The dilemma of diagnosing coenzyme Q_{10} deficiency in muscle

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Highlights

- We quantified CoQ10 in 600 xg muscle supernatants in an ethnically diverse cohort.
- A central 95% reference interval is reported.
- CoQ10 was normalized to citrate synthase or protein.
- The normalization strategies showed different diagnostic yield of CoQ10 deficiency.
- We recommend normalization of muscle CoQ10 on both citrate synthase and protein.

ABSTRACT

Background: Coenzyme Q_{10} (CoQ_{10}) is an important component of the mitochondrial respiratory chain (RC) and is critical for energy production. Although the prevalence of CoQ_{10} deficiency is still unknown, the general consensus is that the condition is under-diagnosed. The aim of this study was to retrospectively investigate CoQ_{10} deficiency in frozen muscle specimens in a cohort of ethnically diverse patients who received muscle biopsies for the investigation of a possible RC deficiency (RCD).

Methods: Muscle samples were homogenized whereby 600 x *g* supernatants were used to analyze RC enzyme activities, followed by quantification of CoQ_{10} by stable isotope dilution liquid chromatography tandem mass spectrometry. The experimental group consisted of 156 patients of which 76 had enzymatically confirmed RCDs. To further assist in the diagnosis of CoQ_{10} deficiency in this cohort, we included sequencing of 18 selected nuclear genes involved with CoQ_{10} biogenesis in 26 patients with low CoQ_{10} concentration in muscle samples.

Results: Central 95% reference intervals (RI) were established for CoQ_{10} normalized to citrate synthase (CS) or protein. Nine patients were considered CoQ_{10} deficient when expressed against CS, while 12 were considered deficient when expressed against protein. In two of these patients the molecular genetic cause could be confirmed, of which one would not have been identified as CoQ_{10} deficient if expressed only against protein content.

Conclusion: In this retrospective study, we report a central 95% reference interval for 600 x g muscle supernatants prepared from frozen samples. The study reiterates the importance of including CoQ_{10} quantification as part of a diagnostic approach to study mitochondrial disease as it may complement respiratory chain enzyme assays with the possible identification of patients that may benefit from CoQ_{10} supplementation. However, the anomaly that only a few patients were identified as CoQ_{10} deficient against both markers (CS and protein), while the majority of patients where only CoQ_{10} deficient against one of the markers (and not the other), remains problematic. We therefore conclude from our data that, to prevent possibly not diagnosing a potential CoQ_{10} deficiency, the expression of CoQ_{10} levels in muscle on both CS as well as protein content should be considered.

Keywords: Coenzyme Q10 deficiency; complex II+III; electron transport chain; OXPHOS; reference range

Abbreviations

CI to CIV:	Respiratory chain enzyme complexes I to IV, respectively
CS:	Citrate synthase
RCD:	Respiratory chain deficiency
CRC:	Clinically referred controls
PDSS1:	Prenyl (decaprenyl) diphosphate synthase, subunit 1
PDSS2:	Prenyl (decaprenyl) diphosphate synthase, subunit 2
COQ2:	Coenzyme Q2, polyprenyltransferase
COQ3:	Coenzyme Q3, methyltransferase
COQ4:	Coenzyme Q4
COQ5:	Coenzyme Q5, methyltransferase
COQ6:	Coenzyme Q6, monooxygenase
COQ7:	Coenzyme Q7, hydroxylase
COQ8A:	Coenzyme Q8A
COQ8B:	Coenzyme Q8B
COQ10A:	Coenzyme Q10A
COQ10B:	Coenzyme Q10B
APTX:	Aprataxin
ETFDH:	Electron Transfer Flavoprotein Dehydrogenase
ETFA:	Electron Transfer Flavoprotein Alpha subunit
ETFB:	Electron Transfer Flavoprotein Beta subunit
BRAF:	B-Raf proto-oncogene, serine/threonine kinase
APOE:	Apolipoprotein E

