

Quantification of coenzymeQ₁₀ in South African pediatric patients with electron transport chain deficiencies

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Kwantifisering van KoënsiemQ₁₀ in Suid-Afrikaanse pediatriese pasiënte met elektrontransportketting defekte

Deur

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For in Him all things were created: things in heaven and on earth, visible and invisible, whether thrones or powers or rulers or authorities; all things have been created through Him and for Him.

- Colossians 1:16

Yet God has made everything beautiful for its own time. He has planted eternity in the human heart, but even so, people cannot see the whole scope of God's work from beginning to end.

- Ecclesiastes 3:11

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ABSTRACT

CoenzymeQ₁₀ (CoQ₁₀) is an important component of the mitochondrial electron transport chain (ETC). CoQ₁₀ deficiency mainly affects children, and patients present with heterogeneous clinical phenotypes. Patients frequently respond to treatment with CoQ₁₀ supplementation, but with disparate responses. This study was aimed at quantifying muscle CoQ₁₀ concentrations in patients with ETC deficiencies. The samples of the experimental group of this study were received from muscle biopsies performed to determine underlying mitochondrial disorders. These biopsies were performed on patients with a Mitochondrial Disease Criteria ≥ 6 or when they had two or more unrelated organ systems involved, or any of the classical phenotypes associated with muscle mitochondrial disorders. After homogenization of the samples, 600 x g supernatants were used for analyses of the ETC enzyme activities. These same homogenate supernatants were used for CoQ₁₀ quantification.

A liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) method was standardized for the quantification of CoQ₁₀ in muscle samples, using a stable isotope dilution approach. The experimental group that was investigated in this study consisted of one hundred and fifty five patients of which seventy nine were clinically referred controls and seventy six had confirmed mitochondrial disorders. Statistical inference of the clinically referred controls showed that not race, age, or gender had any influence on the measured CoQ₁₀ concentrations. A central 95% reference interval was established for CoQ₁₀ concentrations in 600 x g supernatants using the clinically referred controls. After comparing all the ETC deficient patients to the newly established reference interval, it was found that eight patients, all with combined (CoQ₁₀ dependent) complex II+III (CII+III) deficiencies, had deficient CoQ₁₀ concentrations (below the 2.5th % of the reference interval). These eight patients are very likely to benefit from CoQ₁₀ supplementation, and this association of CoQ₁₀ deficiency and CII+III deficiency can serve as future selection criteria for patients who may benefit from CoQ₁₀ supplementation.

Key words: CoQ₁₀, CoQ₁₀ deficiency, LC-MS/MS, skeletal muscle, respiratory chain deficiencies, pediatric patients.

OPSOMMING

KoënsiemQ₁₀ (CoQ₁₀) is 'n belangrike komponent van die mitochondriale elektrontransportketting. CoQ₁₀ tekort affekteer hoofsaaklik kinders en pasiënte toon heterogene fenotipes. Pasiënte reageer dikwels op behandeling met CoQ₁₀ aanvulling, maar met uiteenlopende reaksies. Hierdie studie was daarop gemik om CoQ₁₀ konsentrasies te kwantifiseer in spiermonsters van pasiënte met elektrontransportketting defekte. Die monsters in die eksperimentele groep van hierdie studie is verkry vanaf spier biopsies uitgevoer om moontlike onderliggende mitochondriale defekte te bepaal. Biopsies was uitgevoer op pasiënte met 'n Mitochondriale Siektes Kriteria ≥ 6 , of wanneer twee of meer onverwante orgaanstelsels betrokke was, of wanneer enige van die klassieke fenotipes, wat verband hou met die mitochondriale siektes, teenwoordig was. Na homogenisering van die monsters is 600 x g supernatante gebruik vir analyse van die elektrontransportketting se ensiem aktiwiteite. Hierdie selfde homogenaat supernatante is gebruik vir CoQ₁₀ kwantifisering.

Vir die kwantifisering van CoQ₁₀ in spiermonsters is 'n metode met vloeistofchromatografie en tandem massaspektrometrie (LC-MS/MS) gestandaardiseer, met behulp van 'n stabiele isotoop verdunnings benadering. Die eksperimentele groep wat ondersoek is in hierdie studie het bestaan uit een honderd vyf en vyftig pasiënte waarvan sewe en negentig klinies verwysde kontroles was en ses en sewentig gediagnoseer is met mitochondriale defekte. Statistiese inferensie van die klinies verwysde kontroles het getoon dat nie ras, ouderdom, of geslag 'n invloed op die CoQ₁₀ konsentrasies gehad het nie. 'n Sentrale 95% verwysingsinterval is bereken vir CoQ₁₀ konsentrasies in 600 x g supernatante deur van die klinies verwysde kontroles gebruik te maak. Nadat al die pasiënte met elektrontransportketting defekte vergelyk is met die nuut berekende verwysingsinterval, is agt pasiënte geïdentifiseer met verlaagde CoQ₁₀ konsentrasies (onder die 2.5^{de} persentiel van die verwysingsinterval). Al agt pasiënte het ook gekombineerde (CoQ₁₀ afhanklike) mitochondriale komplekse II+III (CII + III) defekte gehad. CoQ₁₀ aanvulling mag heel waarskynlik tot voordeel wees in hierdie agt pasiënte, en hierdie assosiasie van CoQ₁₀ tekort en CII+III defek kan dien as toekomstige seleksie kriteria vir pasiënte wat mag voordeel trek uit CoQ₁₀ aanvulling.

Sleutel woorde: CoQ₁₀, CoQ₁₀ tekort, LC-MS/MS, skeletspier weefsel, respiratoriese ketting defekte, pediatriese pasiënte.

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LIST OF SYMBOLS AND ABBREVIATIONS

LIST OF SYMBOLS

%	percentage
$[M + H]^+$	protonated analyte adduct
$[M+NH_4HCO_2]^+$	ionized ammonium formate analyte adduct
<	Lesser than
>	Greater than
≥	Greater and/or equal to
®	Registered trademark
µg/mL	microgram per milliliter
µl	microliter
mL	milliliter
µM	micro molar
µm	micrometer
°C	degrees Celsius
Ω	ohm
g	gravitational force
x	multiplied by
µg/g	microgram per gram
™	Trade Mark

LIST OF ABBREVIATIONS

#	
4-HB	4-hydroxybenzoic acid
4-OH-benzoate	parahydroxy-benzoquinone
A	
Acetyl-CoA	acetyl-coenzyme A
ADP	adenosine diphosphate
ANT	adenine nucleotide translocator
AOA1	ataxia-oculomotor-apraxia 1
APT _X	aprataxin
ATP	adenosine triphosphate
B	
BHT	butylated hydroxytoluene
Bin	Binary
C	
Cat	Catalog
C	Celsius

