and maternally inherited diabetes and deafness. The reverse is also true in that specific syndromes can have a diverse genetic aetiology. For instance, Leigh syndrome can be caused by an array of mtDNA and nDNA mutations in several genes (1,17,18).

For all these reasons mentioned above, diagnosis of MD is both challenging and demanding. It is necessary to take into account the particular family and personal history, the course of the disease, the comprehensive clinical examination, the results of specialized examinations (especially cardiology, visual fundus examination, brain imaging and electromyography), and laboratory testing of body fluids (lactate, pyruvate, aminoacids [alanine], organic acids, fibroblast growth factor-21 [FGF-21], growth differentiation factor-15 [GDF-15], etc.). Outcomes of these examinations help tailor targeted molecular genetic testing, but if it is not possible to target direct gene sequencing, next-generation sequencing (NGS) technologies can be used (16). Performing genetic testing might avoid the need for an invasive tissue biopsy, but some cases will still require it for biochemical confirmation of the consequences of mutations of unknown significance (1).

Finally, in addition to the diagnostic challenge, clinicians also encounter difficulty in the management of MD due to lacking of effective disease-modifying therapy. Because they are clinically and genetically heterogeneous disorders, no single therapeutic approach can address the diverse biochemical pathogenic mechanisms. Several treatments have been used, mostly nutritional supplements such as CoQ, carnitine, creatine, dichloroacetate and 'vitamin cocktails' (2,19). The physiological basis of vitamin supplementation is to remove toxic metabolites or promote ATP production by using electron-transport chain mediators, bypassing the metabolic defect (13). Despite these treatments have been widely used, the available sparse evidence suggests that they have modest beneficial effects (1,20).

4.2. CoQ deficiencies: a group of potentially treatable mitochondrial diseases

4.2.1. CoQ structure and functions

CoQ or ubiquinone is the only endogenously synthetized redox-active lipid that is found in virtually all eukaryotic cells, being especially abundant in mitochondria (21). It was isolated and characterized by Festenstein *et al.* in 1955, and it was established in 1957 by Crane *et al.* that this compound functions as a member of the MRC. Wolf *et al.* determined its complex structure in 1958, which is composed of a redox-active

benzoquinone ring and a polyisoprenoid tail of different lengths: ten isoprene units in humans (CoQ_{10}), nine in mice (CoQ_9) and six in yeast (CoQ_6) (Figure 4.2.) (22,23).

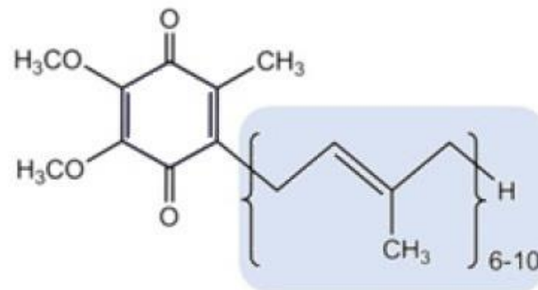

Figure 4.2. Chemical structure of CoQ. Adapted from Ref. (21).

CoQ is permanently going through oxidation-reduction cycles. It can be found in a completely reduced form (ubiquinol), after receiving two electrons, or in a completely oxidized form (ubiquinone). When this redox cycle occurs by a two-step transfer of one electron each, a semiquinone (semi-ubiquinone) intermediate is produced (21).

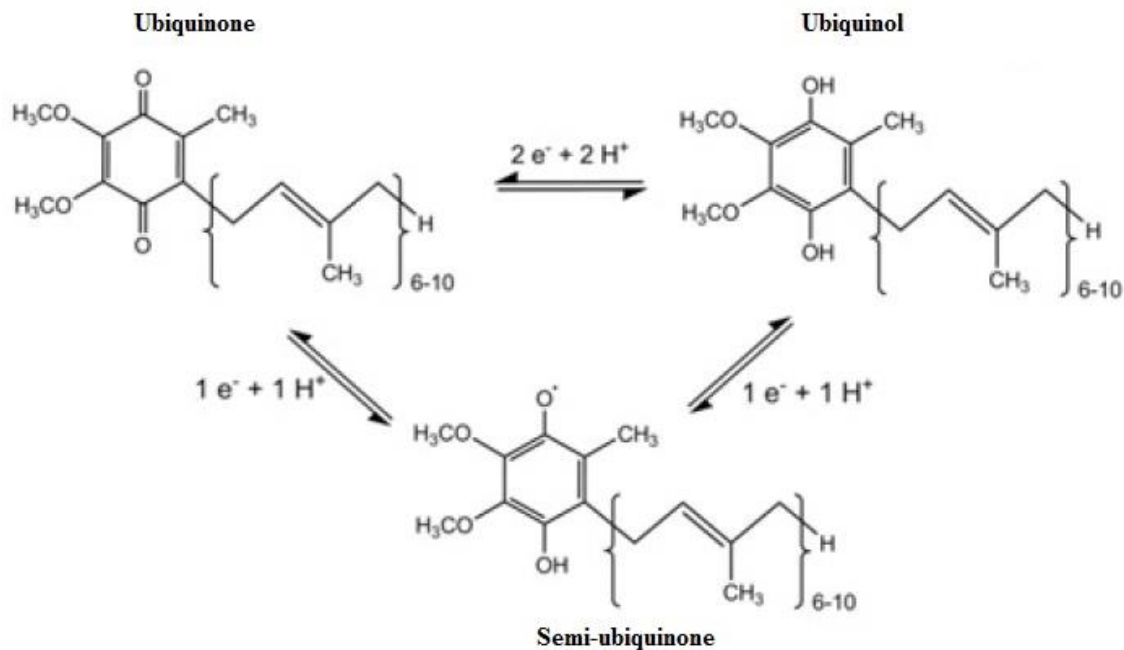

Figure 4.3. Redox cycle of CoQ. Adapted from Ref. (21).

The ability of this peculiar molecule to sustain continuous redox cycles makes it an excellent electron carrier in many crucial cellular pathways (24). In this sense, as previously mentioned, CoQ constitutes an essential element of the mitochondrial

electron-transport chain, shuttling electrons from complexes I and II to complex III at the inner mitochondrial membrane (Figure 4.1.) (25).