1. Introduction

The mitochondrion is at the center of energy production and referred to as the “energy hub” of the eukaryotic cell. The oxidative phosphorylation (OXPHOS) system is situated in the inner mitochondrial membrane and incorporates the four complexes (CI-IV) of the respiratory chain (RC) as well as complex V (ATP-synthase) in the transduction of energy molecules into the energy releasing adenosine triphosphate (ATP) (Saraste 1999). CoQ_{10} plays a vital role in this process by acting as an electron transport molecule from CI and CII (and other dehydrogenases) to CIII of the RC (Lenaz et al 2007). Therefore a CoQ_{10} deficiency may result in RC dysfunction with diverse clinical manifestations (Quinzii et al 2008). Within the group of respiratory chain enzyme deficiencies, which has a minimum live birth prevalence of 1 in every 5000 - 10000 (Applegarth et al 2000, Darin et al 2001, Skladal et al 2003, Schaefer et al 2008, Hargreaves 2014, Gorman et al 2015), the presence of the combined CI+III and/or CII+III RC deficiencies may be indicative of a CoQ_{10} deficiency (Emmanuele et al 2012). CoQ_{10} deficiencies are the most readily treatable subgroup of mitochondrial disorders, and CoQ_{10} supplementation has also shown therapeutic benefit in patients with mitochondrial RCDs (Hargreaves, 2014). But, although CoQ_{10} mainly localizes in the mitochondria (Saito et al., 2009), the exact distribution of CoQ_{10} between the different organelles, membranes and cytoplasm remains elusive. Furthermore, no universally accepted units on how to represent muscle CoQ_{10} status exists between researchers as CoQ10 is expressed in units against mass of fresh tissue, per protein content or per unit of citrate synthase (CS).

The aim of this study was therefore to quantify CoQ_{10} and to determine the diagnostic differences when CoQ10 is expressed against CS and protein content in a cohort of patients who was previously included for investigations into underlying mitochondrial disorders. Furthermore, we aimed to determine if there is an association between CoQ_{10} deficiency and other RC enzyme deficiencies. A panel of selected nuclear genes involved with CoQ_{10} biosynthesis and genes involved in possible secondary CoQ_{10} deficiencies were sequenced and the most important findings are briefly discussed. A new reference interval for CoQ_{10} levels in 600 x g muscle supernatants, the working material often used when investigating RCDs in patients with suspected mitochondrial disease, is reported as well as the diagnostic difference for CoQ_{10} deficiency when expressing muscle CoQ_{10} against CS and protein.

2. Materials and methods

2.1 Patient samples

All muscle samples from the cohort of 156 pediatric patients referred to the Pediatric Neurology Unit at the Steve Biko Academic Hospital, Pretoria, South Africa, were obtained from the *Vastus lateralis* muscle and stored immediately at -80 ºC until use. Patients with a Mitochondrial Disease Criterion score (Wolf and Smeitink 2002) of six or higher, or that presented with a severe clinical phenotype suggestive of one of the syndromic mitochondrial disorders, were included for OXPHOS analysis (n = 156) and genetic analysis (n = 26) in this study. The cohort consisted of 94 African, 54 Caucasian and eight Indian patients.

2.2 Muscle sample preparation

Frozen muscle samples (30 - 100 mg) were thawed and homogenized in 10 volumes of isotonic buffer [mannitol 210 mM; sucrose 70 mM; 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) 5 mM; ethylene glycol tetra-acetic acid (EGTA) 0.1 mM; pH 7.2)] with 15 stokes in a tight-fit Potter-Elvehjem tissue grinder. Samples were centrifuged for 10 minutes at 600 x *g* at 4 ºC. Supernatants were transferred to new tubes and frozen at -80 ºC prior to enzyme analyses and CoQ_{10} quantification.

2.3 Analyses of respiratory chain enzymes

Mitochondrial RC enzymes (CI to IV; EC 1.6.5.3, EC 1.3.5.1, EC 1.10.2.2, EC 1.9.3.1, respectively, as well as CII+III, i.e. succinate: cytochrome c reductase; EC. 1.3.5.1 + 1.10.2.2) and citrate synthase (CS, EC 2.3.3.1) activities were measured in muscle as described previously (Smuts et al 2010). Protein content was determined with the bicinchoninic acid assay (Smith et al 1985). A respiratory chain deficiency was diagnosed when an RC enzyme activity was lower than reference values when expressed against at least two of three enzyme markers (CS, CII, or CIV), providing that these were not deficient.

2.4 Quantification of coenzyme Q_{10}

CoQ_{10} was quantified with a modified version of the assay described by Itokonen et al (2013). In short, 30 µl muscle sample (600 x g supernatant) was mixed with 30 µl internal standard (0.5 µg/mL $CoQ_{10}[^2H_6]$ dissolved in acetonitrile) in a glass Kimax tube for 30 seconds. Then 1 mL ice-cold ethanol: isopropanol (95/5; v/v) was added to precipitate protein before the sample was mixed for 10 seconds. The sample mixture was then extracted twice with 2 ml *n*-hexane. The organic phase from the second extraction was added to the organic phase from the first extraction before 30 µl of ethanolic 1,4-benzoquinone (0.4 mg/ml) was added for the complete oxidation of CoQ_{10}. The sample was dried under nitrogen gas at room temperature in a light protection glass vial for 20 minutes. The dried residue was re-dissolved in 60 µl acetonitrile prior to LC-MS/MS analysis.