CI	complex I
CI+III	combined complex I and complex III
CII	complex II
CII+III	combined complex II and complex III
CIII	complex III
CIV	complex IV
CV	complex V
C18	18 carbon
CID	collision induced dissociation
CLSI	Clinical and Laboratory Standards Institute
cm	centimetre
COQ	ubiquinone biosynthesis protein
COQ2	decaprenyl-4OH-benzoate transferase
COQ4	Coenzyme Q4
CoQ ₁₀	CoenzymeQ ₁₀
CoQ ₁₀ H ₂	ubiquinol/ reduced form of CoQ ₁₀ /hydroquinone
CoQ ₁₀ [² H ₆]	deterium labeled CoQ ₁₀
CoQH•	ubisemiquinone/free radical form of CoQ ₁₀
CRC	clinically referred control
CS	citrate synthase
CV	coefficient of variance
D	
ddH ₂ O	double distilled water
D-loop	displacement-loop
DNA	deoxyribonucleic acid
E	
ECD	electrochemical detection
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ETC	electron transport chain ETC ETC
ETF	electron transfer flavoprotein
ETF dehydrogenase	electron-transferring-flavoprotein dehydrogenase
ETF-QO	electron transfer flavoprotein:ubiqionone oxidoreductase
ESI	electrospray ionization
ESI(+)	positive ionization mode
<i>et al</i>	and others
ETFDH	electrontransferring-flavoprotein dehydrogenase
EtOH	ethanol
F	
FAD	flavin adenine dinucleotide
FAD ⁺	oxidized flavin adenine dinucleotide

FADH ₂	reduced flavin adenine dinucleotide
FMN	flavin mononucleotide
FMNH ₂	reduced flavin mononucleotide
FPP	farnesyl-pyrophosphate
G	
g/mol	gram per mole
GA II	glutaricaciduria type II
GPP	geranyl pyrophosphate
H	
H ⁺	hydrogen adduct
H ₂ O	water
H ₂ O ₂	hydrogen peroxide
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HPLC	high performance liquid chromatography
HPLC-MS/MS	high performance liquid chromatography tandem mass spectrometry
I	
IFCC	International Federation of Clinical Chemistry
IMM	inner mitochondrial membrane
IPP	isopentenyl pyrophosphate
K	
KOH	potassium hydroxide
L	
L/min	litre(s) per minute
LC	liquid chromatography
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
LDL	low density lipoprotein
LHON	Leber's hereditary optic neuropathy
M	
MADD	multiple acyl-CoA dehydrogenase deficiency
MDDS	mitochondrial DNA depletion syndromes
MeCN	acetonitrile
MELAS	mitochondrial encephalopathy with lactic acidosis and stroke-like episodes
MERRF	myoclonic epilepsy with ragged-red fibers
MeOH	methanol
mg/mL	milligram per millilitre
MIDD	maternally inherited diabetes and deafness
min	minutes
mL	millilitre
ml/min	millilitre per minute
mm	millimeter
mM	millimolar

MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
mtDNA	mitochondrial DNA
N	
N ₂	nitrogen gass
NaBH ₄	sodium borohydride
NADH	nicotinamide adenine dinucleotide
ND	NADH dehydrogenase
nDNA	nuclear DNA
NH ₄ HCO ₂ ⁺	ammonium adduct
NO	nitrogen oxide
NOX	NADH oxidase
O	
OH	hydroxyl
OH [•]	hydroxyl radical
OXPHOS	oxidative phosphorylation
P	
P	phosphate
PP	pyrophosphate
PDSS1	prenyldiphosphate synthase, subunit 1
PDSS2	prenyldiphosphate synthase, subunit 2
PPHB	polyprenyl-4-hydroxybenzoate
psi	per square inch
Q	
Q1	first mass analyzer
Q2	collision cell
Q3	third mass analyzer
QC	quality control
Q-pool	mobile pool of CoQ ₁₀
R	
R ²	linear regression coefficient of determination
RCD	respiratory chain deficiency
RF	response factor
RI	reference interval
ROS	reactive oxygen species
RNA	ribonucleic acid
RRF	ragged-red fibres
rRNA	ribosomal RNA
RSD	relative standard deviation
RT	retention time

S

SB	stable bond
SDS	sodium dodecyl sulphate

T

TCA	tricarboxylic acid
TIC	total ion chromatogram
tRNA	transfer RNA

U

UCP	uncoupling protein
UPLC	ultra high pressure liquid chromatography
UQ	ubiquinone
UV	Ultraviolet

V

V	Volt
v/v	volume to volume ratio
v/v/v	volume to volume ratio of three solutions

W

w/v	weight to volume ratio within a solution
-----	--

CHAPTER 1: INTRODUCTION

At the center of energy metabolism and the etiology of human mitochondrial disease is the mitochondrion, often referred to as the “energy hub” of every eukaryotic cell in the body. Situated in the inner mitochondrial membrane is the oxidative phosphorylation (OXPHOS) system. This system incorporates the four complexes (CI-IV) of the electron transport chain (ETC) and complex V (ATP-synthase) in the transduction of energy molecules into the energy releasing adenosine triphosphate (ATP) (Saraste, 1999). CoQ₁₀ plays a vital role in the production of ATP as an electron transport molecule from the ETC CI and CII (and other dehydrogenases) to CIII (Lenaz *et al.*, 2007). It is also a potent lipid soluble antioxidant (Turunen *et al.*, 2004). CoQ₁₀ deficiency in patients has shown diverse clinical symptoms and various mitochondrial ETC deficiencies (Quinzii *et al.*, 2008a). These ETC deficiencies are recognized as a more widespread subgroup of mitochondrial disease with a minimum live birth prevalence of 1 in every 5000 (Hargreaves, 2014). The presence of the combined CI+III and/or CII+III ETC deficiencies is especially indicative of a CoQ₁₀ deficiency, because these ubiquinone dependent activities are frequently impaired in patients with CoQ₁₀ deficiency (Emmanuele *et al.*, 2012).

CoQ₁₀ deficiencies are one of the most readily treatable subgroups of mitochondrial disorders, and CoQ₁₀ supplementation has also shown therapeutic benefit in patients with mitochondrial ETC disorders (Hargreaves, 2014). The already available enzymatic data of the experimental group investigated in this study revealed that of the patients diagnosed with ETC deficiencies (n=76), 38% (n= 29) had combined CII+III deficiencies. With CoQ₁₀ being an electron carrier from CI and CII to CIII, it was reasonable to assume that CoQ₁₀ depletion could be a crucial factor in these patients with ETC deficiencies, especially in the patients with CII+III deficiencies (Ogahashara *et al.*, 1989; Quinzii *et al.*, 2008b). The quantification of decreased CoQ₁₀ concentrations in the patients with ETC deficiencies, especially CI+III and/or CII+III deficiencies would identify patients in whom CoQ₁₀ supplementation may restore the supply and flow of electrons within their ETC, and provide antioxidant defense against the resultant oxidative damage to cellular constituents (Hargreaves, 2014).