CoQ is also a potent membrane antioxidant which protects lipids, proteins and nucleic acids from harmful oxidative damage. The high efficiency of CoQ against oxidative stress may be related to its ubiquitous distribution, its localization in the core of membranes and the availability of diverse dehydrogenases, able to efficiently regenerate the molecule (21,26).

In addition to its central role in the MRC and its function as an antioxidant, CoQ is also involved in a number of aspects of cellular metabolism (22,23,27):

- Participation in extra-mitochondrial electron transport
- Regulation of mitochondrial permeability transition pores
- Activation of mitochondrial uncoupling proteins
- Cofactor of several mitochondrial dehydrogenases, including an enzyme involved in pyrimidine nucleotide biosynthesis
- Regulation of the physicochemical properties of membranes
- Modulation of the amount of β_2-integrins on the surface of blood monocytes
- Improvement of endothelial dysfunction

4.2.2. CoQ biosynthesis

All mammalian cells have the capacity to synthesize CoQ, but its availability also comes up from dietary sources, which influence plasmatic levels up to 25% of the total amount. However, the distribution of plasma CoQ through cells and organs seems limited. Thus, every mammalian cell produces CoQ, likely because this molecule is poorly absorbed into cells and tissues (28,29).

CoQ is synthesized by a set of nuclear-encoded proteins through a pathway that is not completely understood. Most of the work on CoQ biosynthesis has been done in yeast, and at least thirteen yeast genes have been identified as players of this process. Information about the human pathway is very scarce, but orthologues of almost all of these genes have been already identified (21,30). In yeast, Coq proteins assemble in a multi-subunit complex which requires the presence of all its components for its stability. This complex seems to be present also in mammalian cells, however, the exact composition and organization of this complex are not completely clear yet (27).

In mammals, the precursor of the benzoquinone ring is 4-hydroxybenzoate (4-HB), which is derived from tyrosine through an uncharacterized set of reactions. The

polyisoprenoid tail is synthesized through the mevalonate pathway (which is also common to cholesterol and dolichol biosynthesis), which comprises the reactions that starting from acetyl-coenzyme A (acetyl-CoA) produce farnesyl-pyrophosphate (FPP) (24). The latter, after conversion to decaprenyl-pyrophosphate (decaprenyl-PP), condenses with 4-HB to decaprenyl-4-hydroxybenzoate (decaprenyl-4-HB), which is then converted in a number of additional reaction steps to CoQ (Figure 4.4.). While the most of the early steps take place in the cytosol, the steps specific to CoQ biosynthesis, starting with the generation of the side chain from FPP, occur in the mitochondria (25).

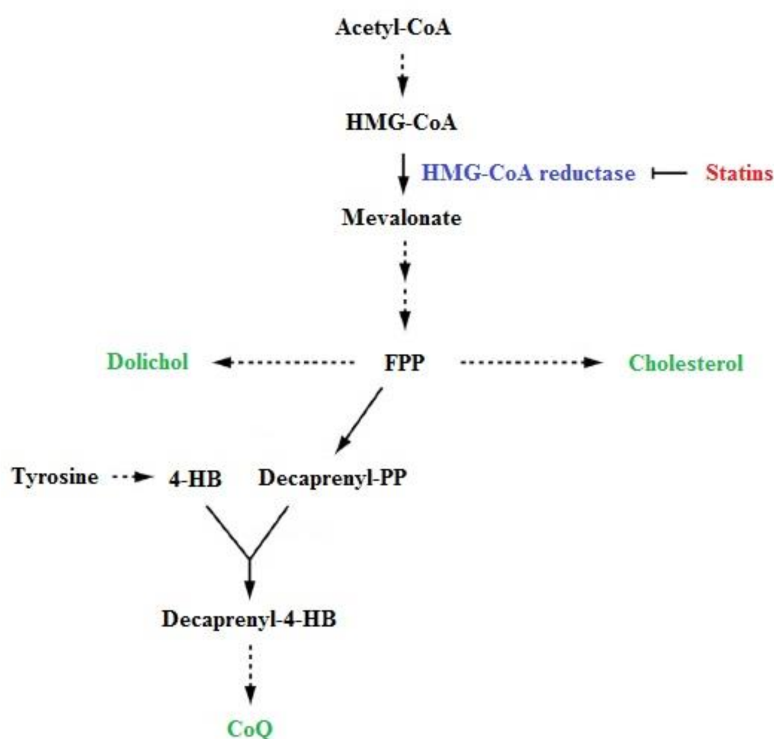

Figure 4.4. The mevalonate pathway produces isoprenoid precursor units, which are required for the biosynthesis of a variety of important molecules, including CoQ, cholesterol and dolichol. Adapted from Ref. (25).

Whereas many aspects of CoQ biosynthesis have been unveiled, little is known about CoQ biosynthesis regulation, which may occur at the transcriptional, post-transcriptional and post-translational level, or even during the assembly of the putative multi-subunit complex (21). In the same way, there is still little information about the CoQ degradation pathway. The tissue half-life of CoQ is remarkably long (in the order of several days), and its catabolites are found in urine and feces; they consist of the

ring and a short side chain, which are phosphorylated, but the enzymes that catalyse these processes are still unknown (23).

4.2.3. CoQ deficiencies

CoQ deficiency is a biochemical abnormality associated with markedly different clinical presentations, and it is not expected to occur in healthy individuals because endogenous production is usually sufficient. Given the essential functions of CoQ, a deficit in this molecule leads to a number of MD with an unexplained heterogeneous clinical spectrum that encompasses at least five major phenotypes: 1) an encephalomyopathy, characterized by recurrent myoglobinuria, 2) a severe infantile multisystem disorder with encephalopathy, 3) an ataxic syndrome with cerebellar atrophy, 4) an isolated myopathy, and 5) a steroid-resistant nephrotic syndrome (SRNS) (25,31,32).

Although the first patients with very low levels of CoQ were described in 1989 by Ogasahara *et al.*, the genetic bases of these discoveries remained elusive until 2006, when the first molecular causes of primary CoQ deficiency were described (9,10,33). The identification of the molecular defects allowed to propose a genetic classification, which distinguishes primary deficiencies, in which the reduction in CoQ content is due to mutations in genes controlling CoQ biosynthesis, and secondary forms, which are due to defects in genes unrelated to the CoQ biosynthetic pathway (23). The existence of secondary forms, which are probably much more frequent than primary defects, is important because it demonstrates how the CoQ biosynthetic pathway can be easily perturbed (24). It should be noted that the majority of patients with a biochemical diagnosis of CoQ deficiency lack a definite genetic diagnosis and, therefore, it is not possible to classify them into primary or secondary forms (27).

