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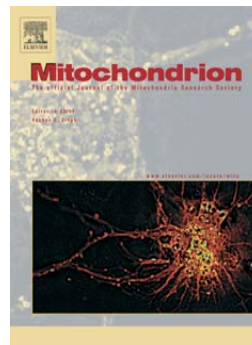
Coenzyme Q<sub>10</sub> deficiency in mitochondrial DNA depletion syndromes

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**Coenzyme Q<sub>10</sub> deficiency in mitochondrial DNA depletion syndromes.**

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**Abstract**

We evaluated coenzyme Q<sub>10</sub> (CoQ) levels in patients studied under suspicion of mitochondrial DNA depletion syndromes (MDS) (n=39). CoQ levels were quantified by HPLC, and the percentage of mtDNA depletion by quantitative real-time PCR. A high percentage of MDS patients presented with CoQ deficiency as compared to other mitochondrial patients (Mann-Whitney-U test: p= 0.001). Our findings suggest that MDS are frequently associated with CoQ deficiency, as a possible secondary consequence of disease pathophysiology. Assessment of muscle CoQ status seems advisable in MDS patients since the possibility of CoQ supplementation may then be considered as a candidate therapy.

**Keywords**

Mitochondrial DNA depletion syndrome; coenzyme Q<sub>10</sub> deficiency; mitochondrial disorders.

**Abbreviations**

Coenzyme Q<sub>10</sub> (CoQ); mitochondrial DNA depletion syndromes (MDS); high pressure liquid chromatography (HPLC); mitochondrial DNA (mtDNA); mitochondrial respiratory chain (MRC); citrate synthase (CS);

## 1. Introduction

Mitochondrial DNA depletion syndromes (MDS) are a heterogeneous group of disorders characterized by low number of mitochondrial DNA (mtDNA) copies in different tissues (Spinazzola, 2011). These syndromes are frequently associated with severe infant and childhood mitochondrial respiratory chain (MRC) deficiencies and patients may present with different phenotypes. Myopathic (OMIM: 609560), encephalomyopathic (OMIM: 612073 and 612075) and hepatocerebral (OMIM 251880) are common forms, although a wide range of clinical presentations are being profiled (Nogueira et al., 2011; Rotig and Poulton, 2009; Suomalainen and Isohanni, 2010). MDS have been related to impaired mtDNA replication (mutations in *POLG1* and *C10orf2/PEO1* genes), to altered mitochondrial metabolism of deoxynucleotide pools (mutations in *TK2*, *DGUOK*, *MPV17*, *RRM2B*, *SUCLA2*, *SUCLG1* and *TYMP* genes) (Nogueira et al., 2011; Rotig and Poulton, 2009; Suomalainen and Isohanni, 2010). However, in a high percentage of the cases the aetiology of the disease remains to be elucidated (Alberio et al., 2007).

Coenzyme Q<sub>10</sub> (CoQ) is a mobile molecule that acts as an electron carrier in the MRC transferring electrons from complex I and complex II to complex III (Ernster and Dallner, 1995). It is also a cofactor for several mitochondrial dehydrogenases, including dihydroorotate dehydrogenase (EC 1.3.3.1), an enzyme involved in pyrimidine biosynthesis. A link between CoQ deficiency and impaired pyrimidine biosynthesis has previously been demonstrated (Lopez- Martín et al., 2007), although only anecdotic reports have studied CoQ status in MDS patients (Montero et al., 2009). Mutations in 6 genes involved in CoQ biosynthetic pathway have been reported in association with a range of clinical phenotypes: mutations in *COQ2*, *PDSS1*, *PDSS2* and *COQ4* have been reported in cases of severe infantile multisystemic disease (Quinzii et al., 2010;

Sacconi et al., 2010; Salviati et al., 2012), mutations in the *ADCK3* gene have been associated with cerebellar ataxic form of CoQ deficiency (Langier-Tourenne et al., 2008) and a mutation in the *COQ6* gene that causes nephrotic syndrome with sensorineural deafness (Heeringa et al., 2011). Interestingly, several studies have reported the presence of a secondary CoQ deficiency in diseases such as ataxia with oculomotor apraxia (Quinzii et al., 2005) and glutaric aciduria type II (Gempel et al., 2007). CoQ deficiency has also been frequently detected in mitochondrial disorders (DiMauro et al., 2007; Miles et al., 2008; Sacconi et al., 2010, Quinzii et al., 2011, Emmanuele et al., 2012). In our global experience, CoQ deficiency is present in a similar percentage of patients with mitochondrial disorder to that reported in the study by Montero et al., 2005. Furthermore, secondary CoQ deficiency may be very difficult to differentiate from that of primary deficiency, since in most cases the molecular basis of the disease remains elusive (Emmanuele et al., 2012).