The methodology for analytical CoQ₁₀ measurement in biological samples is well advanced. Yet even with the application of established methods, the verification of precise quantification thereof is still important in clinical measurements, because this would aid in the correct interpretation and discrimination of pathologic conditions, such as CoQ₁₀ deficiency (Barshop & Gangoti, 2007).

High performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has recently become a popular method in CoQ₁₀ quantification. However, only two reports used a non-physiological stable isotope as internal quantification standard (Duberley *et al.*, 2013a; Itkonen *et al.*, 2013). Hence, in this study, an analytical research approach using LC-MS/MS was designed for the precise and reproducible quantification of total CoQ₁₀ (sum of the oxidized and reduced forms of CoQ₁₀) in patient muscle samples using a non-physiological stable isotope, deuterium labeled CoQ₁₀ (CoQ₁₀[²H₆]), as internal standard. The LC-MS/MS method was utilized to accomplish the aim of this study, namely, to quantify total CoQ₁₀ levels in a cohort of South African patients with ETC deficiencies. The objectives were i) to optimize the quantification of total CoQ₁₀ using LC-MS/MS, and ii) to investigate the levels of CoQ₁₀ in ETC deficiencies by quantifying the levels of CoQ₁₀ in South African patient muscle samples with an optimized LC-MS/MS analytical method.

In Chapter 2, a Literature overview is presented, describing the human mitochondrion, the activities of the ETC, and the causes and effects of an ETC deficiency. This is followed by a description of the characteristics of the CoQ₁₀ molecule, its synthesis and metabolism, its role in the ETC, as well as its other functions. CoQ₁₀ deficiency and the levels of CoQ₁₀ in patients with ETC deficiencies are also discussed. Finally, the experimental approach of this study is conferred. Chapter 3 then presents and discusses the results obtained from the standardization and validation of the LC-MS/MS method for the quantification of CoQ₁₀ in muscle samples. Chapter 4 gives the results, discussion, and the statistical inferences of the CoQ₁₀ concentrations measured in the patient groups. Chapter 5 then formulates conclusions of the method and the patient data to verify whether the study objectives were successfully met, followed by a summarizing conclusion of whether the aim was accomplished.

CHAPTER 2: LITERATURE OVERVIEW

2.1 The mitochondrion and the OXPHOS system

2.1.1 Structure and functions

Mitochondria are the eukaryotic cell's main energy production site (Stucki, 1980) and are present in thousands of copies in most eukaryotic cells (Saraste, 1999; reviewed by DiMauro & Schon, 2003). The mitochondrion is composed of five sections: i) the outer membrane and ii) inner membrane enclosing the iii) intermembrane space, the iv) cristae which are really the invaginated inner membrane protruding into, and defining v) the matrix (Mitchell, 1979). The inner mitochondrial membrane (IMM) contains the five enzyme complexes of the oxidative phosphorylation (OXPHOS) system (Saraste, 1999). The OXPHOS system uses the enzymatic complexes I – IV, designated as the electron transport chain (ETC) and a fifth complex, ATP-synthase, to generate cellular energy (ATP) by oxidizing energy-rich molecules according to the respective cellular energy demands (reviewed by Anderson *et al.*, 1981).

In addition to energy production, the mitochondrion is involved in diverse activities like adaptive thermogenesis (reviewed by Cannino *et al.*, 2007), pyrimidine and steroid synthesis, calcium and iron homeostasis, innate immune responses (reviewed by Van Houten *et al.*, 2006; reviewed by Koopman *et al.*, 2012), as well as modulation and generation of reactive oxygen species (ROS) which assist in cell signaling (reviewed by Van Houten *et al.*, 2006; Rhee, 2006). Excessive ROS production or decreased ROS scavenging leads to oxidative damage and eventual cell death (reviewed by Van Houten *et al.*, 2006).

2.1.2 Mitochondrial genetics and gene expression

Within the human nucleated cell's mitochondrial matrix there are multiple copies of the 16,569 base pair circular mitochondrial DNA molecules (Figure 2.1). Mitochondrial DNA is transcribed and translated within the mitochondrion (reviewed by Taanman, 1999). This mitochondrial DNA (mtDNA) genome consists of 37 genes encoding 13 polypeptides, 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs) (reviewed by Taylor & Turnbull, 2005). The 13 polypeptides form part of the OXPHOS system of which 7 are constituents in complex I (ND1, 2, 3, 4, 4L, 5 and ND6; ND is short for NADH dehydrogenase), none in complex II (CII), 1 in complex III (cytochrome *b* subunit), 3 in complex IV (COX I, II, and COX III), and 2 in complex V (*A₆* and *A₈*) (reviewed by Wallace, 1999). The two mitochondrially encoded rRNAs and 22 tRNAs are involved in the translation process and the synthesis of the 13 polypeptides of the ETC (reviewed by DiMauro & Schon, 2003; reviewed by Taylor & Turnbull, 2005).

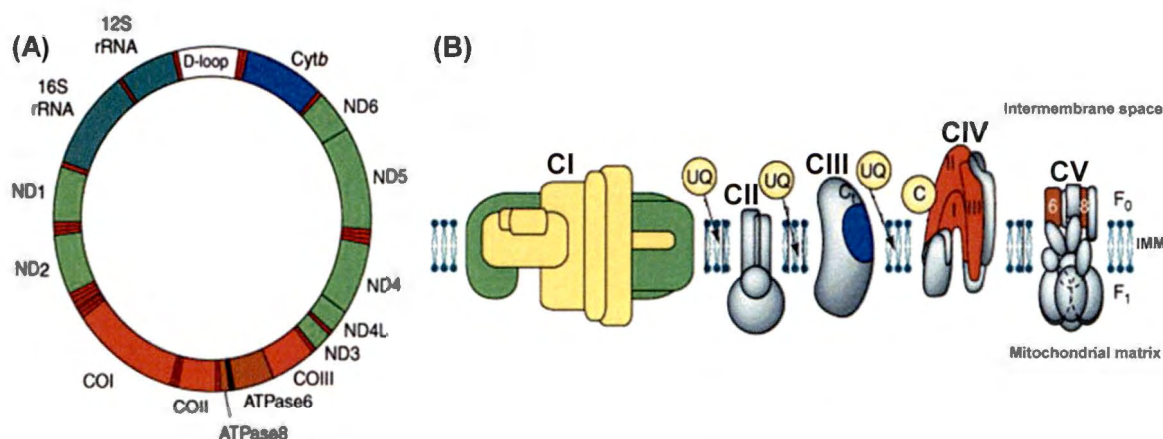


Figure 2.1: Proteins of the OXPHOS system encoded by the mtDNA genome. This figure illustrates A) the 37 genes of the mtDNA genome encoding 22 tRNAs (red stripes distributed all around the genome), 2 rRNAs and 13 polypeptides which form part of B) the OXPHOS system. These 13 genes encode for 7 constituents in CI, none in CII, 1 in CIII, 3 in CIV, and 2 in CV (with the color of the gene in A matching the polypeptide it encodes in B) (adapted from Blier *et al.*, 2001). IMM: inner mitochondrial membrane.