4.2.3.1. Primary CoQ deficiencies

Primary CoQ deficiencies are very rare conditions usually associated with highly variable multisystem manifestations and genetically caused by autosomal recessive mutations. It has been estimated a worldwide total of 123.789 individuals (1 in 50.000 individuals) affected by these disorders, taking into account the frequency of the different known or predicted pathogenic variants in given populations (21,34).

To date, nine genes encoding CoQ biosynthetic proteins (*COQ2*, *COQ4*, *COQ6*, *COQ7*, *COQ8A*, *COQ8B*, *COQ9*, *PDSS1* and *PDSS2*) have been shown to have

pathogenic variants causing human CoQ deficiency with an autosomal recessive inheritance; the association of two other genes (*ADCK2*, *COQ5*) with CoQ deficiency must be confirmed yet. These mutations affect multiple organ systems in a highly variable way, including central nervous system (CNS), peripheral nervous system, kidney, skeletal muscle, heart and sensory system (Table 4.2.). While many signs and symptoms reported in CoQ-deficient patients are common to other MD, some features are typical of some forms of CoQ deficiency, as SRNS (21,24,35).

Among the clinical manifestations of primary CoQ deficiencies, the most frequently encountered features are encephalomyopathy, nephropathy and cerebellar ataxia. These features have been interpreted as indicating that skeletal muscle, kidney and cerebellum have a relatively higher susceptibility to damage under conditions of CoQ deficiency, probably because of a relatively low safety margin of CoQ content (19,36,37).

The clinical variability of CoQ deficiencies concerns the age of onset (from birth to seventh decade), the severity of the disease (from fatal multisystem disorder to milder, tissue specific manifestations), the pattern of tissue involvement (even for patients with mutations in the same gene), and the clinical response to CoQ supplementation (27). This clinical heterogeneity is best illustrated by the patients that all harbour a mutation in the *COQ2* gene yet are seemingly phenotypically divergent. For example, the first patient to be reported with a homozygous missense mutation in *COQ2* was noted to have nystagmus at age 2 months and developed a severe SRNS, progressive encephalomyopathy, hypotonia, seizures and other symptoms at 12-18 months, whereas his young sister developed nephrotic syndrome at 12 months without any clinical signs of neurological involvement (10,36).

The considerable heterogeneity in the clinical expression of CoQ biosynthetic defects could be reflective of differences in the residual activities of the affected proteins and, thus, of variable degrees of CoQ shortage. Moreover, there remains the possibility of other functions of COQ proteins in addition to the biosynthesis of CoQ. Furthermore, it is reasonable to suspect that some CoQ biosynthetic intermediates and defective COQ proteins may have some biological activities, which could contribute to the variation in clinical manifestations of different molecular defects (36).

Gene	Clinical manifestations					
	Renal	**Heart**	**Eye**	**Hearing**	**Neurologic**	**Muscle**
COQ2	SRNS	HCM	Retinopathy	SNHL	Encephalopathy, seizures, other	Myopathy
COQ4		Heart failure, HCM			Encephalopathy, seizures, other	Myopathy
COQ6	SRNS			SNHL	Encephalopathy, seizures	
COQ7					Encephalopathy, ID, peripheral neuropathy	Muscle weakness
COQ8A					Encephalopathy, cerebellar ataxia, dystonia, spasticity, seizures	Exercise intolerance
COQ8B	SRNS				ID	
COQ9	Tubulopathy	HCM			Encephalopathy	Myopathy
PDSS1			Optic atrophy		Encephalopathy, peripheral neuropathy	
PDSS2	SRNS		Retinopathy	SNHL	Leigh syndrome, ataxia	

Table 4.2. Clinical manifestations of primary CoQ deficiencies. Abbreviations: SRNS, steroid-resistant nephrotic syndrome; HCM, hypertrophic cardiomyopathy; SNHL, sensorineural hearing loss; ID, intellectual disability. Adapted from Ref. (35).

4.2.3.2. Secondary CoQ deficiencies

CoQ levels can also be reduced secondary to conditions not directly linked to CoQ biosynthesis but related to OXPHOS, other non-OXPHOS mitochondrial processes, or even to non-mitochondrial functions. Remarkably, secondary CoQ deficiencies are proved to be more common than primary deficiencies, probably because of the diversity of biological functions and metabolic pathways in which CoQ is involved in mitochondrial and non-mitochondrial membranes (21,27,38).

The exact mechanisms by which these genetic defects cause CoQ deficiency remain unknown. Several hypotheses have been proposed, including interference with the signalling pathways regulating CoQ biosynthesis, alteration of the mitochondrial inner membrane milieu, reduction in the stability of the CoQ biosynthetic complex, increased rate of CoQ degradation due to oxidative damage caused by a non-functional respiratory chain, or a general impairment of mitochondrial function. Although none of these hypotheses have been yet demonstrated, a combination of different factors could be the most plausible explanation (21,27).

Specific symptoms of secondary CoQ deficiencies depend on the underlying condition. However, most reports focus on skeletal muscle and the CNS. Muscular manifestations consist of weakness, hypotonia, exercise intolerance or myoglobinuria, while the CNS manifestations include ataxia and general CNS impairment. Although in these situations CoQ deficiency is a secondary phenomenon, it probable exacerbates the symptoms caused by the primary molecular defect, and these patients often benefit from oral CoQ supplementation, even though the response is not as dramatic as in those with the primary forms (27,39).

CoQ deficiency is a common finding in patients with a MD, especially in mitochondrial myopathies (40). In this sense, a comprehensive analysis of muscle and fibroblasts samples from patients affected by a wide range of MD showed that secondary deficiencies were more frequent in mtDNA depletion syndromes than in any other MD (38,41). However, other diseases may display a CoQ reduction, including ataxia with oculomotor apraxia, cardiofaciocutaneous syndrome, multiple acyl-CoA dehydrogenase deficiency (MADD), methylmalonic aciduria, phenylketonuria (PKU) or mucopolysaccharidosis (MPS), among others (38,42). Moreover, secondary deficiency may also occur as a result of the use of certain pharmacotherapeutic agents such as statins, which are inhibitors of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and, thus, interfere not only with the biosynthesis of cholesterol, but also of CoQ (Figure 4.4.) (27,32).

