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ORIGINAL ARTICLE

Characterization of CoQ_{10} biosynthesis in fibroblasts of patients with primary and secondary CoQ_{10} deficiency

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Abstract Primary coenzyme Q_{10} (CoQ_{10}) deficiencies are associated with mutations in genes encoding enzymes important for its biosynthesis and patients are responsive to CoQ_{10} supplementation. Early treatment allows better prognosis of the disease and therefore, early diagnosis is desirable. The complex phenotype and genotype and the frequent secondary CoQ_{10} deficiencies make it difficult to achieve a definitive diagnosis by direct quantification of CoQ_{10}. We developed a non-radioactive methodology for the quantification of CoQ_{10} biosynthesis in fibroblasts that allows the identification of primary deficiencies. Fibroblasts were incubated 72 h with 28 μmol/L $^{2}H_3$-mevalonate or 1.65 mmol/L $^{13}C_6$-p-hydroxybenzoate. The newly synthesized $^{2}H_3$- and $^{13}C_6$-labelled CoQ_{10} were analysed by high performance liquid chromatography-tandem mass spectrometry. The mean and the reference range for $^{13}C_6$-CoQ_{10} and $^{2}H_3$-CoQ_{10} biosynthesis were 0.97 (0.83–1.1) and 0.13 (0.09–0.17) nmol/Unit of citrate synthase, respectively. We validated the methodology through the study of one patient with *COQ2* mutations and six patients with CoQ_{10} deficiency secondary to other inborn errors of metabolism. Afterwards we investigated 16 patients' fibroblasts and nine showed decreased CoQ_{10} biosynthesis. Therefore, the next step is to study the COQ genes in order to reach a definitive diagnosis in these nine patients. In the patients with normal rates the deficiency is probably secondary. In conclusion, we have developed a non-invasive non-radioactive method suitable for the detection of defects in CoQ_{10} biosynthesis, which offers a good tool for the stratification of patients with these treatable mitochondrial diseases.

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Introduction

Coenzyme Q_{10} (CoQ_{10}) is a lipophilic molecule critical for the transport of electrons from complex I and complex II (and also from the β-oxidation pathway via the electron transfer flavoprotein, ETF) to complex III in the mitochondrial respiratory chain (RC) (Festenstein et al 1955; Crane et al 1957; Frerman 1987). It also participates in extra-mitochondrial electron transport and functions as an antioxidant in cell membranes preventing lipid, protein and DNA oxidation. Moreover, CoQ_{10} is involved in the regulation of mitochondrial uncoupling proteins and mitochondrial permeability transition pore; it is also required for pyrimidine nucleoside biosynthesis and may modulate apoptosis (Turunen et al 2004).

In humans CoQ_{10} is synthesized in cells and tissues and no uptake is usually required; 2–4 % of the dietary CoQ_{10} is recovered in the circulation, but its transfer to the organs seems very limited (Turunen et al 2004).

CoQ_{10} is composed of a benzoquinone ring derived from tyrosine and a decaprenyl side-chain coming from the mevalonate (MV) pathway after successive additions of isopentenyl-diphosphate (IPP) molecules to farnesyl-diphosphate (FPP) catalyzed by prenyl-diphosphate synthase (*COQ1*) (Fig. 1) (Dallner and Sindelar 2000). Decaprenyl diphosphate (DPP) and p-hydroxybenzoate (PHB) are condensed by PHB-polyprenyltransferase (*COQ2*), and further modified by at least six enzymes catalyzing methylation, decarboxylation, and hydroxylation reactions to synthesize the final CoQ_{10} molecule. The MV pathway comprises the reactions from acetyl-coenzyme A (acetyl-CoA) to FPP, which is precursor for CoQ_{10}, cholesterol, dolichol and isoprenylated proteins (Turunen et al 2004; Dallner and Sindelar 2000).

Primary CoQ_{10} deficiencies are described as genetic disorders with good response to supplementation with CoQ_{10}. Early treatment based on early diagnosis is critical to maximize the efficacy of ubiquinone supplementation (López et al 2010). These mitochondrial disorders are rare conditions that have been reported in individuals with various clinical phenotypes showing decreased activities of the RC complexes I+III and II+III, and low levels of CoQ_{10} (Rahman et al 2012; Ogasahara et al 1989; Rötig et al 2000; Salviati et al 2005; Horvath et al 2006; Quinzii et al 2007; Rustin et al 2004) in muscle or fibroblasts. The diversity of symptoms along with the large number of genes involved in the synthetic pathway and the frequent secondary CoQ_{10} deficiencies make it difficult to achieve a definitive diagnosis. CoQ_{10} deficiencies are primary when due to mutations in genes involved in CoQ_{10} biosynthesis (*COQ* genes), where even haploinsufficiency for the *COQ4* gene has been described to cause CoQ deficiency (Salviati et al 2012). It can also be secondary to genes not directly involved in it, such as *APTX* (aprataxin) (Quinzii et al 2005), *ETFDH* (electron-transferring-flavoprotein dehydrogenase) (Gempel et al 2007; Liang et al 2009) or *BRAF* (Aeby et al 2007). Secondary deficiencies have also been reported in patients with mitochondrial DNA (mtDNA) mutations or deletions (Rahman et al 2012; Sacconi et al 2010; Matsuoka et al 1991), and some specific genetic factors may confer susceptibility to develop secondary CoQ_{10} deficiency (Sacconi et al 2010).