The displacement-loop (D-loop) is the only non-encoding region in the mtDNA genome (Sbisà *et al.*, 1997). This shows very little redundancy by the mtDNA genome when encoding these polypeptides and ribonucleotides (Schaefer *et al.*, 2001). The D-loop contains two major sites for regulation of intramitochondrial transcription and translation of the ETC subunits/polypeptides (Sbisà *et al.*, 1997). In correspondence with nucleic regulatory proteins, the D-loop controls mtDNA maintenance, *i.e.*, mitochondrial replication, transcription and translation (reviewed by Tuppen *et al.*, 2010). So it is clear that mitochondria are not independent entities but are reliant on imported nuclear gene products for proper expression, assembly and functionality of the ETC polypeptides. The nuclear genome also has a key role in every other aspect of mitochondrial respiratory chain (OXPHOS system) biosynthesis (reviewed by Cannino *et al.*, 2007). The 13 polypeptides encoded by the mtDNA interact with more than 60 imported nuclear gene products to form the five multisubunit enzymatic complexes of the OXPHOS system (Figure 2.1). These five complexes facilitate cellular energy production by means of oxidative phosphorylation (Schaefer *et al.*, 2001).

2.1.3 The role of mitochondria in cellular energy production

During aerobic respiration in eukaryotes, cellular energy is generated in the mitochondria when extracted electrons from nutrient complexes undergo a series of coupled redox reactions as they are sequentially transferred along the ETC chain (Saraste, 1999).

These nutrient compounds from the glycolysis and β -oxidation pathways are oxidized by the tricarboxylic acid (TCA) cycle within the mitochondrial matrix and generate reducing agents nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH_2) that activate the OXPHOS system (Saraste, 1999; Andersson *et al.*, 2002; reviewed by DiMauro & Schon, 2003).

Reoxidation of NADH and FADH_2 occurs during a series of coupled redox reactions as electrons from the respective reducing equivalents are passed along the ETC. Complex I (CI), complex III (CIII) and complex IV (CIV) simultaneously pump protons (H^+) from the mitochondrial matrix into the inner membrane space with the passage of electrons. This creates an electrochemical proton gradient: with the inner membrane suspended between an inside-negative membrane potential within the matrix and a proton motive force directed from the mitochondrial intermembrane space to the mitochondrial matrix. The energy potential of this proton-motive force is then utilized by ATP synthase (also known as the hydrogen pump) to accomplish phosphorylation of adenosine diphosphate (ADP) to ATP (Saraste, 1999). Protons pass through the subunits of ATP-synthase from the intermembrane space to the matrix and cause the condensation of ADP and P_i (inorganic phosphate) to form ATP (reviewed by Wallace, 1999). The final electron acceptor along this respiratory chain is an oxygen molecule, which forms a water molecule with two protons in the mitochondrial matrix (Saraste, 1999). The synthesized ATP is then transported across the IMM into the cytosol and simultaneously exchanged for cytosolic ADP via the adenine nucleotide translocator (ANT) (reviewed by Wallace, 1999). Many of the mitochondrial functions are sustained by this proton-motive force, such as ion transport, protein import, metabolite exchange, and mitochondrial fusion. If the proton-motive force is distorted, defects in OXPHOS as a consequence often induce many cellular aberrations (reviewed by Koopman *et al.*, 2012).

The mitochondrion is responsible for the production of sufficient levels of energy in most physiological conditions to enable normal cellular function. An increase or decrease in energy demand is 'detected' by the mitochondrion in various ways so the mitochondrion can adapt the rate of oxidative phosphorylation in an attempt to avoid energy depletion and resulting cellular dysfunction (Korzeniewski & Zoladz, 2001).

2.1.4 The electron transport chain

Criddle *et al.* (1962) stated that there is a suggested order to the passage of electrons along the ETC (Figure 2.2). The four enzyme complexes of the ETC function as electron carriers and they are electrically connected by electron carriers such as CoQ_{10} and cytochrome *c* (Mitchell, 1979).

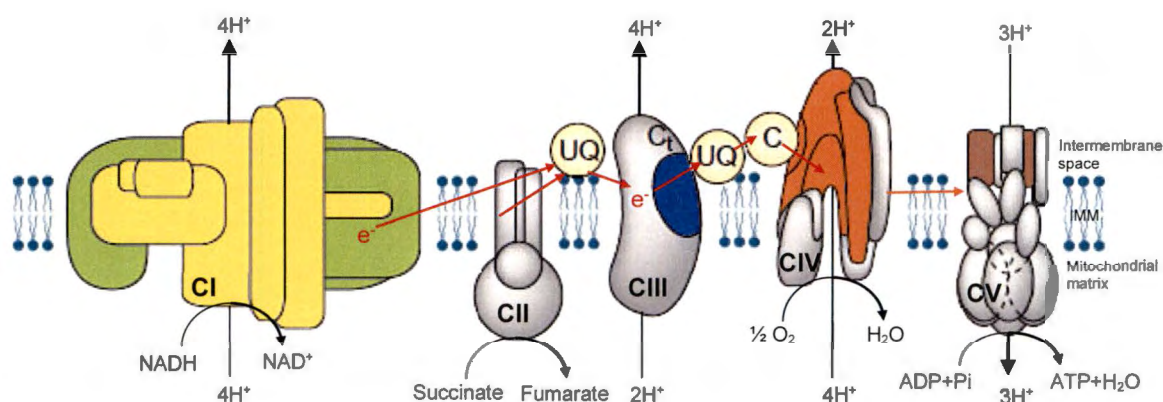


Figure 2.2: The OXPHOS system. This figure illustrates the OXPHOS system with its four membrane-bound enzyme complexes (CI-IV) of the ETC and the last complex (CV) of the OXPHOS system, ATP-synthase, all embedded in the inner membrane of the mitochondrion. The ETC structures are electrically connected by electron(e⁻) carriers CoQ₁₀ (UQ) and cytochrome c (C). The transfer of electrons across the ETC (indicated by the red arrows) provides the energy for proton translocation by CI, CIII, and CIV from the matrix into the intermembrane space. This creates an electrochemical proton gradient utilized by ATP synthase for ATP synthesis when a proton is pumped through the subunits of ATP-synthase back into the matrix (adapted from Blier *et al.*, 2001; Acín-Pérez *et al.*, 2008).