Lastly, it is important to keep in mind that a reduction of CoQ levels is not a consistent feature in these conditions mentioned above, which could suggest different susceptibility to the development of secondary deficiencies among different individuals. Currently, there is not any general explanation for this, although genetic factors, such as certain polymorphisms, have been proposed to be involved (21,27).

4.2.3.3. Pathogenesis of CoQ deficiency

The pathogenesis of CoQ deficiency is complex and not completely understood, but it seems to involve two main aspects: 1) reduced ATP production, and 2) altered oxidative stress levels. In this regard, it has been reported that short-tail ubiquinone analogues such as idebenone (which are good antioxidants but do not rescue mitochondrial respiration) are not effective in the treatment of CoQ deficiencies, indicating that both aspects (the bioenergetics defect and the increased ROS production) are relevant for the pathogenesis of the disorder. However, the wide spectrum of CoQ functions, the unclear roles of some *COQ* gene products and the considerable phenotypic variability suggest that other mechanisms also contribute to the pathogenesis of the disease (21,24).

First of all, because CoQ is an essential component of the MRC, its deficiency (regardless of whether it is a primary or a secondary form) causes an impairment of the transport of electrons to complex III and, therefore, an inhibition of OXPHOS and ATP production in cells (Figure 4.1.), which in turn compromise cellular functions (27).

Secondly, CoQ plays an essential role both in ROS generation and in antioxidant defence. On one hand, CoQ is the only endogenously synthesized lipophilic antioxidant preventing oxidative damage by directly sequestering free radicals or by regenerating other antioxidants (*i.e.* vitamin E and C). On the other hand, CoQ also acts as a pro-oxidant mainly through the semiquinone intermediate formed during electron transport activity (Figure 4.3.), which is believed to be capable of donating its free electron to oxygen at complex III, leading to formation of superoxide anion, which is the precursor of other damaging oxygen species (36). In cultured cells, there appears to be an inverse relationship between the severity of CoQ deficiency and ROS production, such that even relatively mild defects do not significantly impair ATP production but cause a significant increase of ROS production and, therefore, may be harmful to the cell (21,27,43).

Last but not least, there is evidence that other functions of CoQ are also involved in the pathogenesis of the disease. In this sense, a dysfunction of nucleotide metabolism

(CoQ is required for the biosynthesis of pyrimidines), increment of cellular apoptosis and mitophagy, and impairment of mitochondrial sulfide oxidation pathway have been reported in patients with CoQ deficiency (29).

4.3. Diagnosis of CoQ deficiencies

4.3.1. Clinical diagnosis

Since the first established molecular case of CoQ deficiency was described, the progression in the molecular diagnosis has increased the phenotypical spectrum of the disease. As mentioned earlier, a huge clinical variability is observed, including the age of onset, the severity of the phenotype, the degree of CoQ reduction in tissues, or the clinical response to CoQ supplementation (29).

Because of the heterogeneity of clinical presentations and also because there are no pathognomonic manifestations, it is difficult to define precisely the specific subgroups of patients who should be routinely screened for CoQ deficiency. Obvious candidates are patients with respiratory chain defects, those with beta-oxidation defects, those with unexplained ataxia with cerebellar involvement, and those with subacute exercise intolerance and muscle weakness (23). Besides, CoQ deficiency should also be suspected in patients with isolated SRNS or with SRNS and also presenting with deafness or other CNS manifestations (27).

Clinical identification of potential cases is of paramount importance to initiate investigations that may provide early diagnosis and initiation of specific treatment, especially as some CoQ-deficient patients respond well to CoQ supplementation (44).

4.3.2. Biochemical diagnosis

Biochemical measurements play an important role in the diagnostic pathway by providing a fast and reliable demonstration of CoQ deficiency that allows early treatment initiation (44). However, it is important to note that biochemical analyses are not able to distinguish between primary and secondary CoQ deficiencies, as this classification requires a definite genetic diagnosis (45).

The biochemical detection of CoQ deficiency can be done in the patient's tissues or biological fluids (29,46). A common biochemical pattern has been reported in most cases, consisting of a variable degree of CoQ deficiency in tissues (muscle, fibroblasts), which in turn may cause reduced combined activities of the CoQ-dependent MRC enzymes (complex I+III and complex II+III). A strong correlation exists

between these enzyme activities and the total CoQ content in muscle, although this pattern is not always observed; thus, direct quantitative measurement of CoQ levels is the most reliable test for diagnosis (29,44).

4.3.2.1. Tissue assessment

Essential to direct CoQ quantification is the choice of tissue for analysis. The particular CoQ distribution in distinct cellular fractions and the complexity of biological matrices make the biological sample choice and preparation a critical step in the CoQ quantification process. Additionally, since CoQ deficiency may be tissue-specific, invasive procedures are frequently needed in order to assess endogenous CoQ in the target organ, especially in muscle. Thus, it can be of value to analyse CoQ status in a full range of sample types, as a deficiency may remain undetected if the appropriate specimen is not chosen. Table 4.3. summarises the advantages and limitations of different biological specimens that can be used for CoQ analysis (44,47).

Skeletal muscle has been used for diagnosis of CoQ deficiency since the first cases of this deficiency were reported. Routine morphological studies on muscle sections do not usually yield specific findings (*i.e.* the histological picture may be normal, whereas in severe cases there may be signs of mitochondrial proliferation), although a common finding in both primary and secondary forms is the presence of lipid accumulation (23,27). This tissue is considered the gold standard for investigating endogenous CoQ status, and it seems to correlate with the severity of the disease. In addition, it also allows for the measurement of MRC activities, the expression and assembly of mitochondrial complexes, and the analysis of other biomarkers of mitochondrial function, such as citrate synthase (which is a marker of mitochondrial number and volume) (29,38,44,48,49).

However, skeletal muscle has some important limitations. First of all, the muscle biopsy is an invasive procedure (this is particularly evident for paediatric patients), and the amount of tissue required can be significant, especially if a complete biochemical characterization must be performed. Secondly, CoQ measurements are only performed in few selected laboratories and the muscle sample must be shipped in dry ice, therefore, the logistics may be complex and expensive (45).