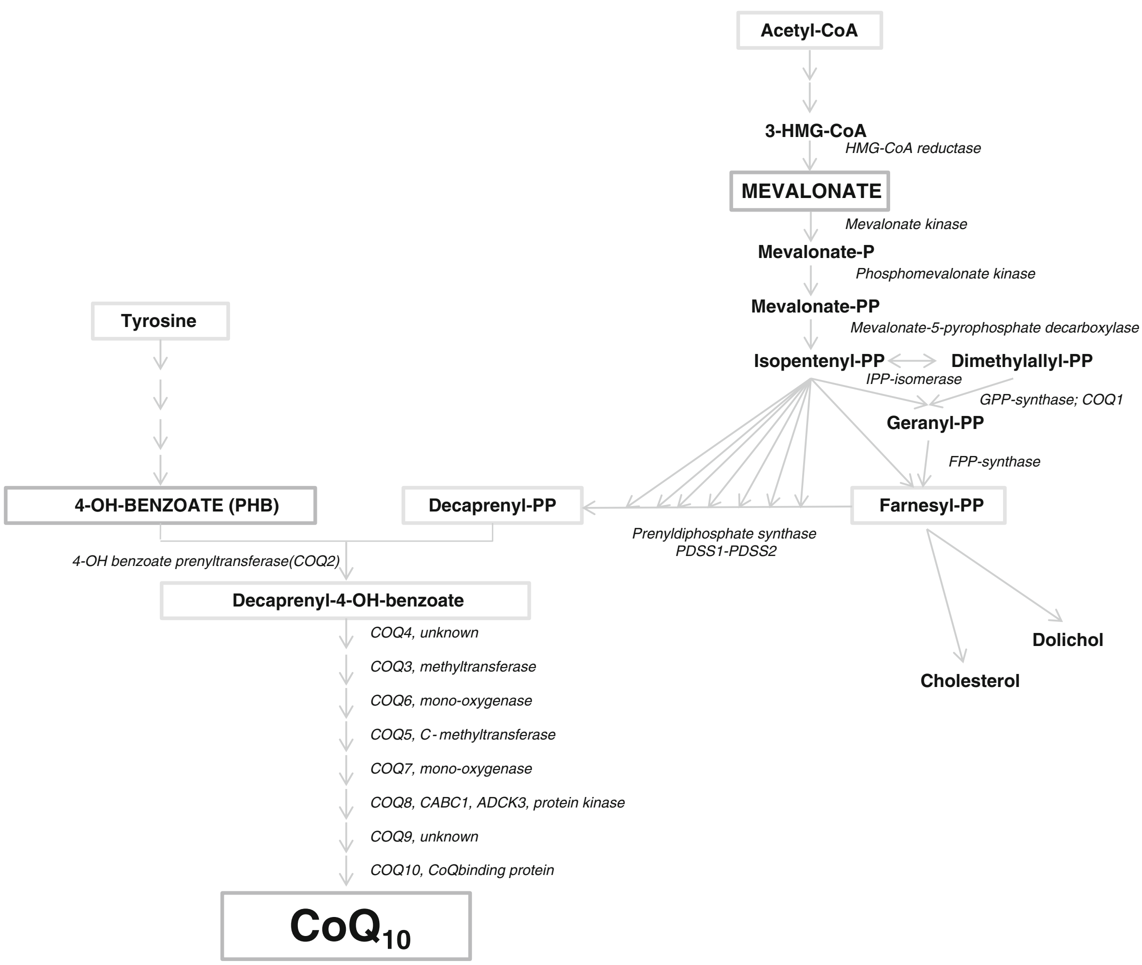

Fig. 1 Biosynthetic pathway of CoQ_{10}. Modified from Dallner and Sindelar (Dallner and Sindelar 2000)

Skeletal muscle is accepted as the tissue of choice for CoQ_{10} evaluation, but obtaining a muscle biopsy is invasive. Less invasive procedures such as obtaining lymphoblastoid cell lines, fibroblasts, or lymphocytes have been used for the diagnosis of CoQ_{10} deficiency (Rahman et al 2012; Montero et al 2008; Arias et al 2012).

For these reasons, our objective was to develop a methodology for the study of the endogenous biosynthesis of CoQ_{10} in fibroblasts that may allow the identification of primary CoQ_{10} deficiencies.

Materials and methods

Reagents

$^{13}C_6$-PHB, $^{2}H_3$-MV, non-labelled PHB, non-labelled MV, cyclodextrine, 5,5′-ditio-bis[−2-nitrobenzoic acid] (DTNB), oxaloacetate, tris(hydroxymethyl)aminomethane (Tris), saccharose, EDTA, coenzyme Q9 (CoQ_9), CoQ_{10}, thiazolyl blue tetrazolium bromide (MTT), sodium dodecyl sulphate (SDS), dimethyl sulfoxide, ammonium bicarbonate and cycloheximide (CHX) were provided from Sigma-Aldrich (Madrid, Spain).

Trypsin was from Thermo Scientific (Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin (10,000 units/mL) and streptomycin (10,000 μg/mL) were from PAA (Pasching, Austria).

DNA and RNA extraction kits, QIAshredder and RNeasy respectively, were from Qiagen (Germany).

All other solvents and chemicals were of analytical or liquid chromatography grade and were obtained from a variety of sources.