Samples were analyzed using an Agilent 6460 Triple Quadropole mass spectrometer (MS) quipped with an Agilent 1290 Infinity Binary Pump for sample handling and mobile phase delivery. The injection volume was 1 µL and the injection needle was washed for 10 seconds in a flush port containing a methanol:isopropanol:water (70:10:20, v/v/v) solution to prevent carryover. The mobile phase used for the isocratic separation was 100% methanol containing 5mM ammonium formate, with a constant flow rate of 0.3 ml/min. An Agilent ZORBAX StableBond SB-C18 narrow-bore column-cartridge (2.1 x 30 mm, 3.5 µm) was used for reversed phase chromatography and the column temperature was kept at 45°C. Samples were delivered to the MS via electrospray ionization (ESI) in positive mode. Multiple reaction monitoring (MRM) was used for the monitoring the ammonium adducts of the target analytes (*m/z* 880.7 → 197.2 for CoQ_{10}, *m/z* 882.7 → 197.2 for $CoQ_{10}H_2$, and *m/z* 886.7 → 203.1 for $CoQ_{10}[^2H_6]$). Sheath gas and nebulizing/drying gas temperature and flow rate were set at 300°C and 6 L/min, respectively. Nebulizer pressure was set at 30 psi, the capillary voltage at 3500 V, the nozzle voltage at 500 V and the dwell time was set at 50 ms. Although CoQ_{10} was oxidized using ethanolic 1,4-benzoquinone, the presence/absence of $CoQ_{10}H_2$ was monitored to confirm that all the CoQ_{10} in the sample was oxidized. CoQ_{10} content was normalized using the internal standard ($CoQ_{10}[^2H_6]$) and expressed against citrate synthase activity as nmol CoQ_{10}/UCS (where U = µmol/min.mg^{-1} CS) or against total protein content as nmol/mg. The linear dynamic range of the CoQ_{10} assay was established as 0.01 – 2 µg/ml for CoQ_{10} ($R^2 > 0.999$). The intra- and inter-day coefficient of variance for the CoQ_{10} assay was 1.96% (determined after sequential analysis of five QC samples) and 2.37% (determined after analysis of 18 QC samples in 18 different batches over a period of 4 months), respectively.

2.5 Genetic analysis on coenzyme Q_{10} deficiency

The coding regions of selected genes involved in CoQ_{10} biosynthesis and secondary CoQ_{10} deficiencies were sequenced in selected patients identified with reduced muscle CoQ_{10} levels, expressed on both protein and CS. The genes included for sequencing were *PDSS1 (COQ1), PDSS2, COQ2-9, COQ10A, COQ10B, APTX, ETFDH, ETFA, ETFB, BRAF, and APOE.* The Ion PGM™ System for Next-Generation Sequencing (NGS) was used with a custom designed Ion AmpliSeq™ Targeted Sequencing Technology panel from ThermoFischer Scientific. The panel of 18 genes had a total of 304 amplicons with a combined size of 61kb with coverage of 98% of exonic target regions. The amplicon library preparation, enrichment and sequencing were done in accordance to manufacturer's protocols.

Primary data analysis (quality assessment, read alignment against GRCh37/hg19 and variant identification) was done using the Torrent Suite™ Software for Sequencing Data Analysis (v5.0.2) and Ion Torrent™ Suite Software Plugins (v.5.0) for variant calling. Secondary data analyses included variant annotation using Ensembl online VEP runner (Ensembl GRCh37 release 85, McLaren et al 2016) and data mining using GEMINI, an open-source light database framework for genome mining (GEMINI v0.19.1, Paila et al 2013). Variants were firstly filtered based on allele frequency for specifically African and Caucasian population groups as reported in the Exome Aggregation Consortium (ExAC; Lek et al 2016) followed by variant classification as either novel (coding variants with or without Loss of Function; LoF) or previously reported (coding variants with

or without LoF). The variant prediction software, SIFT (sorting intolerant from tolerant) and PolyPhen-2 (Polymorphism Phenotyping, v2) were used to sort the intolerant from tolerant variants and predicted the polymorphism impact respectively (Zeng et al 2014). Potential disease-causing variants were further investigated using OMIM and CliniVar (http://www.ncbi.nlm.nih.gov/clinvar/), to determine if a known clinical phenotype (clinical significance) was associated with the identified variants. Variants of importance were confirmed and validated by Sanger Sequencing.