Specimen	Advantages	Limitations
Skeletal muscle	- Good diagnostic yield for CoQ deficiency - Other mitochondrial studies can be performed	- Invasive - No treatment monitoring
Fibroblasts	- Good diagnostic yield for some CoQ deficiencies - Functional studies can be performed - Unlimited biological material for further studies	- False negative results in some cases
Plasma	- Minimally invasive - Identification of some secondary CoQ deficiencies - Treatment monitoring	- Low diagnostic yield for CoQ deficiency in MD - CoQ values modified by external sources
Blood cells	- Minimally invasive - Correlation with CoQ tissue levels - Treatment monitoring	- Fresh preparation - Time-consuming - Few reported experiences in MD
Urine	- Non-invasive - Easily detectable CoQ values - Treatment monitoring	- Correlation with kidney CoQ status remains to be established

Table 4.3. Advantages and limitations of different biological specimens that can be used for CoQ analysis. Adapted from Ref. (44).

In terms of practicability, the best tissue would be cultured skin fibroblasts, as obtaining them is much less invasive and potentially they may provide an almost unlimited amount of biological material (45). Besides, fibroblasts are of great value in functional studies such as uridine biosynthesis, ROS production and CoQ biosynthesis analysis, which are extremely useful tools for discriminating between primary and secondary deficiencies (38). For instance, in these cells it is possible to perform kinetic measurements of the CoQ biosynthetic rate (50) and to demonstrate correction of the CoQ deficiency by supplementation of the culture medium (51), while these analyses cannot be performed in muscle. Furthermore, finding appropriate controls and the logistics of shipping cultured fibroblasts are much simpler than for skeletal muscle samples (45).

Yet, diagnostic pitfalls have been demonstrated when analysing fibroblasts CoQ concentrations, especially in the milder and late-onset forms of CoQ deficiency. While there is specificity in primary CoQ deficiency (meaning that almost all patients with primary CoQ deficiency in muscle also present with a defect in fibroblasts), when a secondary CoQ deficiency is suspected in the presence of a predominant muscular phenotype, a muscle biopsy should be preferred as the pathogenesis is often not clear and fibroblasts may not reproduce the situation in muscle (29).

Regarding plasma samples, it has been reported that they are useful for the identification of diseases that include a deficient CoQ status apparently not related to a primary defect, such as PKU or MPS. Interestingly, a meta-analysis found new associations between serum CoQ levels and genes that are important for the development of neuronal disorders (29,52). Furthermore, plasma CoQ determination has a critical role in CoQ treatment monitoring, as CoQ therapy is commonly used for the treatment of MD and regular plasma CoQ quantification allows for informed adjustment of the oral CoQ dose, control of treatment compliance and confirmation of adequate CoQ intestinal absorption (44).

Even so, CoQ status in plasma can be affected by both dietary intake and by the amount of plasma lipoproteins (which are the major carriers of CoQ in the circulation) (21). For this reason, it has been suggested that plasma CoQ evaluation is not reliable for the diagnosis of primary CoQ deficiencies, as partial correction of CoQ levels may occur due to dietary consumption of CoQ or increases in cholesterol availability. Indeed, in most patients with primary CoQ deficiency, plasma CoQ values are normal (42,44).

Lastly, other biological samples have been reported as useful for CoQ analysis, but the applications in clinical practice have not been applied to large series of patients (29). For example, the analysis of blood mononuclear cells (BMCs) and platelets requires just a few days (in contrast to the several weeks required for fibroblasts growth), and it may reflect changes in cellular status following supplementation (45,53). A second example might be biological samples collected by non-invasive procedures, as buccal mucosa cells or urine. The latter is especially interesting as it could add data about kidney CoQ status in patients with renal diseases in general, and those associated with mitochondrial dysfunction in particular (29,54,55).

4.3.2.2. CoQ quantification

The gold standard procedure for biochemical diagnosis of human CoQ deficiency is the analysis of CoQ concentration in muscle biopsy by high-pressure liquid chromatography (HPLC). Mainly two different detection systems are used (*i.e.* ultraviolet and electrochemical detectors), being both useful to measure CoQ content in human samples and to determine the total CoQ status. In particular, HPLC coupled with electrochemical detection (HPLC-ED) is an interesting approach because it is a more sensitive procedure than the spectrophotometric one (thus, it yields more accurate results and only minimal amounts of muscle homogenates are needed), and because it allows the detection of the two natural CoQ forms (ubiquinol and ubiquinone) (23,45). The simultaneous assessment of both reduced and oxidised forms of CoQ is probably more suitable for research purposes rather than for clinical diagnosis, as the determination of total tissue CoQ status is sufficiently accurate to detect human CoQ deficiencies (53).

The reviewed and updated protocol used currently for the determination of CoQ status in different biological samples at the Inborn Errors of Metabolism Unit of Hospital Sant Joan de Déu is reported in Annex I. Typical HPLC-ED chromatograms are illustrated in Figure 4.5.