Biosynthesis of labelled CoQ_{10} in cultured fibroblasts

Our method was based on the previously reported method for radiolabelled substrates (Tekle et al 2008). Skin fibroblasts were grown in DMEM containing 10 % FBS and 1 % penicillin-streptomycin. After culture, cells were rinsed with phosphate buffered saline (PBS), trypsinized, centrifuged for 10 min at 252×*g* and cultured again in 6 well plates. At 60–70 % confluence the medium was changed for medium containing $^{13}C_6$-PHB or $^{2}H_3$-MV, and incubated for 24, 48 or 72 h. $^{13}C_6$-PHB was tested at 1.65 mmol/L and 3.3 mmol/L and $^{2}H_3$-MV at 14 μmol/L, 28 μmol/L, 42 μmol/L, 56 μmol/L, 112 μmol/L, 140 μmol/L and 280 μmol/L. After incubation, cells were trypsinized and washed twice with saline. Pelleted-cells were resuspended with 300μL of a buffer solution containing 0.25 mmol/L sucrose, 2 mmol/L EDTA, 10 mmol/L Tris and 100 UI/mL heparin, pH 7,4, and sonicated twice for 5 s. These homogenates were used to determine CoQ_{10} biosynthesis, total protein and citrate synthase (CS) activity. For CoQ_{10} determination, 10 μL of CoQ9 (1 μM, as internal standard, IS), and 800_μL of methanol were added to 100 μL of homogenate. The results were expressed in nmol CoQ_{10}/g protein or nmol CoQ_{10}/Unit of citrate syntase (UCS).

Viability test

Viability tests were performed after 24, 48 and 72 h incubation with $^{13}C_6$-PHB or $^{2}H_3$-MV at the concentrations above mentioned. Cells were washed with PBS and were incubated 3 h with 100 μL of MTT solution (0.5 mg/mL MTT in PBS). The purple MTT-formazan products were dissolved in dimethyl sulfoxide and optical densities of the solutions were measured by absorbance at 570 nm in an ELISA plate reader. Cells treated with 0.02 % SDS were used as positive control. Untreated cells correspond to the negative control. Cell viability was expressed as the optical density ratio of the treated cells respect to the negative control (% of control). Experiments were performed in triplicate.

HPLC-MS/MS analysis

CoQ_{10} and $^{13}C_6$-CoQ_{10} or $^{2}H_3$–CoQ_{10} (the two forms of CoQ_{10} synthesized depending on whether the substrate is $^{13}C_6$-PHB or $^{2}H_3$-MV, respectively) were measured by HPLC-MS/MS, as described in Arias et al (2012). The HPLC (Alliance HT 2795, Waters) was equipped with a 2.1×50 mm Symmetry C18 HPLC column (3.5 μm particle size). The mobile phase consisted of 50 % methanol with 5 mM methylamine, 45 % 2-propanol and 5 % water acidified with formic acid (0.5 mL/L), at a flow rate of 0.2 mL/min and isocratic conditions. MS/MS analysis was performed in a Micromass Quattro micro™ (Waters/Micromass, Manchester, UK). The MS/MS was operated in the electrospray positive ion mode with CV and CE of 15 V and 20 eV respectively. The following multiple reaction monitoring (MRM) transitions were selected: m/z 900>203 and 897>197 for $^{13}C_6$-CoQ_{10} or $^{2}H_3$–CoQ_{10} respectively, 894>197 for the physiological CoQ_{10} and 826>197 for CoQ_9 (internal standard). Dwell time for each transition was 200 ms and run-time was 16 min. Nitrogen (at flow rate of 50 L/h) and argon (adjusted to obtain a vacuum of 3×10^{-3} bar) were used as nebulising and collision gas, respectively.

The physiological content of CoQ_{10} for some fibroblast samples and for muscle tissue was determined by HPLC with electrochemical detection as previously described (Montero et al 2008).

Intra-assay precision (CV) was evaluated in six parallel analyses of the same cell culture. To establish the inter-assay variability, one cell line was independently analysed on six different days.

Citrate synthase and protein determinations

CS activity was measured spectrophotometrically according to the method described by Srere (1969), with 0.1 mM DTNB, 0.2 % Triton X100 and 30–50 μg protein in 500 μL total incubation volume. Proteins were quantified using Protein Assay kit (Bio-Rad Laboratories, EEUU) based on the Lowry method.

Subjects

Thirteen control fibroblast cell lines from the repository bank of our hospital were analysed to establish the reference values. In order to validate the methodology, we studied seven patients with a definite diagnosis and CoQ_{10} deficiency in fibroblasts, including one patient homozygous for a *COQ2* (OMIM*609825) mutation (unpublished results) and six patients with other inborn errors of metabolism: multiple Acyl-CoA dehydrogenase deficiency (MADD; OMIM#231680), very long chain Acyl-CoA dehydrogenase deficiency (VLCADD; OMIM#201475), mitochondrial encephalopathy with complex III deficiency and a mtDNA mutation, and Niemann-Pick type C disease (NPC; OMIM#257220) (Table 1). Then, we investigated three further groups of patients (Table 2) with CoQ_{10} deficiency in fibroblasts (and in muscle in some cases) but still with no definite genetic diagnosis: Group 1: three patients with a single mutation in one *COQ* gene; Group 2: five patients responsive to CoQ_{10} supplementation, without mutations in the genes studied; Group 3: eight patients with CoQ_{10} deficiency, without further genetic studies or documented response to treatment. All cell lines were analysed in two independent experiments.

Patients or parents provided informed consent. The study was approved by the Ethics Committee of the Hospital Clinic-Barcelona, Spain. All samples were obtained in accordance with the current revision of the Helsinki Declaration.

Statistical analysis

Statistical analysis was performed using the SPSS version 18.0.0 software. Kolmogorov-Smirnov test was used to check variables which were under a normal distribution. The reference range was calculated as the mean±2 standard deviations. Pearson test was applied to correlate CoQ_{10} biosynthesis between both substrates.