The aim of the present study was to evaluate muscle and liver CoQ levels in a cohort of patients with a clinical phenotype suggestive of MDS.

## **2. Methods**

### **2.1 Patients:**

During the last 3 years, we have recruited 39 patients (age range: 1 month – 24 years; average 2.6 years) with the following inclusion criteria: clinical phenotype suggestive of MDS; no CoQ supplementation therapy at the time of the biopsy; biochemical, histopathological and/or genetic evidence of a mitochondrial disease according to previously established criteria for diagnosis of mitochondrial disease (Bernier et al., 2002). 12 patients were from Coimbra, 4 patients from London and 23 patients from Barcelona. The patients were classified in 2 groups.

Group 1: 14 patients (age range: 1 month – 2 years; average 0.5 years) with the diagnosis of MDS (percentage of mtDNA depletion greater than 70%): 8 patients presented with decreased mtDNA copy number in muscle, 5 patients in liver and 1 patient in brain.

Group 2: 25 patients (range age: 1 month – 24 years; average 3.1 years) with a clinical suspicion of a MDS (they presented with hepatocerebral involvement (n=5), encephalopathy (n=11) and myopathy plus encephalopathy (n=8) who did not show mtDNA depletion in the tissues studied (muscle or liver). The main laboratory findings of both groups of patients are stated in tables I and II, and the clinical details of the MDS patient group are outlined in table II. From the 39 patients, we investigated 30 muscle biopsies, 8 liver biopsies and 1 brain tissue collected after necropsy.

## 2.2 Laboratory studies:

MRC and citrate synthase (CS) enzyme activities were determined by spectrophotometric enzyme assays in muscle biopsies as previously reported (Rustin et al., 1994; Grazina, 2012). We assessed MRC enzyme activities corrected for citrate synthase activity, and MRC deficiencies were considered as described by Grazina et al., 2012 and Bernier et al, 2002.

Muscle and liver CoQ levels were determined by reverse-phase high pressure liquid chromatography (HPLC, Waters, MA, USA) with electrochemical detection (ED; Coulochem II, ESA, MA, USA) (Montero et al., 2008). Briefly, CoQ was separated on a nucleosil C-18 column (5  $\mu$ m, 25 $\times$ 0.4 cm, Teknokroma, Barcelona, Spain). Mobile phase consisted of 20 mmol/L of lithium perchlorate in ethanol/methanol (40/60; v/v). ED cells were attached at –600 mV (conditioning cell, Model 5021) and +600 mV (analytical cell, Model 5010). The muscle CoQ reference interval was established from 37 paediatric patients with no clinical presentation of muscle disease (age range 2-16 years; average 9.2 years). Details of the establishment of this reference interval have

been previously reported (Montero et al., 2008). The muscle CoQ status of patients from the UK was determined by HPLC with UV detection at 275 nm (Jasco UV 975 detector, Jasco, UK) (Duncan et al., 2005). CoQ was separated on a (Techsphere ODS 5 $\mu$ , 150 x 4.6mm) HPLC column. The mobile phase consists of ethanol: methanol: 60 % (v/v) perchloric acid; 700:300:1.2 (v:v) to which is added 7 g of sodium perchlorate]. For the HPLC-UV procedure, the muscle CoQ reference interval was established from 20 patients with no clinical/biochemical evidence of muscle disease or MRC dysfunction (age range 0.6-18 years; average 5.6 years), as previously reported in the study by Duncan et al., 2005.

To establish the liver CoQ reference interval liver samples were collected following necropsy from 7 paediatric patients (age range 1 day-1 year, average 3 months) with no clinical/biochemical evidence of MRC disorder and analyzed by HPLC with ED. The results of CoQ determinations were expressed as nmol/gram of total protein content as measured by the Lowry et al., method (1951). All HPLC determinations were undertaken with internal quality controls to ensure continuity between analyses.