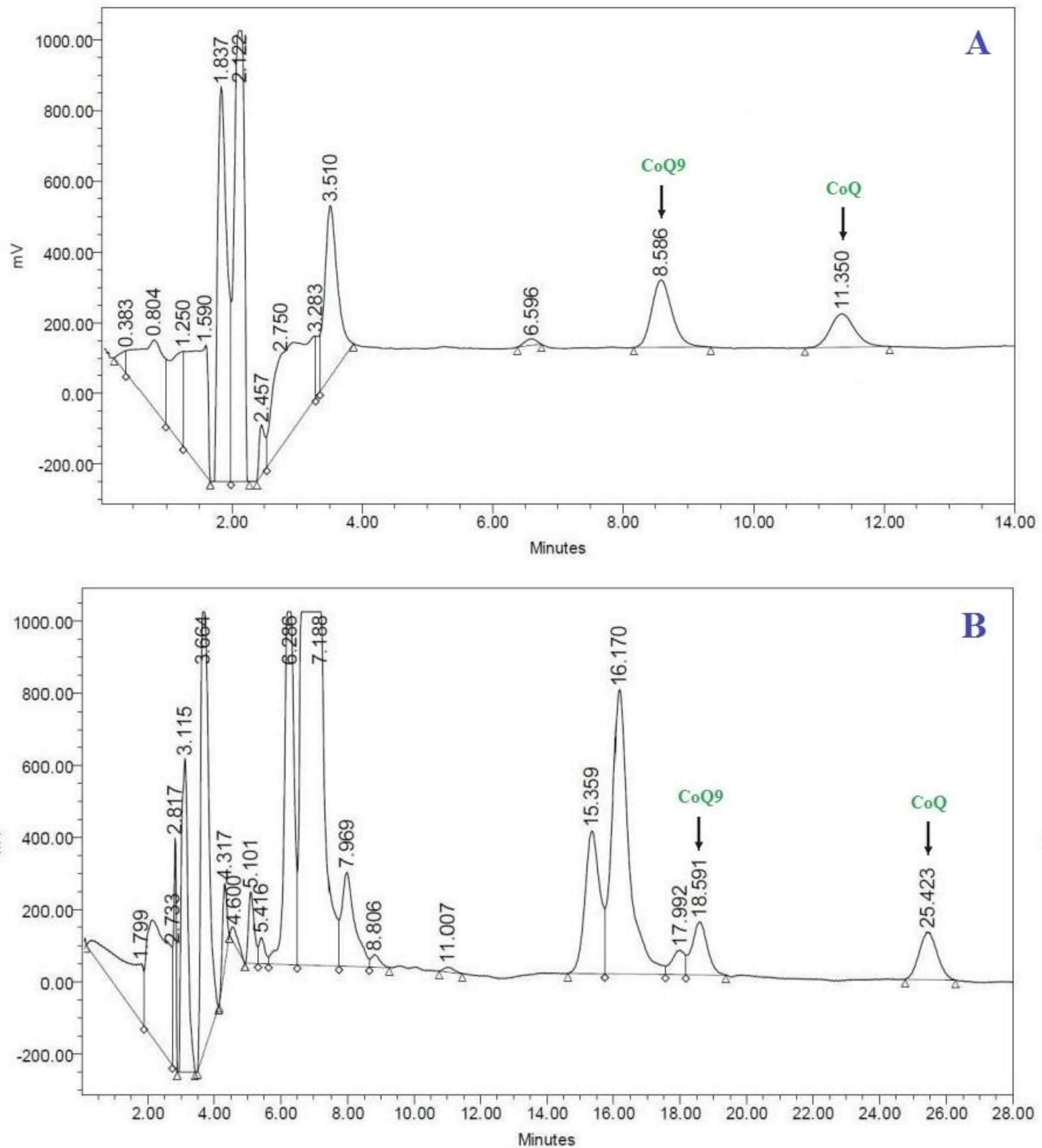

Figure 4.5. Typical CoQ chromatograms of skeletal muscle (A) and plasma (B) samples. CoQ_9 was used as the internal standard. Adapted from Ref. (44).

Recently, new procedures for CoQ determination have been developed based on liquid chromatography-tandem mass spectrometry (LC-MS/MS), allowing not only CoQ quantification but also an estimation of the CoQ biosynthetic rate in fibroblast cell cultures incubated with adequate CoQ precursors (44,50).

4.3.3. Molecular diagnosis

Apart from demonstrating the biochemical deficiency, it is important to provide a molecular diagnosis to the patients by identifying the specific genetic defect, because this is essential for clinical care, enabling assignment of risk, genetic counselling and prognosis (45). The molecular diagnosis of CoQ deficiency is complicated by the fact that a large number of genes are involved in the CoQ biosynthetic pathway (which has yet to be fully elucidated, meaning that several genes remain to be identified), but also by the possibility that the cause of the deficit may result from pathogenic mutations in genes not directly related to CoQ biosynthesis (53). Additionally, CoQ deficiency syndromes are clinically heterogeneous, making it difficult to perform accurate genotype-phenotype correlations (29,56). Taking all together, investigating all the genes related to CoQ synthesis and other possible genes that can lead to similar phenotype by Sanger sequencing is not a realistic option and it is not cost-efficient as well (45).

The incorporation of NGS technologies in hospital laboratories during the last decade has facilitated molecular diagnosis in terms of speed, efficiency and diagnostic yield (29). Either gene panels or exome sequencing based on NGS allow the analysis of very large numbers of genes for a fraction of the cost and of the time required by traditional Sanger sequencing gene-to-gene (45). Therefore, NGS has largely replaced the need to serially sequence individual *COQ* genes and other genes associated with secondary deficiencies and, thus, it has profoundly changed the diagnostic process (44).

Since its appearance, NGS has helped to associate new genes to disease and to establish more defined disease frontiers when multiple genes might cause overlapping phenotypes. For example, Freyer *et al.* described a patient with multiple organ dysfunction who presented at birth with muscular hypotonia, respiratory distress and renal dysfunction, in which whole-exome sequencing (WES) analysis revealed a *COQ7* mutation that implied a severe reduction in CoQ levels in mitochondrial extracts from skeletal muscle (29,57).

The other benefit of NGS application involves assessing secondary CoQ deficiencies, which are more common than primary deficiencies, meaning that in most cases with a biochemical CoQ deficiency the gene which will establish the cause of the disease is not involved in the CoQ biosynthetic pathway. In this sense, multiple studies have demonstrated the efficiency of NGS in MD (58). As not only mitochondrial or OXPHOS diseases are associated with CoQ deficiency, it is important to carefully evaluate the

clinical, biochemical and genetic profiles of each patient, as it is plausible that unexpected findings will arise during the genetic analysis (29,38).

For all these reasons mentioned above, NGS methods are at present the best choice for the molecular diagnosis of CoQ deficiency syndromes and also for MD. As a diagnostic algorithm, targeted NGS gene panels (which are commonly used for SRNS, ataxias and MD) or clinical exome panels (which include 5.000-7.000 genes associated with mendelian diseases) can speed up and simplify the analysis, and that can be the first step prior to assessing other causal genes by WES (which is theoretically the best option, but its costs are higher and its coverage is inferior to targeted panels) (35,45).

Finally, it is important to have a wide collection of functional studies and cell biology techniques for the demonstration of the pathogenicity of the new mutations found by NGS. This may include MRC activity studies, protein expression in tissues, assessment of the CoQ biosynthetic pathway by using labelled substrates, or functional studies in yeast (29).