Genetic studies

Prior to the introduction of the present methodology to evaluate CoQ_{10} biosynthesis, incomplete studies of some

Table 1 CoQ_{10} biosynthesis in patients with definite diagnosis and CoQ_{10} deficiency in fibroblasts

Patient	Diagnosis	Mutation	Protein change	CoQ_{10} concentration (nmol/UCS)		Biosynthesis of CoQ_{10} [a]	
				Fibroblasts	Muscle	$^{2}H_3$-MV	$^{13}C_6$-PHB
1	COQ2	c.437G>A and c.437G>A	p.Ser146Asn and p.Ser146Asn [b]	**0.4**	ND	**0.04**	**0.29**
2	MADD-ETFB	c.124T>C and c.604_606delAAG	p.Cys42Arg and p.Lys202del	**1.2**	ND	0.17	0.87
3	MADD-ETFDH	c.779T>C and c.41_42ins14pb	p.Phe260Ser and p.Gln14Hisfs*11	**1.9**	ND	0.13	0.96
4	VLCAD	c.848T>C and c.1748C>T	p.Val283Ala and p.Ser583Leu	**1.9**	ND	0.18	0.89
5	Complex III deficiency	m.3229A ins		**1.7**	**1.8**	0.17	0.91
6	Niemann-Pick Type C	c.2932C>T and c.983T>C	p.Arg978Cys and p.Phe995Leu [c]	**1.3**	ND	0.13	**0.54**
7	Niemann-Pick Type C	c.2746_2748delAAT and c.3451G>A	p.Asn916del and p.Ala1151Thr [c]	**1.5**	ND	0.10	**0.60**
			Control mean (mean±2SD)	2.4 (2.0–2.8)	5.4 (2.7–8.5)	0.13 (0.09–0.17)	0.94 (0.84–1.0)
			n=number of controls	*n*=66	*n*=37	*n*=13	*n*=13

Altered results are outlined in bold. Each individual value is the mean of at least a duplicate determination

ND not done

[a] Biosynthesis of CoQ_{10} was evaluated by measuring the corresponding labelled CoQ_{10} (nmol/UCS) generated both with $^{2}H_3$-MV or $^{13}C_6$-PHB as substrates

[b] Mutation previously described by Diomedi-Camassei et al (2007)

[c] Macías-Vidal et al (2011)

COQ genes had been performed in some of the patients as described beneath. The continuation of those studies was conditioned to the demonstration of an altered biosynthesis in the patients.

Genomic DNA was extracted from blood, skin fibroblasts or tissues using standard protocols, and mutational screening of 13 *COQ* genes (*PDSS1* (OMIM*607429), *PDSS2* (OMIM*610564), *COQ2* (OMIM*609825), *COQ3* (OMIM*605196), *COQ4* (OMIM*612898), *COQ5, COQ6* (OMIM*614647), *COQ8* (OMIM*606980), *COQ9* (OMIM: *612837), *ADCK1, ADCK2, ADCK4* and *ADCK5* was performed using self-designed oligonucleotides.

Total RNA was isolated from cultured fibroblasts using QIAshredder and RNeasy kits and cDNA was synthesized using standard protocols. We also isolated RNA from patients' fibroblasts that had been treated during 7 h with 500 μg/mL CHX, in order to inhibit possible mRNA degradation by nonsense-mediated decay (NMD). Overlapping segments of the *COQ8, PDSS1, PDSS2* and *COQ4* cDNAs were PCR amplified and sequenced.

Patients from group 1 (Table 2) were studied for the following genes: *PDSS1, PDSS2* and, *COQ2-COQ9* in patient 8, and *PDSS1, PDSS2, COQ2, COQ4, COQ5, COQ8* and *COQ9* in patient 9. In addition, cDNA mutational screening was performed for the mentioned four genes in patients 9 and 10.

Concerning patients of group 2, the 13 mentioned genes were screened in their genomic DNAs.

Results

Method setting up and validation

CoQ_{10} biosynthesis in control fibroblasts (Fig. 2a) increased linearly with time (24–72 h) using either 1.65 mmol/L or 3.3 mmol/L $^{13}C_6$-PHB as precursor, and the synthesized $^{13}C_6$-CoQ_{10} amounts were alike at both concentrations. When using $^2H_3$-MV as precursor, $^2H_3$-CoQ_{10} biosynthesis was also linear with time but increased with increasing $^2H_3$-MV from 14 to 56 μmol/L (Fig. 2b). For higher concentrations (112 μmol/L, 140 μmol/L and 280 μmol/L) during 72 h incubation, CoQ_{10} biosynthesis decreased (Fig. 2c). After those results, the elected conditions were 1.65 mmol/L $^{13}C_6$-PHB, 28 μmol/L $^2H_3$-MV, and 72 h incubation.

The quantity of cells grown in the wells and analysed for each experiment was always similar, which is reflected by the protein concentration measured in the preparations (0.63 ±0.07 mg/mL, n=102). Therefore, the assay conditions are comparable between cell lines.

Viability tests demonstrated that $^2H_3$-MV at 28 μmol/L does not affect fibroblasts stability at any incubation time tested and neither was there effect on viability for 1.65 mmol/L $^{13}C_6$-PHB when the incubation time was 24 h or 48 h. And, although the viability decreased slightly (80 % residual) after 72 h incubation, the peak of $^{13}C_6$-CoQ_{10} was threefold the LLOQ (S/N>10) (data not shown).

When incubating with $^{13}C_6$-PHB, inter-assay variability (CV) of the newly synthesized $^{13}C_6$-CoQ_{10} was 19 % if results were normalized to protein content and 10 % if normalized to UCS. When incubating with $^2H_3$-MV, inter-assay CV of the newly synthesized $^2H_3$-CoQ_{10} was 16 % and 13 % related to protein and CS, respectively. Concerning intra-assay CV, when incubating with $^{13}C_6$-PHB it was 10 % when expressed per g protein and 9 % if expressed per UCS; and, when incubating with $^2H_3$-MV, it was 12 % and 8 % per g protein and UCS, respectively. Due to the lower imprecision when normalizing the results to UCS we decided to use it instead of per protein content.