Recently, we have initiated a pilot quality control scheme between the London and Barcelona laboratories, with a good agreement in the determination of CoQ levels being reported (results available on request).

The mtDNA content was measured by quantitative real-time PCR according to the method of Marcuello et al 2005, Ashley et al. 2008 and Navarro-Sastre et al 2012.

Briefly, the analysis was performed in a Step One Plus real-time PCR system (qRTPCR; PE7500 real-time PCR instrument; Applied Biosystems, Foster City, CA, USA). This method is based on the amplification of the mitochondrial 12S rRNA and m.3130-3301 and the quantity of mtDNA was corrected by simultaneous measurements of a single copy of nuclear RNaseP and APP genes. PCR was performed in a final reaction volume of 20  $\mu$ l with 2 mM of MgCl<sub>2</sub>, 0.5  $\mu$ M each of the mitochondrial



probes (5'-[6FAM]tgccagccaccgcg[BHQ1]-3'), forward and reverse primers (forward primer: 5'-ccacgggaaacagcatgtatt-3', reverse primer: 5'-ctattgactgggtaatcgtgtga-3'), 1  $\mu$ l of TaqMan® RNaseP, 25 ng of DNA and 10  $\mu$ l of TaqMan® Gene Expression Master Mix (Applied Biosystems).

For mtDNA depletion assessment, evidence of mitochondrial DNA depletion was considered when the level of mtDNA was <30% of age matched controls, as described by Rahman et al. (2009). For samples analyzed in Spain, the reference values for mtDNA were established from the 37 muscle biopsies that were used to establish the CoQ reference interval by HPLC assessment and liver biopsies from 6 newborn patients with no clinical/biochemical evidence of a MRC disorder. For samples analysed in the UK muscle mtDNA reference values were established from 2 age groups of patients: (1) Aged less than 2 years: 9 individuals with no clinical/biochemical evidence of a MRC disorder (age range 0.1-1.5 years; mean 0.54); (2) Aged greater than 2 years: 7 individuals with no clinical/biochemical evidence of a MRC disorder (age range 2-24 years; mean 10.4). For samples analyzed in Portugal, the mtDNA reference values were established from 24 control liver biopsies (age range 1 – 21 years) and 261 control muscle samples (age range 1 – 21 years) as previously reported in the study by Dimmock et al, 2010.

Genetic analyses of candidate genes associated with MDS (*POLG1*, *DGOUK*, *MPV17*, *RRM2B*, *TK2*, *C10orf2*, *SUCLG1* and *SUCLA2*) were performed or are under investigation in all patients, belonging to group 1. In group 2, molecular analyses in mitochondrial or nuclear DNA were performed according to the clinical and biochemical phenotype of the patients. Briefly, the mtDNA ATPase gene was sequenced in 2 patients, the mtDNA cytochrome b was sequenced in 2 patients, and 32 different mtDNA point mutations were also assessed in 3 patients. Mitochondrial DNA

rearrangements were investigated in muscle biopsies from 3 patients from the UK patients, 9 from Portugal and 5 patients from Spain, all with negative results.

### 2.3 Ethical issues:

Patients and their parents signed informed consent for genetic studies. The study was approved by the Ethical Committee of the different centres involved in this study and tissue samples from patients and controls were obtained according to the Helsinki Declarations of 1964, as revised in 2001.

### 2.4 Statistical analysis:

The Mann-Whitney-U test was applied to compare muscle CoQ values in patients from groups 1 and 2. The Chi square test was used to search for an association between categorical variables (the presence of MDS, CoQ deficiency and MRC enzyme defects). The Spearman test was used to establish correlations between CoQ muscle content, citrate synthase activity and % mtDNA depletion in the 39 patients. Statistical significance was considered as  $p < 0.05$ . Calculations were performed with the SPSS 20.0 program.