4.4. Treatment of CoQ deficiencies

Primary CoQ deficiencies are unique among MD because an effective therapy is available for patients, which is the supplementation of CoQ. For this reason, ubiquinol was approved as an orphan drug for primary CoQ deficiencies in 2016 (46). Except for *COQ8A* and *COQ9* patients, most individuals with primary forms show a good response to CoQ supplementation, which is usually evident after 10-20 days from starting treatment. Actually, oral CoQ supplementation at high doses (ranging from 5 to 50 mg/kg/day) has been demonstrated to be effective for treatment of both primary and secondary CoQ deficiencies (21,35).

It has been reported that CoQ supplementation can stop the progression of the encephalopathy and of renal manifestations in *COQ2*, *COQ6* and *ADCK4* patients. However, it is essential to institute treatment as early as possible since once damage in critical organs (such as the kidney or the CNS) is established, only minimal recovery is possible (23,24). For example, Montini *et al.* reported a progressive recovery of renal function and reduced level of proteinuria 20 days after the initiation of CoQ supplementation (Figure 4.6.) in a patient with a homozygous missense mutation in the *COQ2* gene who had developed a nephrotic syndrome at 12 months of age (59).

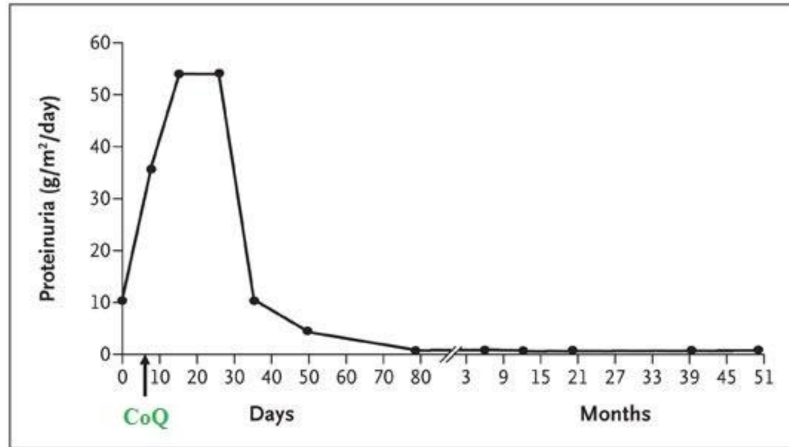

Figure 4.6. Proteinuria in a *COQ2* patient during a 50-month follow-up period. Reduction in proteinuria levels can be observed 20 days after the initiation of CoQ supplementation (indicated by the arrow). Adapted from Ref. (59).

Patients with CoQ deficiency show variable clinical responses to CoQ supplementation, and many different aspects may influence this variability. On one hand, obvious factors are the therapeutic dosages, the pharmaceutical formulation employed, the severity of the underlying illness and the progression of tissue damage, but there are probably many other components (genetic, environmental, and even epigenetic) that modulate the response to treatment. In this sense, it is important to provide adequate doses of CoQ and the appropriate formulations since often patients receive insufficient doses of the compound (27,60). On the other hand, another likely contributing factor to the disparate responses to CoQ is its poor bioavailability. Less than 5% of oral CoQ reaches plasma in humans, and rodent studies have demonstrated low uptake of CoQ by tissues with little or no detectable uptake by brain except in aged rats; hence, the blood-brain barrier appears to impair CNS intake of CoQ. Furthermore, because CoQ is highly lipophilic, exogenously administered CoQ will be integrated into plasma and other cellular membranes before reaching the inner mitochondrial membrane. Taking into account both the poor bioavailability and the delayed mitochondrial uptake of CoQ, it is clear that early rather than late supplementation is likely to successfully treat CoQ deficiency (19,22).

Different doses of CoQ have been employed for the treatment of primary CoQ deficiencies, being 30 mg/kg/day (divided into three doses) effective for both neuromuscular and renal symptoms in children; higher doses are well tolerated and no serious adverse effects have been reported (23). Split doses should be preferred to

single doses, as the efficiency of absorption decreases with the increase of individual dose of CoQ (27,32).

Currently, different formulations of CoQ are available (both in the oxidized and reduced forms), including crystalline CoQ powder, oil emulsions, solubilizates of CoQ and nanoparticulate formulations (61). The bioavailability of the crystalline form of CoQ is low and inconsistent due to the poor solubility and high molecular weight of this form (32); thus, it is recommended that solubilized formulations of CoQ, rather than powder-based CoQ, are used therapeutically as former have enhanced plasma response and, therefore, superior bioavailability (62).

Interestingly, as previously pointed out, primary CoQ deficiencies should not be treated with short-tail ubiquinone analogues such as idebenone, because those are good antioxidants but cannot replace CoQ in the MRC under conditions of CoQ depletion (63).

Finally, novel approaches have been proposed recently. In this regard, it has been reported that probucol, an antioxidant and hypolipidemic drug, has beneficial effects in *Pdss2* mutant mice, but no data on other genetic defects or human subjects are available (24). Also, some 4-HB analogues have been proposed as potential bypass molecules with higher bioavailability than CoQ. These water-soluble precursors of the benzoquinone ring would restore endogenous CoQ production, bypassing enzymatic steps disrupted by mutations in *COQ* genes, but their efficacy may differ depending on the stability of the CoQ biosynthetic complex. This approach is particularly interesting as these compounds are not toxic, have good bioavailability and may cross the blood-brain barrier. Some examples are vanillic acid (VA) and 3,4-dihydroxybenzoate (3,4-dHB), which are able to bypass *COQ6* mutations (21,24).

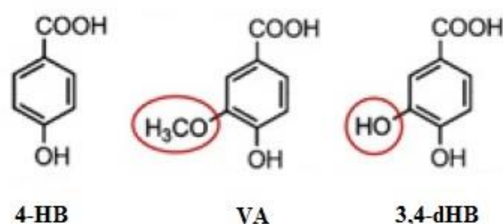

Figure 4.7. Chemical structures of 4-HB and two analogues, VA and 3,4-dHB, which may be used to bypass enzymatic steps disrupted by mutations in *COQ6*. Adapted from Ref. (21).