The mean and reference range for $^{13}C_6$-CoQ_{10} and $^2H_3$-CoQ_{10} biosynthesis were 0.94 (0.84–1.0) nmol/UCS and 0.13 (0.09–0.17) nmol/UCS, respectively (Table 1). The correlation between $^{13}C_6$-CoQ_{10} and $^2H_3$- CoQ_{10} biosynthesis in fibroblasts, in the whole group of controls and patients, showed that the two variables tend to increase together and 63 % of the variance was shared between them (Fig. 3c).

Patients studied to validate the methodology are summarized in Table 1. Patient 1 shows significant CoQ_{10} deficiency in fibroblasts is homozygous for a mutation in *COQ2* and presents deficient CoQ_{10} biosynthesis with both substrates ($^{13}C_6$-PHB and $^2H_3$-MV). In contrast, patients 2–5, with CoQ_{10} deficiency in fibroblasts and diagnosis of other inborn errors of metabolism, showed normal biosynthesis.

Fibroblasts from patients 6 and 7, affected with NPC, showed deficient CoQ_{10} and normal biosynthesis with $^2H_3$-MV as precursor, while the rate was decreased using $^{13}C_6$-PHB as substrate (Table 1).

Patients' results

The investigations in patients suspected of primary CoQ_{10} deficiency are summarized in Table 2, Fig. 3a and b. In group 1 only patient 8 (with a mutation in *COQ4*) showed deficient CoQ_{10} biosynthesis. Table 2 also shows the results for patient 8's parents; her father (number 25) gave normal results for both substrates, while her mother's (number24) biosynthesis was at the lower control range when the precursor was $^{13}C_6$-PHB (0.84 nmol/UCS; controls 0.84–1.0 nmol/UCS).

Concerning group 2, all but patient 15 showed low CoQ_{10} biosynthesis, ranging from 0.04 to 0.09 nmol/UCS with $^2H_3$-MV as substrate and from 0.55 to 0.78 nmol/UCS with $^{13}C_6$-PHB (Table 2).

Finally, CoQ_{10} biosynthesis was in the normal range in four patients of group 3. In patients 16, 22 and 23, using

Table 2 CoQ_{10} biosynthesis in patients with CoQ_{10} deficiency in muscle or fibroblasts. Group 1: patients with a single detected mutation in a *COQ* gene. Group 2: patients with good clinical response to CoQ_{10}. Group 3: patients with CoQ_{10} deficiency in fibroblasts, and no genetic studies or documented response to treatment

	Patient	Clinical data [reference]	Mitochondrial Respiratory Chain activities		CoQ_{10} concentration (nmol/UCS)		Biosynthesis of CoQ10[b]		Altered COQ gene/ genotype
			Fibroblasts	Muscle	Fibroblasts	Muscle	$^2H_3$-Mevalonate	$^{13}C_6$-PHB	
Group 1	8	Muscle hypotonia, weakness, psychomotor retardation, rabdomyolysis, elevated CK, fatty acids and ketones	Normal	Multiple deficiencies	**2.6**[a]	**1.2**	**0.06**	**0.54**	*COQ4* p.[Glu161Asp]+[=]
	9	Ataxia	ND	Normal	**2.5**[a]	**1.3**	0.23	0.91	*COQ8* p.[Leu609Val]+[=]
	10	Rabdomyolysis, myopathy; elevated CK	Normal	ND	**1**	ND	0.14	0.99	*PDSS1* p.[Ala380Thr]+[=]
Group 2	11	Ataxia, muscle weakness, cerebellar atrophy [32]	Normal	↓CI+III, ↓CII+III	**0.6**[a]	**2**	**0.04**	**0.63**	NF
	12	Ataxia, psychomotor retardation, myoclonias, cerebellar atrophy [31]	Normal	ND	**1.8**[a]	ND	0.09	**0.77**	NF
	13	Ataxia, psychomotor retardation, myoclonias, cerebellar atrophy [31]	Normal	ND	**0.9**[a]	ND	**0.07**	**0.55**	NF
	14	Ataxia, psychomotor retardation, epilepsy, convulsions [31]	Normal	Normal	**1.8**[a]	**1.6**	**0.08**	**0.78**	NF
	15	Cerebellar atrophy, clumsiness, frequent falls, nistagmus [32]	ND	Normal	**1.2**[a]	7.9	0.14	1.05	NF
Group 3	16	Hepatopathy, lactic and metabolic acidosis 78 % mtDNA depletion [33]	Normal	↓CII+III	**1**	**1.6**	0.15	**0.7**	NF
	17	Psychomotor retardation, ketosis and lactic acidosis	ND	ND	**0.9**	ND	0.12	**0.82**	NF
	18	Ataxia, cerebellar syndrome	ND	↓CI+III, ↓CII+III	**1.1**[a]	3.8	0.14	0.91	ND
	19	Psycomotor retardation, myoclonias, elevated lactate and alanine, corpus callosum hypoplasia	ND	↓CI+III, ↓CII+III, ↓CIV	**2.9**[a]	**1.2**	0.18	1.01	ND
	20	Myopathy with exercise intolerance, high lactate and lactate/pyruvate ratio	Normal	↓CI+III, ↓CII+III	**1.3**[a]	ND	0.12	0.96	ND
	21	Psychomotor retardation, cerebellar atrophy, hypotonia, ataxia, hypertrophic cardiomyopathy	ND	Multiple deficiencies; CS	**2.8**[a]	**1.3**	0.11	0.86	ND
	22	Psychomotor retardation, ataxia, epilepsy, cerebellar atrophy. Retrospectively diagnosed of neuronal ceroid lipofuscinosis type 2	ND	ND	**1.6**	ND	0.12	**0.65**	ND
	23	Psychomotor retardation, convulsions, cerebellar atrophy, epilepsy	ND	↓CI	**1.1**	ND	0.09	**0.64**	ND
Parents	24	Asymptomatic	ND	ND	4.9[a]	ND	0.16	0.84	NF
	25	Asymptomatic	ND	ND	5.2[a]	ND	0.16	1.02	*COQ4* p.[Glu161Asp]+[=]
		Control mean (mean±2SD) n=number of controls			2.4 (2.0–2.8) *n*=66	5.4 (2.7–8.5) *n*=37	0.13 (0.09–0.17) *n*=13	0.94 (0.84–1.0) *n*=13	