3. Results and discussion

3.1 The effect of ethnicity, gender and age on 600 x g muscle CoQ_{10} levels

Samples (n = 156) were randomized and CoQ_{10} analyzed in 18 batches with a quality control (QC) sample included in every batch. The QC sample was prepared by mixing a small volume of numerous samples used in the study. Only data from the clinical referred control (CRC; patients who initially presented with symptoms usually associated with mitochondrial disease that were referred to the clinic, but where no respiratory chain deficiency on enzymatic level was detected; n = 80) were used to investigate the potential influence of ethnicity, gender and age on muscle CoQ_{10}. As illustrated in Fig S1, CRC samples were divided into ethnic groups [Black African (n = 38), Caucasian (n = 35) and Indian (n = 7)], gender groups (39 female and 41 male) and the following age groups: < 1 years (n = 16), 1 - 10 years (n = 57), 11 - 25 years (n = 5) and 26 - 60 years (n = 2). Muscle CoQ_{10}/UCS were not significantly influenced by ethnicity (P = 0.07; Kruskal Wallis test), gender (P = 0.63; Mann Whitney U test) or age (P = 0.60; ANOVA). When expressing the CoQ_{10} against protein content (nmol/mg protein), no significant effect were found either for ethnicity (P = 0.52; Kruskal Wallis test), gender (P = 0.80; Mann Whitney U test) or age (P = 0.34; ANOVA). Thus the results indicate that muscle CoQ_{10} normalized to CS or protein was not influenced by ethnicity, gender or age in our cohort. Itokonen reported that gender and age affected muscle CoQ_{10} when normalized to mass of wet tissue, but when they normalized the CoQ_{10} to CS, no significant effect of either age or gender was found (Itokonen et al 2013).Thus although Itokonen used isolated mitochondria (15 000 x g pellet), while we used 600 x g supernatant, the same outcome was obtained: when normalizing CoQ_{10} to CS, age group and gender had no significant effect on muscle CoQ_{10}.

3.2 CoQ_{10} in respiratory chain deficient (RCD) patients

CoQ_{10} levels from the CII+III defect group (which included patients with combined CII+III deficiencies; n = 29), other RCD group [which included patients diagnosed with RCD (excluding CII+III defects); n = 47] and the CRC group (n = 80) were compared (Fig 1). Table 1 summarizes the mean, median and interquartile (25th percentile - 75th percentile) ranges for CoQ_{10} in these groups expressed against CS activity (UCS) and protein content.

Both the median CoQ_{10}/UCS ratios of the CRC group and the Other RCD group differed significantly from the CII+III defect group (P < 0.001 and P = 0.036, respectively), although no difference was found between the other RCD group and the CRC group. When expressing the CoQ_{10} against protein content (nmol/mg protein), the median CoQ_{10} of the CII+III deficient group differed significantly form the CRC group (P = 0.018) but not from the other RCD group (P = 0.216). The CRC and other RCD groups also did not differ significantly from one another. This significant lower CoQ_{10} detected in the CII+III group relative to the controls (CRC) was expected, since low CoQ_{10} availability in the mitochondria would impair electron transfer essential for ATP production (Rahman et al 2012, Yubero et al 2016). However, it is likely that CoQ_{10} must decrease to a certain threshold before oxidative phosphorylation would be impaired.