## 3. Results

### 3.1 Biochemical and molecular studies

The results of the biochemical and genetic investigations in muscle and liver biopsies from the whole group of patients are shown in table 1. The biochemical, genetic and clinical details of the 14 patients diagnosed with MDS are outlined in table 2. In 5 out of the 14 patients with MDS (Group 1), mutations in *SUCLA* (n=1), *MPV17* (n=1) and *DGOUG* (n=3) genes were detected (Navarro-Sastre et al., 2012, Tavares et al., 2013). The remaining 9 patients with MDS are still under investigation for an underlying genetic cause (no mutations have been found in 6 patients selected for *POLG* analysis). In 1 of the 25 patients with no MDS (Group 2), a novel mtDNA mutation in tRNA has

recently been identified (O'Callaghan et al., 2012), while the other patients in this group are still under investigation.

### 3.2 Statistical findings

Significant differences were observed when a comparison was made between muscle CoQ status and CS activity in patients from groups 1 and 2 (Mann-Whitney-U test:  $p=0.001$  and  $p=0.003$  respectively). The Chi square test indicated that a decreased CoQ status in muscle was associated with the diagnosis of mtDNA depletion (table 3). In deed, 75% patients with mtDNA depletion presented with a decreased level of muscle CoQ (range: 4 – 82 nmol/g prot; average: 53). In contrast, only 21% patients with no MDS showed decreased muscle CoQ levels (range: 74 – 106 nmol/g protein; average: 91). CS and complex IV activities in muscle were also associated with the diagnosis of mtDNA depletion (table 3). This association was not observed when other MRC enzyme activities (complex I+II and II+III) that were assessed in patients from groups 1 and 2 ( $X^2 = 0.491$   $p=0.483$ ;  $X^2 = 0.778$ ,  $p=0.378$  respectively). Finally, the Spearman test revealed a high significantly positive correlation between muscle CoQ status and CS activity ( $r=0.708$ ;  $p<0.0001$ ) and a negative correlation between muscle CoQ status and the % mtDNA depletion ( $r= -0.597$ ;  $p<0.001$ ). CS activity was also found to be negatively correlated with the % mtDNA depletion ( $r= -0.561$ ;  $p<0.0001$ ).

In the liver samples, no differences were observed in CoQ levels between groups 1 and 2, and only 1 patient with MDS was found to have a liver CoQ status (132 nmol/g) below the reference interval (table 2).

## 4. Discussion

To our knowledge, no previous studies have determined the CoQ status in patients with MDS, besides a single case study (Montero et al., 2009). In the MDS cohort we found a higher percentage of patients presenting with CoQ deficiency in comparison to the cohort of patients with other mitochondrial diseases investigated in the present study or reported in previous studies (DiMauro et al., 2007; Emmanuele et al., 2012; Miles et al., 2008; Montero et al., 2005; Quinzii et al., 2011; Sacconi et al., 2010;). Furthermore, quantitatively, CoQ deficiency was more severe in MDS patients compared with the levels of CoQ in patients with other mitochondrial disorders. Nevertheless, the deficit in CoQ status might be a secondary consequence of disease pathophysiology since it has been proposed that non-specific inhibition of the CoQ biosynthetic pathway or increased CoQ degradation may occur in the context of generalized mitochondrial dysfunction (Sacconi et al., 2010). In Deed, in a study by Yen et al., 2011 CoQ levels were found to be lower in 143B-rho0 cells (with no mtDNA) compared to that in 143B cells. The authors concluded that the mitochondrial energy deficiency resulted in this secondary CoQ deficiency possibly as the result of impaired import of COQ proteins into the mitochondria. Furthermore, the possibility arises that some as yet unknown genetic factor may confer susceptibility to develop secondary a CoQ deficiency since not all patients with MDS were found to have a deficit in CoQ10 status. In addition, the degree of the diminution in CoQ status was found to be variable in our series of patients with MRC disorders as has also been reported in other studies (Miles et al., 2008; Montero et al., 2005; Sacconi et al., 2010). In view of the involvement of CoQ in pyrimidine biosynthesis, where the reduced form of CoQ, ubiquinol plays as an essential cofactor role for dihydroorotate dehydrogenase, (Lopez-Martín et al., 2007), the possibility arises that a deficit in CoQ status may contribute to MDS due to a perturbation of pyrimidine synthesis. Among the patients examined in the present study,

a good candidate would be case 11, who presented with a severe neonatal encephalopathy associated with MDS and a profound CoQ deficiency. However, in this case CoQ-related genes were sequenced but no pathogenic mutations were detected (data not shown). Moreover, to our knowledge no evidence of mtDNA depletion has been reported in patients with primary genetic CoQ deficiency, although the close relationship between CoQ status and pyrimidine biosynthesis deserves further investigation. Unfortunately, in the preponderance of patients with MDS the definite molecular diagnosis remains elusive. An investigation into the molecular basis of MDS in these patients will be essential to understand the association between CoQ deficiency and mtDNA depletion.