ND not done. *NF* not found

[a] Determined by HPLC as previously described [18]

[b] Biosynthesis of CoQ_{10} was evaluated by measuring the corresponding labelled CoQ_{10} (nmol/UCS) generated, both with $^2H_3$-Mevalonate or $^{13}C_6$-PHB as substrates. Altered results are outlined in bold. Each individual value is the mean of at least a duplicate determination

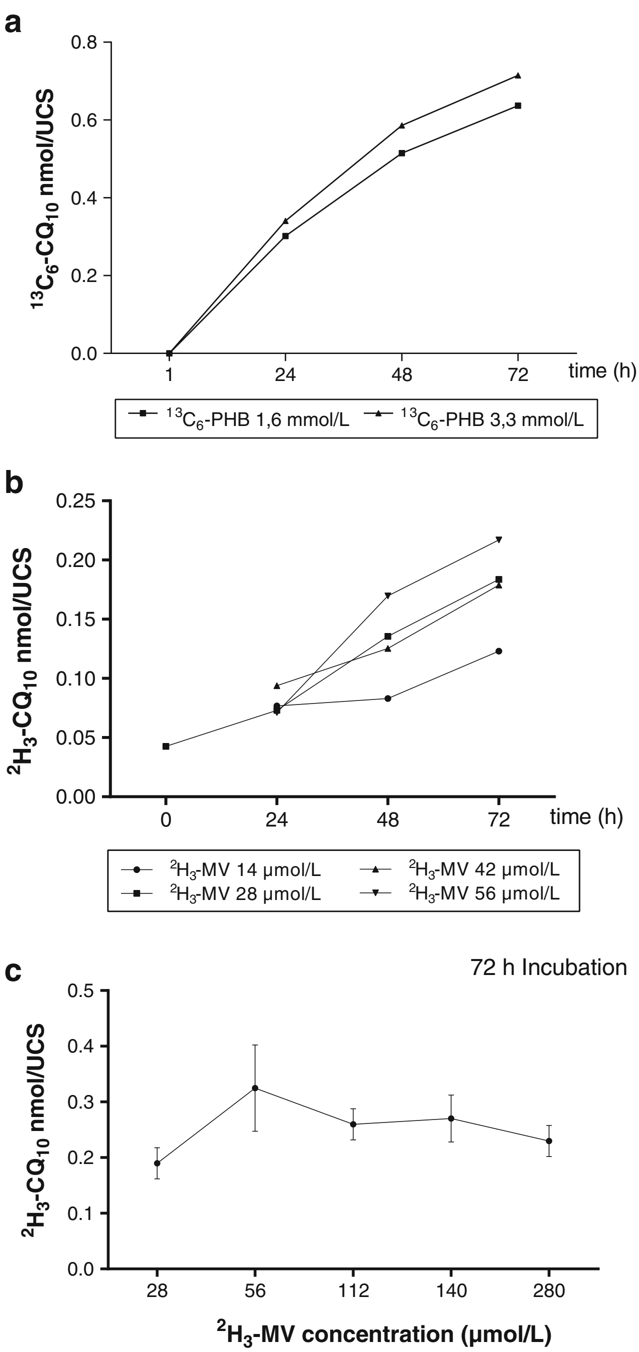

Fig. 2 CoQ_{10} biosynthesis using $^{13}C_6$-PHB and $^{2}H_3$-MV as substrates at different concentrations and periods of incubation. **a** $^{13}C_6$-CoQ_{10} biosynthesis when using $^{13}C_6$-PHB as the precursor, at 1.65 mmol/L or 3.3 mmol/L, it increases linearly with time. **b** $^{2}H_{3-}$ CoQ_{10} biosynthesis when using $^{2}H_3$-MV as the precursor at concentrations 14 to 56 μmol/L, it increases linearly with time. **c** $^{2}H_{3-}$ CoQ_{10} biosynthesis with higher concentrations of $^{2}H_3$-MV (28 μmol/L, to 280 μmol/L) and 72 h incubation; it decreases for concentrations greater than 56 μmol/L