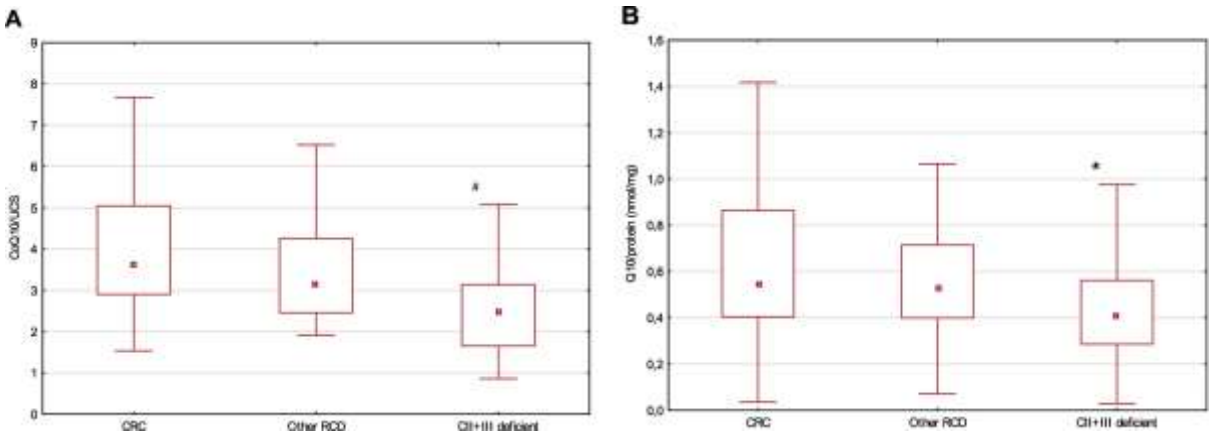

Fig. 1. Muscle CoQ_{10} concentrations in a cohort of South African patients who received muscle biopsies for the investigation of a possible RCD. Patients were divided into clinically referred controls (CRC, n = 80), Other RCD (patients diagnosed with a muscle RCD on enzyme level, but excluding CII+III deficient patients, n = 47) and patients with CII+III deficiency (n = 29). (A) When expressing the CoQ_{10} against CS activity (CoQ_{10}/UCS), the median CoQ_{10} levels of the CII+III deficient group differed significantly (#) form the CRC group (P < .001) and the Other RCD group (P = .019). CRC and Other RCD groups did not differ significantly from one another. (B) When expressing the CoQ10 against protein content (nmol/mg protein), the median CoQ_{10} of the CII+III deficient group differed significantly (*) form the CRC group (P = .018) but not from the Other RCD group (P = .216). CRC and Other RCD groups also did not differ significantly from one another.

Table 1. The mean, median and interquartile ranges calculated for patient muscle CoQ_{10} levels expressed against CS activity and protein content.

RCD group	CoQ_{10}/UCS mean (nmol/UCS)	CoQ_{10}/UCS median (nmol/UCS)	CoQ_{10}/UCS Interquartile range (25th % - 75th %)	CoQ_{10}/protein mean (nmol/mg)	CoQ_{10}/protein median (nmol/mg)	CoQ_{10}/protein Interquartile range (25th % - 75th %)
CRC	4.133	3.606	2.902–5.048 nmol/U	0.642	0.547	0.405–0.864 nmol/mg
Other RCD	3.483	3.171	2.460–4.259 nmol/U	0.561	0.528	0.400–0.715 nmol/mg
CII+III defect	2.552	2.474	1.654–3.140 nmol/U	0.432	0.410	0.287–0.562 nmol/mg

CRC: clinically referred controls, n = 80; Other RCD: respiratory chain deficient patients excluding CII+III defects, n = 47; CII+III defect n = 29.

3.3 Correlation of muscle CoQ_{10} concentrations with complex II+III RC enzyme activity

Firstly, no significant correlation was found between CoQ_{10}/UCS levels and complex II+III enzyme activity in the CII+III defect group (Spearman's correlation = 0.02, $P < 0.92$). This was however expected since (i) the group of CII+III patients was relatively small (only 29 patients) and (ii) not all CII+III defects are caused by deficient CoQ_{10}, some patients might have a genetic defect resulting in altered structure/function of CII and/or CIII. However, when investigating the Other RCD and CRC groups, muscle CoQ_{10}/UCS strongly correlates with CII+III enzyme activity (Spearman's correlation = 0.62) as illustrated in Fig S2. The correlation was also significant ($P < 0.001$). This finding is consistent with the report by Yubero et al (2016), where they showed a strong correlation between CII+III (normalized to CS) and CoQ_{10} (also normalized to CS) in a large cohort (n=435) of patients investigated for mitochondrial disease. However, when expressing CoQ_{10} against protein (nmol/mg), no significant correlation was found in our cohort (Spearman's correlation = 0.202).

3.4 A central 95% reference interval for CoQ_{10} in 600 x g muscle supernatants

A central 95% reference interval (i.e. the 2.5th percentile to 97.5th percentile) for 600 x g muscle supernatants was calculated according to the International Federation of Clinical Chemistry (IFCC) guidelines (using the CRC samples; n = 80) and determined to be 1.71 – 8.46 nmol/UCS, with a median ratio of 3.606 nmol/UCS. This observed reference interval for muscle CoQ_{10} was similar to the reference interval (2.68 - 8.40 nmol/UCS) reported for skeletal muscle 600 x g supernatants in a large cohort (n=436) (Yubero et al 2016). When CoQ_{10} was expressed against protein, a reference interval of 0.213 – 1.387 nmol/mg protein was calculated with a median of 0.547 nmol/mg protein.