Classically, primary CoQ deficient patients present with a reduction in the activities of both MRC complexes I + III and II + III, since the activities of these enzymes is dependent on endogenous CoQ (Ogasahara et al., 1989). A proportion of our MDS CoQ deficient patients presented with a generalized MRC defect as expected, but not with a specific reduction in the activities of the CoQ-dependent MRC complexes. These phenomena may be explained by the fact that the global loss of MRC enzyme complex activities may mask the specific reduction of I + III or II + III activities associated with CoQ deficiency (Sacconi et al., 2010). Interestingly, decreased MRC complex IV activity appears to be associated with CoQ deficiency as indicated by the Chi-Square test. However, this finding may be explained by a primary loss of MRC complex IV activity in MDS (COX negative fibers are usually observed in the myopathic form of MDS; Nogueira et al., 2011; Spinazzola, 2011).

Interestingly, a significant correlation was determined between mtDNA depletion and CoQ status. This correlation reinforces the hypothesis that a CoQ deficiency may be involved in the pathophysiology of MDS. Furthermore, CoQ levels were also associated

with CS activity, a well known marker of mitochondrial number and volume (Montero et al., 2008). This association enforces the possibility that as has been reported for CS activity (Navarro-Sastre et al., 2012), that the CoQ deficiency associated with MDS may be a secondary consequence of a reduction in mitochondrial number and volume. CoQ deficiency is a treatable condition (Trevisson et al., 2011). However, the use CoQ therapy in the treatment of MRC disorders has not generally been found to be efficacious. In the Sacconi (2010) series of patients with MRC disorders, some patients with myopathy were found to respond positively to CoQ supplementation. This supports the hypothesis that an early identification of CoQ deficiency may improve the therapeutic potential of CoQ supplementation in the treatment of this disorder (even in secondary CoQ deficiencies), especially in the mild clinical phenotypes. Unfortunately, in a preliminary study (results not shown) we found no evidence of clinical improvement in patients with MDS and CoQ deficiency following CoQ supplementation in contrast to that observed for patients with primary CoQ deficiencies (Quinzii and Hirano, 2011). However, further work will be required before this can be confirmed or refuted.

## **5. Conclusions**

In conclusion, in this study we have found evidence of an association between CoQ deficiency and MDS. In the absence of a definite molecular diagnosis in most cases, we cannot at present determine whether a CoQ deficiency is a primary or a secondary factor in the disease pathophysiology of MDS. However, the results of the present study indicate that a CoQ deficiency may be a secondary consequence of mitochondrial DNA depletion.

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Table 1. Biochemical and molecular data of the 39 patients under the suspicion of MDS. Data are expressed as range, average (SD), and median.

		% mt DNA depletion	CoQ (nmol/g)	Patients with impaired MRC
Muscle	<b>Group 1: mtDNA depletion (n=8)</b>	75 – 99 85 (10), 86	4 - 151 75 (47), 69	↓ CI + III (n=1) ↓ CII + III (n=2) ↓ CII (n=2) ↓ CIII (n=3) ↓ CIV (n=5)
	<b>Group 2: no mtDNA depletion (n= 22)</b>	4.1 – 66 37 (21), 36	74 – 319 165 (65), 148	↓ CI + III (n=5) ↓ CII+III (n=11) ↓ CII (n=7) ↓ CIII (n=10) ↓ CIV (n=9)
	<b>Reference values</b>	< 70%	<b>121 – 451*</b> <b>229 (105)</b> <b>140-580**</b> <b>241 (95)</b>	<b>n.a.</b>
Liver	<b>Group 1: depletion (n=5)</b>	74 – 96 88 (8), 90	223 – 617 419 (19), 418	n.a
	<b>Group 2: no depletion (n=3)</b>	6.1 – 38 % 29 (19), 38	312 – 464 398 (78), 420	n.a.
	<b>Reference values</b>	< 70%	<b>220 – 920*</b> <b>614 (228)</b>	<b>n.a</b>

Case 13, patient with pathologic % mtDNA depletion in brain, was excluded of this table.