5. CONCLUSIONS

1) MD represent a clinically, biochemically and genetically heterogeneous group of diseases associated with dysfunction of the OXPHOS system. The multiple clinical phenotypes and the involvement of both the mitochondrial and nuclear genome make them particularly challenging for the clinicians.
2) CoQ is an endogenously synthesized redox-active lipid that plays crucial biological functions, including an essential role in energy production. The knowledge on its biosynthetic pathway and its regulation is still limited.
3) CoQ deficiencies have been associated with different clinical phenotypes and genetic conditions. They can occur due to defects of CoQ biosynthesis (primary deficiencies) or due to other causes (secondary forms), highlighting the importance of CoQ homeostasis in human health.
4) Due to the increasing number of genetic conditions that are being associated with CoQ deficiency, it is necessary to apply NGS techniques as a first-line investigation. Yet, this is possible only after meticulous clinical and biochemical characterization of patients.
5) Biochemical CoQ quantification by HPLC-ED allows for a rapid identification of CoQ deficiency. Moreover, biochemical analysis of CoQ status in a full range of biological specimen types can be of value as a deficiency may remain undetected if the appropriate sample is not chosen.
6) The management of patients with MD remains a challenge, due to lacking of effective disease-modifying therapy. CoQ deficiencies are unique among MD because an effective therapy is available for patients, which is the supplementation of oral CoQ at high doses. However, early diagnosis of the CoQ-deficient status is essential to improve the clinical outcome of patients.
7) In summary, despite the advances in the last decades, several interesting and challenging aspects of MD remain unclear. Further elucidation of the biochemistry and genetics of these complex diseases is needed to set the stage for improving the care of patients and the development of novel treatments.

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ANNEX I

The following is the current protocol used for the biochemical determination of CoQ status in different biological samples at the Inborn Errors of Metabolism Unit of Hospital Sant Joan de Déu. It was reviewed, updated and discussed with a clinical biochemist from the mentioned department before being reported here. Relevant articles were used for this purpose (48,55,64).

1. Sample preparation

a) Blood samples: EDTA blood samples must be drawn to obtain plasma, BMCs or platelets.

- Plasma samples are obtained by centrifugation of blood at 1.500 x *g* for 10 minutes at 4 ºC. Resulting samples which are highly haemolysed, icteric or lipemic should not be used. Since CoQ is related to cholesterol, total plasma cholesterol values are analysed by the automated cholesterol oxidase procedure in an Architect autoanalyzer (Abbott Laboratories).
- For BMCs' isolation, the Histopaque-1077 procedure is used. Briefly, 3 mL of Histopaque-1077 solution are added to a 15-mL conical centrifuge tube, and 5 mL of blood are carefully layered onto it. After centrifugation at 500 x *g* for 30 minutes at room temperature (with no brake applied), BMCs form a distinct layer at the plasma-Histopaque interface. This layer is carefully aspirated with a Pasteur pipette and transferred into a clean conical centrifuge tube, and then the cells are washed two times with 5 mL of phosphate-buffered saline (PBS) solution. The resulting cell pellet is resuspended in 200 µL of PBS solution.
- The first step in isolating platelets consists of preparing platelet-rich plasma (PRP) by centrifugation of blood at 60 x *g* for 15 minutes at room temperature (with no brake applied). Then, two thirds of the top layer are transferred into a new tube, without disturbing the buffy coat layer. Platelet counting is performed using an ADVIA 2120 haematology analyser (Siemens Healthineers). Finally, in order to pellet the platelets, the tube is centrifuged at 1.000 x *g* for 20 minutes at room temperature and the supernatant is discarded.
- All the blood-derived samples must be stored at -80 ºC until CoQ analysis.

b) Urine samples: first morning urine samples must be collected in standard urine containers. Ideally, a minimum volume of 30 mL of urine should be collected. Samples containing red or white blood cells or bacteria should not be used. After centrifugation at 1.500 x *g* for 10 minutes at 4 ºC, the urinary pellet is washed with 5 mL of 9 mg/mL saline solution, and then it is centrifuged in order to remove urinary proteins. The

resulting urinary pellet is resuspended in 100 µL of saline solution per 10 mL of total urine, and it is stored frozen at -80 ºC until CoQ analysis.

c) <u>Muscle samples</u>: skeletal muscle samples are weighed and homogenized with cold SETH buffer in an ice bath. The mixture is then vortexed, sonicated and transferred to a polypropylene tube, followed by vortexing for 2 minutes, sonication for 5 minutes and centrifugation at 1.500 x *g* for 10 minutes at 4 ºC. The supernatant must be frozen at -80 ºC until CoQ analysis.

d) <u>Fibroblasts samples</u>: cultured skin fibroblasts are homogenized and the resulting suspension must be frozen at -80 ºC until CoQ analysis.

2. CoQ extraction

50 µL of samples (100 µL in case of urine samples) are transferred into 10-mL Pyrex glass tubes. After internal standard solution in ethanol containing 2.01 µmol/L of CoQ_9 is prepared and added to the samples (20 µL), deproteinization is undertaken by addition of 500 µL of ethanol. Then, 2 mL of hexane are added to the tubes, and these are closed with screw caps and vortexed for 10 minutes. After centrifugation at 1.500 x *g* for 10 minutes, the hexane phase (top layer) is collected, filtered using a 0.22 µm filter, evaporated to dryness under a stream of nitrogen and redissolved in 200 µL of methanol/ethanol (60:40, v/v). Calibrators, controls and samples are prepared in the same way.

3. Instrumentation and chromatographic conditions

The CoQ content in the different biological specimens is analysed by HPLC (Waters) coupled to electrochemical detection (Coulochem II, ESA). The mobile phase consists of 1.06 g/L lithium perchlorate in methanol/ethanol (60:40, v/v), and CoQ is separated in a Nucleosil C-18 column (250 x 4 mm, 5 µm particle size, Teknokroma). The flow rate is 1 mL/min, and the injection volume is 50 µL (100 µL in case of urine samples). Once the CoQ is separated, it is quantified by electrochemical detection (the analytical cell, model 5010, is set to -600 mV and +600 mV) using CoQ_9 as the internal standard. The chromatographic data are integrated using Waters Breeze HPLC software.

4. CoQ content calculations

CoQ concentrations in the different biological samples are determined by calculating the peak height ratios between CoQ and the internal standard (CoQ_9). Plasma CoQ values are reported as µmol/L and µmol/mol cholesterol. In order to estimate the cell

content of the BMCs, urine, muscle and fibroblasts samples, the total protein concentration is determined by the Lowry method. In these specimens, the CoQ values are normalized to the total protein concentration and reported as nmol/g of total protein. Regarding to platelets, final results are reported as pmol/10^9 platelets.