Complex I (CI, NADH:ubiquinone oxidoreductase) is one of the entry points of electrons into the ETC and it has 45 - 46 subunits of which 7 are encoded by the mtDNA genome (Lenaz *et al.*, 2007). This enzyme can be divided into three parts, namely, a flavoprotein [subdivided into three units containing the flavin mononucleotide, NADH, and iron-sulfur (Fe-S) binding sites], an Fe-S protein (subdivided into several iron-sulfur cluster binding sites), and the hydrophobic fraction (membrane part containing among others, the mitochondrial gene encoded subunits) (Loeffen *et al.*, 1998). The flavin mononucleotide (FMN) of CI catalyzes the oxidation of NADH to NAD⁺. The electrons from this reaction are then transferred from the reduced flavin (FMNH₂) to the iron-sulfur centers of CI. The iron-sulfur centers are able to undergo oxidoreduction reactions with the iron molecule during which CoQ₁₀ is reduced to CoQ₁₀H₂ with the electrons from the iron-sulfur centers (reviewed by Loeffen *et al.*, 2000). The main reactions in CI during electron transfer are explained by an encapsulated illustration in Figure 2.3. The number of binding sites for CoQ₁₀ within CI is still a subject of controversy as well as the mechanism by which electrons are transferred from CI to ubiquinone. The hydrophobic component N2 iron-sulfur cluster in the subunits of CI is the proposed electron donor to membrane bound CoQ₁₀ and most likely linked to the proton translocation in this enzyme (Lenaz *et al.*, 2007). Also, FAD in the electron-transferring-flavoprotein dehydrogenase is the electron donor to ubiquinone (UQ).

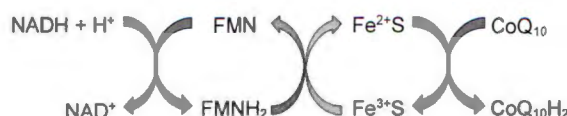


Figure 2.3: Illustration of the oxidoreduction reactions between NADH, FMN, Fe-S, and CoQ₁₀. These reactions occur during the transportation of electrons in the subunits of CI of the ETC.

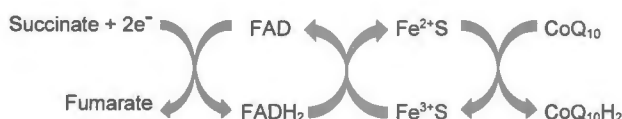


Figure 2.4: Illustration of the oxidoreduction reactions between the TCA cycle, FAD, Fe-S, and CoQ₁₀. These reactions occur during the transportation of electrons in CII of the ETC.

Complex II (CII, succinate dehydrogenase) has a dual function as the enzyme that catalyzes the $2e^-$ oxidation of succinate to fumarate during the TCA cycle and as part of the ETC it facilitates the $2e^-$ reduction of ubiquinone (CoQ₁₀) to ubiquinol (CoQ₁₀H₂) (Lenaz *et al.*, 2007; Lancaster, 2002). As part of the ETC, CII is more accurately referred to as succinate-CoQ oxidoreductase, because its subunits imbedded in the IMM are structurally composed to reduce CoQ₁₀ (Lancaster, 2002). Complex II is the only enzymatic complex of the ETC with no subunits encoded by the mtDNA genome (reviewed by Wallace, 1999). It has a flavoprotein subunit with a linked coenzyme FAD and iron-sulfur (Fe-S) centers contained within its Fe-S protein subunit. When succinate is oxidized to fumarate during the TCA cycle (Figure 2.4) by succinate dehydrogenase (CII), two electrons are extracted from succinate to the flavoprotein subunit of CII. This flavoprotein subunit is located near the mitochondrial matrix side where the binding site for CoQ₁₀ is situated. The iron-sulfur centers of CII accept electrons from FADH₂ derived from the TCA cycle and/or β -oxidation for the subsequent reduction of CoQ₁₀ (reviewed by Hägerhäll, 1997; Lenaz *et al.*, 2007). As illustrated in Figure 2.2 to 2.4, it is evident that CoQ₁₀ serves as a merging point for electrons from both CI and CII (Ernster *et al.*, 1969). Therefore, the transfer of electrons from CI and II to CIII is made possible with CoQ₁₀ freely available in a homogenous pool (Q-pool) as a mobile interconnecting mediator, laterally diffusing between dehydrogenases such as CI and CII to CIII within the lipid bilayer (Kröger & Klingenberg, 1973). This is why the combined complex enzyme activities of CI+III and CII+III (denoted NADH:cytochrome c oxidoreductase and succinate:cytochrome c oxidoreductase, respectively) are referred to as CoQ₁₀ dependent enzyme activities (Heron *et al.*, 1978; Trumpower & Edwards, 1979).

Complex III (CIII, ubiquinol cytochrome c oxidoreductase) is also known as the cytochrome *bc*₁ complex implying its main redox components, *i.e.* its electron transferring subunits namely the di-haem of cytochrome *b* (*b*_L haem and *b*_H haem), cytochrome *c*₁, and the Rieske iron-sulfur protein (involving a high-potential 2Fe-2S cluster) and lastly, ubiquinone CoQ₁₀ (Xia *et al.*, 1997). The subunits of CIII are all encoded by nuclear DNA (nDNA) genes except for cytochrome *b*, which is encoded by the mtDNA genome (Koene *et al.*, 2011). Within CIII the transfer of two electrons occurs in two stages in a pool function through an underlying mechanism denoted the protonmotive Q-cycle (Mitchell, 1975a; reviewed by Link, 1997).