\* Reference interval for muscle CoQ determined by HPLC-electrochemical detection.

\*\* Reference interval for muscle CoQ determined HPLC-UV detection.

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Table 2. Clinical, biochemical and molecular details of 14 patients diagnosed with MDS.

Patients

	Sample	% depletion	CoQ (nmol/g protein)	Citrate synthase nmol/min.mg prot.	MRC deficient complexes	Clinical picture
Case 1	Muscle	99 %	129	95	CII+III and CIV	Miclonus epilepsy, myopathy, hypotonia, developmental delay and nistagmus.
Case 2	Muscle	73 %	50	76	CIV	Pretrem boy with congenital lactic acidosis and sepsis. Dead by cerebral haemorrhage and renal failure.
Case 3	Liver	96 %	617	212	CII+III, II, IV	Liver failure and nistagmus. Pathogenic mutation in <i>DGUOK</i> gene c.677A>G in homozygosity.
Case 4	Liver	90 %	552	101	CIV	Nistagmus, cholestasis, liver failure. Pathogenic mutation in <i>DGUOK</i> gene: c.749T>C in homozygosity.
Case 5	Liver	91 %	223	114	CII+III, II, IV	Nistagmus, cholestasis, liver failure. Pathogenic mutation in <i>DGUOK</i> gene c.677A>G in homozygosity.
Case 6	Liver	89 %	285	103	CII+III, II, IV	Lactic acidosis and liver insufficiency with cirrhosis. Multiorgan involvement.
Case 7	Muscle	75 %	46	63	CI+III, II+III	Multiorgan failure, neonatal encephalopathy.
Case 8	Muscle	85 %	71	98	CI+III, II+III, II, III,IV	Multiorgan failure, neonatal encephalopathy.
Case 9	Muscle	87 %	82	121	CI+III, II, III, IV	Neonatal encephalopathy, dystonia. Pathogenic mutation in <i>SUCLA2</i> gene: c.[1048G>A] and [1049G>T]



Case 10	Muscle	90 %	67	29	CII+III	Miopathy, hypotony, mental retardation.
Case 11	Muscle	93 %	4	9	CI+III, II+III, IV	Multiorganic failure, neonatal encephalopathy.
Case 12	Muscle	75 %	151	Not done	Not done	Hypotonia, failure to thrive, delayed development and hypothyroidism. <i>MPV17</i> mutation detected: c.[461+G>C]hom.
Case 13	Muscle	60 %	131	88	No deficiencies	72% depletion cortex frontal, 60% hippocampus. Liver failure, neonatal encephalopathy.
Case 14	Liver	74%	132	109	No deficiencies	Liver and brain involvement.
Reference values	Muscle Liver	< 70 % < 70 %	121 – 451* 140-580" 220 – 920	71-200		

\* Reference interval for muscle CoQ determined by HPLC-ED

"Reference interval for muscle CoQ determined HPLC-UV

**Table 3.** Chi-Square test results of muscle CoQ, citrate synthase and MRC complex IV activity from patients with MDS (group 1) compared with patients with no MDS (group 2). Decreased CoQ status, CS and MRC complex IV activities in muscle were associated with the diagnosis of mtDNA depletion. Decreased CoQ levels were defined as those which were below the lowest limit of each reference interval depending on the method applied (121 nmol/L for HPLC-ED and 140 nmol/L for HPLC-UV).

<b>Muscle</b>	<b>Group 1</b> (%mtDNA < 30%)	<b>Group 2</b> (%mtDNA > 30%)	<b>Chi Square</b>
Decreased CoQ values	6/8	5/24	$X^2 = 7.804$ , p = 0.005
Decreased citrate synthase activity	3/8	1/24	$X^2 = 7.219$ , p = 0.007
Decreased MRC complex-IV activity	5/8	6/22	$X^2 = 7.565$ , p = 0.006

### Highlights

Muscle CoQ deficiency detected in mitochondrial DNA depletion. CoQ deficiency is probably secondary to mitochondrial DNA depletion. Association between CoQ10 deficiency and mitochondrial DNA depletion requires investigation.

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