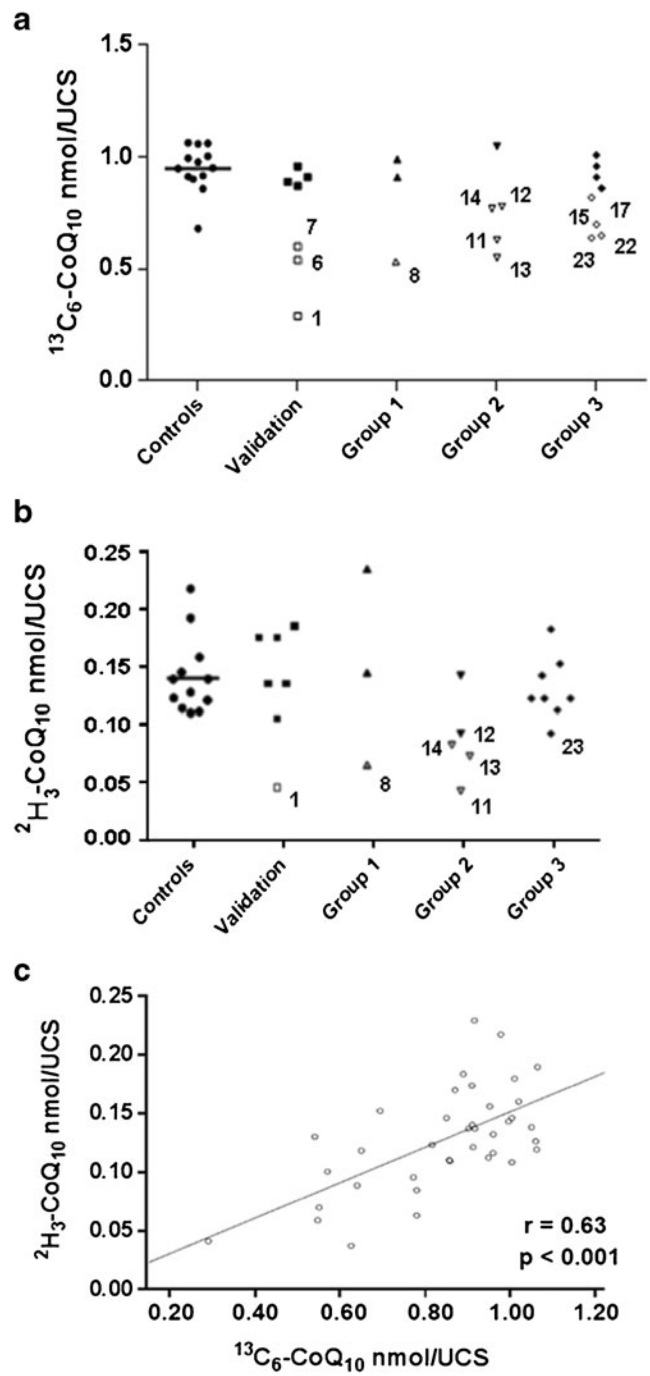

Fig. 3 Graphic representation of CoQ_{10} biosynthesis in controls' and patients' fibroblasts using $^{13}C_6$-PHB and $^{2}H_3$-MV as substrates. **a** $^{13}C_6$-CoQ_{10} biosynthesis. **b** $^{2}H_3$-CoQ_{10} biosynthesis. **c** Correlation between $^{13}C_6$-CoQ_{10} and $^{2}H_3$-CoQ_{10} biosynthesis in fibroblasts (Pearson test, r=0.63; p<0.01). Numbers into the figure represent the corresponding patient in the tables

$^{2}H_3$-MV the amount of $^{2}H_3$-CoQ_{10} synthesized was in the normal range, but with $^{13}C_6$-PHB the biosynthesis was deficient (0.70, 0.65 and 0.64 nmol/UCS, respectively). The same happened with patient 17 though his CoQ_{10} biosynthesis with $^{13}C_6$-PHB was only slightly reduced (0.82 nmol/UCS) (Fig. 3a).

Discussion

Method setting up and validation

We have developed a non-invasive, non-radioactive and sensitive method by HPLC-MS/MS to study the biosynthesis of CoQ_{10} in fibroblasts. Our results indicated that CoQ_{10} biosynthesis increased linearly with time for both substrates, but $^{2}H_{3}$-MV needed higher concentrations. We finally established 1.65 mmol/L $^{13}C_{6}$-PHB, 28 μmol/L $^{2}H_{3}$-MV and 72 h of incubation. The concentration of $^{13}C_{6}$-PHB used was as described by Tekle et al (2008) and for $^{2}H_{3}$-MV it was doubled. Although the conditions for MV were not saturating they were judged adequate to obtain quantifiable peaks of the labelled CoQ_{10} with limited substrate costs and did not significantly affect cells' viability. Correlation studies between $^{13}C_{6}$-CoQ_{10} and $^{2}H_{3}$-CoQ_{10} showed that the biosynthesis of both products tend to increase together (Fig. 3c), for that reason and because they measure different steps in the biosynthesis of CoQ_{10} we maintained the incubation with both substrates.

As expected for primary CoQ_{10} deficiency (Fig. 1), our results showed clearly decreased rates of CoQ biosynthesis in patient 1 either with $^{13}C_{6}$-PHB or $^{2}H_{3}$-MV as substrates (Table 1, Fig. 3a, b). Similar rates of CoQ_{10} biosynthesis were described for a patient harbouring a homozygous mutation in *COQ2* with either ^{14}C-PHB or ^{3}H-decaprenyl-diphosphate (Quinzii et al 2006). As expected, the rates of biosynthesis were normal for patients 2–5 with CoQ_{10} deficiency secondary to other inborn errors of metabolism (MADD, VLCAD and complex III deficiency). MADD is caused by defects in components of flavin metabolism or transport (Frerman and Goodman 2001) that, in mitochondria, mediates the transfer of electrons from flavin to ubiquinone and the RC. Mutations in *ETFDH*, encoding for electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO), have been reported to cause secondary CoQ_{10} deficiency (Gempel et al 2007; Liang et al 2009). We also studied fibroblasts from a patient with VLCADD. The association between VLCADD and CoQ_{10} deficiency had previously been noted in one patient (Laforêt et al 2009). Finally, some patients with mtDNA deletions or mt-tRNA point mutations may show secondary CoQ_{10} deficiencies (Rahman et al 2012; Sznajer et al 2007; Matsuoka et al 1991), as happens with patient 5, with deficient complex III and a homoplasmic mutation in mtDNA. All the mentioned diseases are mitochondrial dysfunctions that may hypothetically increase degradation of CoQ_{10} or decrease its ATP-dependent transport in some patients, but the actual mechanism of the secondary deficiency in all these conditions remains unknown (Rahman et al 2012).