The Q-cycle is based on the principal that the oxidized form, ubiquinone (CoQ_{10}) can undergo one or two electron reductions which form ubisemiquinone ($\text{CoQH}\cdot$) and ubiquinol ($\text{CoQ}_{10}\text{H}_2$), respectively, and these redox reactions are reversible (El-Najjar *et al.*, 2011). In short, CIII catalyzes electron transfer in the mitochondria from ubiquinol to the cytochrome *c*1 subunit of CIII (Mitchell, 1975a). Electrons enter CIII at the (positive, P) intermembrane space side of the inner mitochondrial membrane through ubiquinol. There ubiquinol is oxidized via the ubiquinol oxidation site (Q_P) to ubisemiquinone at the Rieske-iron sulfur center (Xia *et al.*, 1997). One electron is then transferred through the Rieske iron-sulfur protein to cytochrome *c*1. Cytochrome *c*1 then transfers the electron to mobile cytochrome *c* which transfers the electron to complex IV (Figure 2.2). Bifurcation of electron transfer occurs as the other electron from oxidation of ubisemiquinone at the Q_P site is transferred to the low potential cytochrome b_L haem and then rapidly to the high potential b_H haem, which forms part of the quinone reduction site (Q_N) at the (negative, N) matrix side of the IMM. The reduced b_H haem oxidizes either ubiquinone or ubisemiquinone bound to the Q_N site (Xia *et al.*, 1997) or it reduces ubisemiquinone to ubiquinol (reviewed by Link, 1997). Coupled to electron transport in CIII is the simultaneous proton translocation from the mitochondrial matrix to the intermembrane space as a result of the protonation of ubiquinone at the Q_N site or deprotonation of ubiquinol at the Q_P site (Xia *et al.*, 1997). Complex IV (CIV, cytochrome *c* oxidase) is the terminal catalytic enzyme of the ETC. Complex IV enables the transfer of electrons from its component flavins and cytochromes *a* and a_3 (Keilin & Hartree, 1939) to oxygen, the final electron acceptor along this ETC. In CIV, electron transfer is initiated when cytochrome *c* passes electrons to subunit II of CIV and then to the electron acceptor cytochrome *a*. Cytochrome *a* then transfers it to the cytochrome a_3 haem and then to molecular oxygen in conjunction with redox functional copper, leading to the formation of water (Mitchell, 1979).

Hatefi *et al.* (1962) established that, in organic solution, the redox properties of appropriate combinations of the ETC complexes were essentially the same as the redox properties of the individual mitochondrial membrane bound ETC complexes. Thus, whether in solution or membrane bound, the four enzyme complexes of the ETC function as electron carriers (Mitchell, 1979). This implies that, individually (membrane bound) or in association (in solution), CI, CIII and CIV can drive ATP synthesis by functioning as 'coupling' sites for the exergonic synthesis of ATP as they are still electrochemically connected by mobile electron transport molecules such as CoQ_{10} and cytochrome *c* (Hatefi *et al.*, 1962; Mitchell, 1979). Whilst reducing equivalents transfer electrons to any of CI, CIII or CIV, they translocate hydrogen (H^+) atoms from the mitochondrial matrix into the innermembrane space to contribute to the electrochemical proton gradient and can therefore initiate ATP synthesis (Mitchell, 1979).

A review by Fernández-Vizarra *et al.* (2009) explained that the OXPHOS system is partly organized in supermolecular associations between the isolated respiratory enzyme complexes whereby appropriate respiratory supercomplexes are created. These combinations are however denoted “appropriate” because they are not mere random aggregates, but have a distinct architecture (reviewed by Vonck & Schäfer, 2009). Though the functional relevance of respiratory supercomplexes have not yet been established (reviewed by Fernández-Vizarra *et al.*, 2009), respiratory function impaired by mtDNA mutations was retrieved after employing sufficient supercomplex assembly through functional complementation using hybrid cells (D’Aurelio *et al.*, 2006).

It was also reported that the mere presence of CIII is essential for the assembly and the stability of CI (Shägger *et al.*, 2004). This has significant implications in pathological studies of combined complex CI+III ETC deficiencies in humans (reviewed by Fernández-Vizarra *et al.*, 2009).

2.1.5 ETC deficiencies

The unique dual genetic foundation of the ETC for normal mitochondrial function is derived from the fact that the 13 mtDNA encoded subunits of the ETC interact with the greater part of ETC subunits encoded by the nDNA (reviewed by Andersson *et al.*, 2002). Additionally, the mtDNA is dependent on nDNA for the expression of polypeptides involved in the assembly of the ETC subunits, and the transcription, translation, replication and repair of mtDNA (Leonard & Schapira, 2000; reviewed by Cannino *et al.*, 2007). Thus, pathogenic mutations/alterations residing in either the mtDNA genome or the nDNA genome can cause dysfunction in the ETC, which results in a mitochondrial disorder, such as an ETC deficiency (reviewed by DiMauro & Schon, 2003).

Oxidative phosphorylation (OXPHOS) encompasses the careful adjustment of various metabolic processes and cellular functions both preceding and following electron transport through these five enzymatic complexes. A deficiency of OXPHOS would therefore also affect these pathways, though the effect of the individual enzyme complexes would have variable biochemical consequences (Table 2.1). Ineffective electron transfer by either of the ETC enzymes or electron transport molecules (*e.g.* CoQ₁₀ and cyochrome *c*) would result in the generation of ROS when the excessive electrons leak from the ETC to react with oxygen molecules (reviewed by Reinecke *et al.*, 2009). The major sites of superoxide formation are at CI and CIII of the ETC (reviewed by Van Houten *et al.*, 2006). The steady state of ROS production in the human body is considered to be much less than the considered 1 – 2% of the present oxygen species.

This percentage for the formation of ROS in ETC deficiencies would be dependent on the site of deficiency as well as the amount of electron transfer via the ETC enzymes. ROS production may also arise from the TCA cycle (α -ketoglutarate dehydrogenase) and CII (subunit SDHB) (reviewed by Reinecke *et al.*, 2009). A significant defect or loss of activity in the enzymatic complexes of the ETC consequently results in impairment of OXPHOS, causing a wide spectrum of devastating clinical manifestations because of the overall dependence of different tissue types on OXPHOS metabolism for ATP production (Greaves *et al.*, 2012). ETC deficiencies most often affect tissue types with high energy expenditure such as post-mitotic neurons and skeletal muscle, because these tissue types have a lower disease threshold, and are thus most earnestly affected by ETC deficiencies (reviewed by DiMauro & Schon, 2003). Biopsies of skeletal muscle are therefore usually performed (Thorburn, 2004) for research and diagnostic purposes due to its common involvement in ETC deficiencies and its relative ease of access (Shaefer *et al.*, 2001).