3.5 Patients identified with muscle CoQ_{10} deficiency

The new reference intervals were subsequently used to identify patients in the cohort with decreased CoQ_{10} levels. Decreased CoQ_{10} levels were considered as "deficient" in those samples which were below the lowest limit (2.5th %) of the reference interval. Using this interval, CoQ_{10} levels was found to be deficient (< 1.710 nmol/UCS) in six Black African patients and three Caucasian patients for this particular cohort when expressed against CS (Table 2). However, when expressing CoQ_{10} against protein content, 12 patients were identified to have deficient CoQ_{10} levels (below 0.213 nmol/mg protein). A striking observation when comparing the two tables is that there are only four patients that overlaps between the two groups (P2, P14, P24, P29), thus being detected as CoQ_{10} deficient when expressed against both CS and protein. It is also notable, but possibly coincidental, that none of the Caucasian patients were identified as deficient when expressing CoQ_{10} levels against total protein (Table 3).

Table 2. 2CoQ_{10}/UCS ratios and RCDs in patients identified with CoQ_{10} deficiency.

Patient number	Ethnicity	Gender	CoQ_{10}/UCS concentration (nmol/UCS)	RCD diagnosed in patient	Sample group	CoQ_{10} genetic variant		
						Gene	Variant	RefSNP
P2	African	Female	1.559	CII, CII+III	CII+III defect	*COQ6***	c.41G > A (het) + c.859G > T (het)	rs17094161 rs61743884
P14	African	Female	1.638	CII+III	CII+III defect	*ND*		
P24	African	Female	1.204	CI, CII, CIII, CIV, CII+III	CII+III defect	*COQ6* *APTX*	c.41G > A (het) c.597C > T (het)	rs17094161 rs150886026
P29	African	Male	1.607	CII+III	CII+III defect	*COQ6* *COQ9*	c.283G > A (het) c.304C > T (het)	rs61743864 rs143043228
P54	Caucasian	Male	1.535	–	CRC	*ND*		
P78	Caucasian	Female	0.861	CI, CIII, CIV, CII+III	CII+III defect	*ETFDH***	c.1067G > A (het) + c.1448C > T (het)	novel
P95	African	Female	1.654	CIV, CII+III	CII+III defect	*ND*		
P98	African	Female	1.229	CII+III	CII+III defect	*COQ6*	c.41G > A (het)	rs17094161
P99	Caucasian	Male	1.391	CIII, CII+III	CII+III defect	*ETFA* *COQ10A*	c.513G > A (het) c.523C > T (het)	rs1801591 novel

95% central reference interval for 600 ×*g* muscle CoQ_{10}/UCS = 1.710–8.460 nmol/UCS. CRC = clinical referred control

*Possible disease-causing variants of genes involved in CoQ10 biosynthesis

**Protein deficiency confirmed. ND = none detected.

Table 3. 2CoQ_{10}/protein concentrations and RCDs in patients identified with CoQ_{10} deficiency.

Patient number	Ethnicity	Gender	CoQ_{10}/protein concentration (nmol/mg)	RCD diagnosed in patient	Sample group	CoQ_{10} genetic variant*		
						Gene	Variant	RefSNP
P2	African	Female	0.032	CII, CII+III	CII+III defect	*COQ6***	c.41G > A (het)+ c.859G > T (het)	rs17094161 rs61743884
P4	African	Female	0.199	CIII	Other RCD	*ND*		
P13	African	Female	0.103	–	CRC	*ND*		
P14	African	Female	0.132	CI, CII+III	CII+III defect	*ND*		
P24	African	Female	0.066	CI, CII, CIII, CIV, CII+III	CII+III defect	*COQ6* *APTX*	c.41G > A (het) c.597C > T (het)	rs17094161 rs150886026
P29	African	Male	0.176	CII+III	CII+III defect	*COQ6* *COQ9*	c.283G > A (het) c.304C > T (het)	rs61743864 rs143043228
P37	African	Male	0.211	CIII, CIV, CII+III	CII+III defect	*ND*		
P47	African	Female	0.111	CI	Other RCD	*ND*		
P69	African	Male	0.157	CIII, CIV, CII+III	CII+III defect	*ND*		
P72	African	Male	0.028	CII+III	CII+III defect	*Not performed (insufficient sample)*		
P107	African	Female	0.210	–	CRC	*ND*		
P158	African	Male	0.212	–	CRC	*APOE*	c.824 T > C (het)	Novel

95% central reference interval for 600 ×*g* muscle CoQ_{10}/protein = 0.213–1.387 nmol/mg. CRC = clinical referred contro

*Possible disease-causing variants of genes involved in CoQ10 biosynthesis

**Protein deficiency confirmed. ND = none detected.

The conundrum as to why only four patients were CoQ_{10} deficient against both CS and protein, while the rest of the patients were only CoQ_{10} defective against one of the normalization markers, persisted until the CS activity of the homogenates were examined. Citrate synthase, an enzyme found in the mitochondrial matrix, is routinely used as a normalization factor for mitochondrial content (Reisch et al 2007). Protein content, on the other hand, as measured in this study, is not due to mitochondrial content, but all proteins in the sample/homogenate.