Additionally, to validate our method, we have studied patients 6 and 7 affected with NPC (Macías-Vidal et al 2011). Previous authors (Schedin et al 1998) had observed that the accumulation of cholesterol in a NPC murine model was paralleled by increased dolichol-P and decreased CoQ_{10} concentrations. The mevalonate pathway involves condensation of three molecules of acetyl-CoA to 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA). HMG-CoA reductase then converts HMG-CoA to mevalonate (Fig. 1). Cholesterol regulates HMG-CoA reductase by feedback inhibition. In NPC, accumulation of cholesterol may down-regulate HMG-CoA reductase decreasing mevalonate and, consequently, CoQ_{10} synthesis (Turunen et al 2004). Accordingly, patients 6 and 7 showed normal CoQ_{10} biosynthesis with $^{2}H_{3}$-MV as precursor of the pathway, while it was decreased using $^{13}C_{6}$-PHB, as expected if the MV pathway was partially inhibited (Table 1, Fig. 3a, b). In these cases we tried to improve CoQ_{10} biosynthesis by adding non labelled mevalonate in the $^{13}C_{6}$-PHB media but, surprisingly, no amelioration was obtained. Further experiments should investigate this lack of response.

Biosynthesis of CoQ_{10} in patients' fibroblasts

As the previous results indicated that our method was suitable for recognizing alterations of CoQ_{10} biosynthesis, we applied it to different patients with decreased CoQ_{10} in fibroblasts (Table 2, Fig. 3a and b).

The three patients of group 1 carry a mutation in one allele in one of the *COQ* genes; we failed to detect a second mutation even after study of cDNA extracted from CHX treated fibroblasts. Only patient 8 (with a mutation in *COQ4)* showed deficient CoQ_{10} biosynthesis. Deficient CoQ_{10} content with diminished biosynthetic rate in cultured fibroblasts has been reported in a patient with haploinsufficiency of *COQ4* (Salviati et al 2012). As the father of patient 8 also carries the mutation, we studied the biosynthesis in both parents. Results were normal for her father (number 25), while the mother's rate (number 24) was at the lower control range with $^{13}C_{6}$-PHB as substrate. Therefore, the *COQ4* mutation in our patient may not be enough to reduce CoQ_{10} production. Our observations might be hypothetically explained by an additional maternal mutation in an unknown point of the pathway that lowers the rate of synthesis without clinical effect in heterozygotes. Consequently, patient 8 would carry two genetic alterations (the mutation in *COQ4* and that hypothetical mutation) that together cause the deficiency. Whole exome sequencing is on course in this family. The other two patients of group 1 (patients 9 and 10) showed normal RC activities and CoQ biosynthesis. We may conclude that these patients' CoQ_{10} deficiency is secondary and that their heterozygous mutations are not the cause of the deficiency.

Therefore, a normal CoQ_{10} biosynthesis rules out a primary defect but, as exemplified with patients of group 2, the opposite is not always true. In fact, all patients of this group but patient 15 showed deficient CoQ_{10} biosynthesis. However, screening for COQ genes failed to detect pathological changes. Patient 15 is the father of patient 11; they have previously been described (Pineda et al 2010; Artuch et al 2006). He presented slight clinical alterations including action tremor, mild modification of fluency, transient nystagmus and slight saccadic pursuit that were corrected with treatment. CoQ_{10} levels were low in his fibroblasts, but muscle CoQ_{10} and RC activities, as well as fibroblast CoQ_{10} biosynthesis, were normal. In contrast, his daughter (patient 11), with a more severe ataxia and biochemical alterations, showed clearly deficient CoQ_{10} biosynthesis (Artuch et al 2006). The disease in this family may be caused by a heterozygous mutation in an unidentified gene that causes mild alterations as seen in the father while the more severe disease in the girl is due to homozygosity (or compound heterozygosity). We cannot exclude that their CoQ_{10} deficiency is secondary and, in this case, it might only be a modulator of the phenotype, exacerbating the clinical picture in the daughter.

Four patients of group 3 presented CoQ_{10} biosynthesis in the normal range. Therefore, they are most probably secondary CoQ_{10} deficiencies. Conversely, patient 23's biosynthesis was in the lower control range with $^{2}H_{3}$-MV and decreased with $^{13}C_{6}$-PHB. We could infer that this patient's deficiency is primary, and mutations in *COQ* genes should be investigated. Results in patients 16 (Montero et al 2009), 17 and 22 are difficult to conclude because the amount of CoQ_{10} synthesized using $^{2}H_{3}$-MV is in the normal range, but with $^{13}C_{6}$-PHB, the biosynthesis is slightly low or deficient. This points to some altered or inhibited step in the biosynthesis of mevalonate, as observed in NPC disease. In fact, patient 22 has recently been diagnosed with neuronal ceroid lipofuscinosis type 2 (OMIM#204500). To our knowledge, the relationship between this disease and CoQ_{10} deficiency had not been reported previously and should be further investigated.

In conclusion, we have developed a non-invasive non-radioactive method suitable for the detection of defects in CoQ_{10} biosynthesis, which offers a good tool for the stratification of these treatable mitochondrial diseases. Additionally, our method might be of interest to study unknown aspects about the subcellular turnover of newly synthesized CoQ.

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Conflict of interest None.

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