The high dependence of brain and muscle tissue on ATP may in part account for the high frequency of neuromuscular (encephalomyopathies) and neurological clinical manifestations in mitochondrial disorders (Rahman & Hanna, 2009). Dysfunction of the mitochondrial ETC enzymes can also arise from environmental influence such as viral infection or drug effects (reviewed by Koopman *et al.*, 2012), but deletions and common pathogenic mutations of mtDNA account for most of the ETC deficiencies in patients (reviewed by DiMauro & Schon, 2003). Despite the fact that the tRNA regions only make up 5% of the mtDNA genome, more than half of the reported pathogenic mutations related to mitochondrial disease are located in the tRNA genes (Greaves *et al.*, 2012). Mutations in the rRNA, tRNA protein encoding genes, and mtDNA deletions may affect the synthesis of mitochondrial proteins in general or specific proteins of the ETC which then results in deficiency (reviewed by DiMauro & Schon, 2003). Point mutations in the mitochondrial tRNA and rRNA genes are often maternally inherited, as opposed to the large-scale mtDNA deletions that often arise sporadically (Rahman & Hanna, 2009). The mtDNA genome is nearly exclusively inherited down the maternal lineage. A maternal pedigree of mitochondrial disease inheritance can thus be particularly suggestive of an ETC deficiency (DiMauro *et al.*, 2003). Mutations in nDNA genes involved in mitochondrial diseases (Mendelian mitochondrial disorders) have been referred to as “indirect hits” (reviewed by DiMauro, 2010) because these mutations do not affect the ETC directly. Rather, these mutations alter proteins necessary for maintenance (reviewed by El-Hattab & Scaglia, 2013), assembly/stability, function of the ETC complexes (Diaz *et al.*, 2011), and aminoacylation, a process described as the “charging” of tRNA for both cytosolic and mitochondrial translation (McMillan *et al.*, 2014).

Mitochondrial DNA depletion syndromes (MDDS) are caused by mutations in nDNA genes functioning in either mitochondrial nucleotide synthesis or mtDNA replication, and are recognized by severe decrease in mtDNA content. Mitochondrial DNA depletion can cause defects in all 13 mitochondrially encoded subunits of the respiratory chain complexes due to inadequate synthesis thereof. Therefore, if a patient has multiple ETC chain deficiencies, it is most likely caused by an mtDNA mutation that affects intra-mitochondrial protein synthesis or an MDDS (reviewed by El-Hattab & Scaglia, 2013). Isolated ETC deficiencies often involve an nDNA mutation or a mutation in protein encoding genes (rRNA, or tRNA) of mtDNA (Rahman & Hanna, 2009). Table 2.1 gives a simplified overview of ETC deficiencies in humans with their predominant associated genetic mutations and resulting clinical features (Koene *et al.*, 2011). DiMauro & Schon (2003) stated that, even with point mutations in a single gene, there is no straightforward relation between the clinical phenotype of a mitochondrial disease and its causative gene mutation. A prime example of this ambiguous correlation between genotype and phenotype is the well-known mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS) syndrome predominantly caused by the m.3243A>G transition in the mitochondrial tRNA^{Leu(UUR)} gene (reviewed by DiMauro & Schon, 2003). This same mutation (m.3243A>G) may give rise to different mitochondrial diseases (Greaves *et al.*, 2012), while different gene mutations may give rise to the same (MELAS) disease (Moreas *et al.*, 1992; Manfredi *et al.*, 1994; Dunbar *et al.*, 1995).

Table 2.1: Human ETC deficiencies and associated predominant genetic mutations and resultant clinical features.

Deficiency	Clinical and biochemical information
Complex I	Caused by mutations in replication genes in either mtDNA or nDNA, and mutations in certain mitochondrial tRNA genes and nuclear assembly genes. Frequent reports are given of mtDNA mutations in pediatric patients, but more so of nDNA mutations, e.g. subunits NDUFS4 and NDUFV1. A greater percentage caused by mtDNA mutations rather than nDNA mutations occur in adult patients. MELAS syndrome and Leber hereditary optic neuropathy (LHON) disease have greater prevalence in adult patients and are accompanied by mtDNA mutations. The majority of pediatric patients develop Leigh syndrome.
Complex II	Caused by mutations in the SDHA gene and the SDHAF1 gene, encoding the assembly factor of CII. Mutations cause heterogeneous clinical features with neurological features being predominant.
Complex III	Caused by mutations in BCS1L functioning as an assembly factor associated with Leigh and GRAULE syndrome. Milder phenotype in patients with sporadic or maternally inherited mutations in the Cyt b gene with LHON, neuropathy, cardiomyopathy, encephalomyopathy and MELAS/Parkinsonism overlap syndrome. Wide spectrum of clinical features and adaptive organ involvement. Early childhood mortality is common, though some show extended survival.
Complex IV	Mutations are mostly harboured in the nDNA. The most common cause is a mutation in the assembly genes, namely a SURF1 mutation. Patients with mutation in nDNA have a broad spectrum of symptoms mainly caused by severe encephalomyopathies. Patients with mtDNA mutations have a wide phenotype with conditions such as MELAS syndrome, encephalomyopathy, exercise intolerance, Leigh syndrome, and ataxia.

Combined complex	Combined complex deficiency is a case where the activities of two or more enzyme complexes are decreased. Combined complex deficit patients form a clinically heterogeneous group with differing clinical features caused by multiple gene defects. In theory, any mutation in any gene product involved in the function and maintenance of mtDNA can result in combined complex deficiency. These gene products include the necessary proteins for mtDNA maintenance, transcription, posttranscriptional or posttranslational processes, and its replication. Also including the proteins necessary for conveyance of proteins (encoded by nDNA) into the mitochondrial matrix, and the biosynthesis of the mitochondrial membrane. Mutation of the mitochondrial tRNAs causes dysfunction thereof and consequential suboptimal composition of the individual enzyme complexes of the ETC. The most frequent tRNA point mutations in the mtDNA genome include m.3243A>G (a.k.a. MELAS mutation) and m.8344A>G (a.k.a. MERRF mutation). A mutation in a replication gene eventually causes a build-up of multiple deletions or it can lead to depletion syndromes in the mtDNA which is often the cause of the well-known mitochondrial myopathy Kearns-Sayre syndrome (commonly presents before the age of 20 years).
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The m.3243A>G mutation may either cause MELAS syndrome, which is the more severe manifestation, or maternally inherited diabetes and deafness (MIDD), which is the more frequent manifestation in skeletal muscle (Greaves *et al.*, 2012). Also, the degree of mutational load in the affected (skeletal muscle) tissue has been documented to be in line with the clinical severity (reviewed by DiMauro, 2010). Thus the classification of ETC deficiencies based on clinical abnormalities proves to be difficult (Greaves *et al.*, 2012), and established syndromes do not always fit the description of every patient with an ETC deficiency as some patients can present a combination of clinical features of these syndromes (Adams & Turnbull, 1996; Greaves *et al.*, 2012). Childhood phenotypes of mitochondrial diseases are more often marked by nDNA mutations while adult onset mitochondrial diseases are mainly caused by mtDNA mutations (Rahman & Hanna, 2009). This predominance for nDNA mutations may contribute to the fact that diagnoses of mitochondrial syndromes in pediatric patients are particularly difficult as they present with very heterogeneous clinical features that result in ambiguous correlations between the genotype and the respective phenotype of the patients (Van Der Walt *et al.*, 2012).