When we investigated the patients CoQ_{10} deficient against protein, but not CS, many of these homogenates displayed very low CS activity (in nmol/min.mg^{-1} protein). These homogenates can be considered as having a *low mitochondrial content.* Therefore, when the CoQ_{10} concentration is normalized to the low CS (a small value), a higher value is obtained for CoQ_{10}/UCS, falling within the reference range and the patient is thus not flagged as being deficient. On deeper inspection of the patients being CoQ_{10} deficient against CS but not protein, most homogenates (>80%) displayed very high CS values. Normalization to CS thus resulted in very low CoQ_{10}/UCS, indicating defective CoQ_{10} levels, but still normal CoQ_{10}/protein. No significant correlation (Spearman's correlation < 0.3) was detected between CS and protein. Therefore it cannot be assumed that a CoQ_{10} deficiency would inevitably be identified when using only one of the two normalization approaches. The question remains as to why the homogenates display such a wide range in CS activity, and why CS does not correlate with protein content. This could be due to a number of reasons, including: (1) excessive proliferation of mitochondria observed in mitochondrial disease (DiMauro et al 2004), resulting in relative high CS activity; (2) massive rhabdomyolysis in patients, leading to muscle protein depletion (Montero et al 2008), also resulting in elevated CS activity relative to protein content; (3) mitochondrial DNA depletion syndrome is expected to lead to reduced CS activity relative to protein content; (4) the quality of the muscle biopsy; and (5) the efficiency of the homogenization process of the tissue. It is thus evident that expression of CoQ_{10} levels and choice of normalization should be mindful of pathology and sample preparation.

Another aspect to keep in mind is subcellular localization: CoQ_{10} is not only found in mitochondria, but also in the Golgi vesicles, lysosomes, microsomes, peroxisomes, plasma membranes and cytosol (Ernster and Dallner 1995). Although the exact subcellular distribution of CoQ_{10} remains elusive, some reports mentions that approximately 50% of cellular CoQ_{10} is present in the mitochondria (Yubero et al 2014). Therefore the mitochondrial content of a homogenate might greatly influence the eventual [CoQ_{10}] of the homogenate. This, as well as the great variation in CS might, in part, explain the anomaly why most patients are only CoQ_{10} deficient against one of the two markers used in this study.

When considering correlation of CoQ_{10} levels with enzymology, more often than not, combined CI+III and CII+III enzyme deficiencies are impaired by lower CoQ_{10} levels and therefore the presence of these combined RCDs in skeletal muscle are especially predictive of a CoQ_{10} deficiency (Rahman et al 2012). The results in this study support this notion since eight of the nine (89%) patients identified with CoQ_{10} deficiency when expressed against CS, (< 1.710 nmol/UCS, i.e., below the 2.5th % of the reference interval) also had CII+III deficiency (Table 2). Three of the nine patients with decreased CoQ_{10}/UCS ratios had isolated CII+III deficiency,

while two patients had associated multiple RCDs, and three patients had CII+III deficiencies with a concomitant CII, CIII and CIV deficiency, respectively. This reiterates the importance of determining CoQ_{10} even when a complex II-III deficiency is accompanied by other respiratory chain enzyme defects. Only one patient found with deficient CoQ_{10}/UCS was not diagnosed with CII+III deficiency. This particular patient however displayed very low CII+III activity that was just above the cut-off value used to diagnose a CII+III deficiency. However, when the CoQ_{10} levels are expressed against protein levels (Table 3), only seven of the twelve patients (58%) also had a CII+III deficiency. From the remaining five patients, we were able to identify and detect another RCD in two patients but not in the remaining three patients.

3.6 Genetic analysis

We further investigated the coding regions of selected genes involved in CoQ_{10} biosynthesis and secondary CoQ_{10} deficiencies in selected patients (n = 26). The most significant variants detected in this cohort are summarized in Tables 2 and 3. All variants were detected as heterozygous, while only two patients (P78, P2) presented with compound heterozygous variants, reported here as disease-causing. The remaining variants could not contribute in resolving the etiology since autosomal recessive inheritance patterns require disease-causing variants on both alleles and not only affecting one allele. From this data is was clearly evident that more variants were found in the group where CoQ_{10} was expressed against CS (6 of 9 cases) compared to CoQ_{10} expressed against protein (4 of 11 cases). This warrants further investigation.

From the two patients that presented with disease-causing variants, the first patient (P78) presented with compound heterozygous variants (c.1067G>A and c.1448C>T) in the gene *ETFDH* as recently described by van der Westhuizen et al (2018). This patient had the lowest CoQ_{10} level when expressed against CS, but were not classified as deficient when CoQ_{10} was expressed against protein content. This phenomenon was described before (Montero et al 2008) for a patient found to be CoQ_{10} deficient when expressed against CS, but not protein content. The investigators ascribed this discrepancy to the fact that the patient presented with massive rhabdomyolysis, leading to muscle protein depletion. In such a case the relatively low protein content in muscle could have led to a false negative result for CoQ_{10} when normalized to protein. It is however disconcerting that P78 in our cohort would not have been diagnosed as CoQ_{10} deficient if the CoQ_{10} levels were only expressed against protein content like many studies do.

The second patient, P2, presented with two variants in the gene *COQ6.* Both variants, c.41G>A (p.Trp14Ter, LoF with high confidence) and c.859G>T (p.Ala49Ser) were previously reported and classified as benign and tolerated according to ClinVar and OMIM. These variants were however classified as benign from a single submitter, with no evidence of any functional tests done to support the classification. From ExAC, minor allele frequencies of 0.005 and 0.002 are reported respectively. However, to further investigate this patient, we performed functional tests on 600 x g muscle sample supernatant from this patient, including SDS page and Western Blot analyses. Results revealed lower relative total protein when compared to control samples, and lower expression of *COQ6* when compared to the controls (Fig S3). Biochemically, this neonatal female was

diagnosed with CoQ_{10} deficiency on both CS and protein (Tables 2 and 3). We therefore classify these compound heterozygous variants as a risk factor for this patient, due to the supporting evidence mentioned. To fully understand the etiology of these compounding variants in the gene *COQ6*, we suggest segregation analysis on the non-consanguineous parents. All variants of interest were confirmed with Sanger sequencing and genetic variations of importance could not be identified in the remaining 24 patients. Since only two patients presented with disease-causing variants, future studies might focus on distinguishing primary and secondary CoQ_{10} deficiencies with the incorporation of radiolabeled substrates into CoQ_{10}, such as [^{3}H]-mevalonate and [^{14}C]-4-hydroxybenzoate, or that of stable isotopes in fibroblasts (Yubero et al 2017).

4. Conclusion

Although CoQ_{10} plays a central role in energy metabolism, the prevalence of CoQ_{10} deficiency is still unknown and the condition is at present under-diagnosed (Rahman et al 2012). Here we report on 600 x g muscle supernatant CoQ_{10} levels in a cohort of ethnically diverse patients who received muscle biopsies to diagnose a possible RCD. Results indicate that muscle CoQ_{10} was not significantly influenced by ethnicity, gender and age. Nine patients were identified with decreased muscle CoQ_{10}/UCS, eight of these patients were also CII+III deficient. A strong correlation was detected between CoQ_{10} levels and CII+III activity, supporting the notion that CII+III deficiencies are a good indication of a CoQ_{10} deficiency (Montero et al 2005, Miles et al 2008, Trevisson et al 2011, Yubero et al 2016). However, when CoQ_{10} was normalized to protein content, 12 patients were identified to be CoQ_{10} deficient. Only four patients were CoQ_{10} deficient when normalized against CS and protein, thus five patients were deficient when normalized against CS only and not when normalized against protein. Even worse – eight patients were only CoQ_{10} deficient when normalized to protein and not CS. This anomaly remains to be further investigated, with variation in adaptive responses such as mitochondrial biogenesis and mitophagy in muscle possible having an impact on the outcome of expressing CoQ_{10} levels.

This study is, as far as we know, the first to report a central 95% reference interval for 600 x g muscle supernatants prepared from frozen samples in an ethnically diverse cohort. The study reiterates the importance of including CoQ_{10} quantification as part of a diagnostic approach to study mitochondrial disease as it may complement respiratory chain enzyme assays with the possible identification of patients that may benefit from CoQ_{10} supplementation. We conclude from our data that, to prevent possibly not diagnosing a potential CoQ_{10} deficiency, the expression of CoQ_{10} levels in muscle on both CS as well as protein content should be considered.

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Conflict of interest:

All authors declare that they have no conflict of interest

Human and Animal Rights and Informed Consent:

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Author contributions:

Roan Louw: conception and design; drafting the article

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