The wide spectrum of clinical syndromes associated with mtDNA can be attributed to the three unique features of mitochondrial genetics namely, maternal inheritance, heteroplasmy, and also mitotic segregation (reviewed by DiMauro & Schon, 2003). Because of the large amount of mtDNA copies generated within each cell, pathogenic mutations frequently occur in some genomes, resulting in a condition known as heteroplasmy (DiMauro & Hirano, 2005). Heteroplasmy is a condition whereby the mtDNA copies within a mitochondrion are a mixture of mutant and normal mtDNAs (reviewed by Taylor & Turnbull, 2005), and can occur on an organellar level to differ greatly between tissue types and/or adjacent cells (Shaefer *et al.*, 2001). More often than not, a patient with an ETC defect has a proportion of heteroplasmic mtDNA (reviewed by DiMauro & Schon, 2003).

However, with heteroplasmy a patient may be periodically asymptomatic or oligosymptomatic, because there is a critical percentage of mutation that has to be reached before the cell becomes functionally affected by the biochemical defect (threshold effect) and only then do clinical symptoms become evident (Litellier *et al.*, 1994; reviewed by DiMauro & Schon, 2003). Transferred mtDNA point mutations may have a totally different clinical manifestation in the offspring. This interesting occurrence exists because of the randomized redistribution of organelles during mitotic segregation. Mitotic segregation can alter the degree of heteroplasmy through subsequent cell generations, which allows changes in the proportions of mutant mtDNA received by the daughter cells (Shaefer *et al.*, 2001). During early development of the embryo, the inherited mtDNA genome count is first reduced before a selected few are subsequently amplified to as much as $\pm 100\,000$ in a mature oocyte (Shoubbridge, 2000). This 'bottleneck' mechanism underlying the dilution of the mtDNA during oogenesis contributes to the decreased occurrence and increased variability of mutants when maternal mtDNA are transferred to the offspring (Shaefer *et al.*, 2001). Consequently, the pathogenic threshold of the mutant mtDNA may then be surpassed within the newly developed tissue so that the resulting offspring present with a different phenotype (reviewed by DiMauro & Schon, 2003). These unique features of mtDNA genetics also serve to explain in part the tissue-related and age-related variability in the observed clinical phenotypes of mtDNA-related diseases (reviewed by DiMauro, 2010).

The mtDNA genome also has other specific properties that contribute to the relevance of its association with disorders of the ETC chain (Adams & Turnbull, 1996). Due to its proximity to the mitochondrion, mtDNA are very susceptible (3-10 fold more than nDNA) to oxidative damage caused by mutagenic ROS. This oxidative damage can cause mtDNA lesions which prevent RNA polymerase from commencing mtDNA transcription (reviewed by Van Houten *et al.*, 2006). Thus oxidative damage to mtDNA amplifies ROS production through improper expression of the critical proteins (such as the enzymatic complexes of the respiratory chain) that normally perform electron transport to prevent leakage of electrons from generating excessive ROS during OXPHOS (reviewed by Van Houten *et al.*, 2006). The mitochondrion is loaded with Ca^{2+} ions and is juxtaposed to the OXPHOS system which generates excessive ROS through the Fenton reaction (and other mechanisms) during impaired OXPHOS (Ježek & Hlavatá, 2005) and thus results in impaired intracellular buffering through Ca^{2+} ions (Manfredi & Beal, 2000). Although the mtDNA has its repair mechanisms for such damage, prolonged exposure will lead to fatal mutation and subsequent cell death (reviewed by Van Houten *et al.*, 2006).

Cellular consequences, which are either directly related or secondary to ETC deficiency, include alterations in the homeostasis of metabolites such as ROS, Ca^{2+} ions, ATP/ADP and NAD^+/NADH ratios. Alterations in the homeostasis of these ratios modulate the mitochondrial permeability transition pore. Unregulated accumulation of these alterations can lead to apoptosis. The NAD^+/NADH redox balance is an important mediator in many biological processes like Ca^{2+} homeostasis, cellular redox balance, gene expression, immunological response, energy production and, in deficiency of OXPHOS the redox status is converted to NADH. OXPHOS deficiency inherently decreases ATP production and disturbs the ADP/ATP homeostasis. Both the disruption of ROS and Ca^{2+} balance within the cell will disrupt the inner membrane potential to increase the occurrence of apoptosis (reviewed by Reinecke *et al.*, 2009).

In response to these alterations in cell homeostasis, the mitochondrion initiates certain stress-response pathways that compensate for any detected mitochondrial dysfunction on cellular level, which include mitochondrial neogenesis, increased expression of the proteins involved in OXPHOS, removal of mitochondria with impaired function via quality control systems, and a preference switch towards more glycolytic ATP production pathways (reviewed by Koopman *et al.*, 2012). In addition, the rate of OXPHOS, the substrate supply, the ATP generation mode, and the ATP demand all differ among cells and between tissue types (Benoist *et al.*, 2003). These aspects all influence the sensitivity of the specific tissue types to mitochondrial dysfunction (reviewed by Koopman *et al.*, 2012).

Looking at some of the general biochemical features of an ETC deficiency, the first and most apparent effect of a defect anywhere in the ETC would be a decrease in ATP production. The end products of glycolysis will not be metabolized by the impaired TCA cycle because of a lack of the required NAD^+ and FAD^+ provided by the ETC. Lactic acidosis is a common feature of ETC deficiencies because pyruvate cannot be metabolized by dehydrogenase enzymes requiring the limited availability of NAD^+ and FAD^+ , and is therefore converted to lactate by the unhindered activity of lactate dehydrogenase in anaerobic respiration (Adams & Turnbull, 1996; Greaves *et al.*, 2012). These dehydrogenase enzymes are also present in two steps in β -oxidation, and therefore this metabolic pathway is also inhibited. The two inhibited steps in β -oxidation are the acyl-CoA dehydrogenase reaction which involves electron transfer to the electron-transfer flavoprotein and electron-transfer ubiquinone oxidoreductase (ETF-QO), and the second step involves the reduction of NAD^+ to NADH by 3-hydroxyacyl-CoA dehydrogenase (Adams & Turnbull, 1996). The ETF-QO component provides a short pathway for electron transfer between mitochondrial flavoprotein dehydrogenases and the protein bound Q-pool (reviewed by Watmough & Ferman, 2010).

The Q-pool in the inner mitochondrial membrane serves as a mobile interconnecting mediator for electron transfer through freely diffusing ubiquinone (CoQ₁₀) between various dehydrogenases and the ETC (Kröger & Klingenberg, 1973). The Q-pool is also the merging point for electrons from CI and CII which are then transferred to CIII via the laterally diffusing CoQ₁₀ electron transport molecule. This pattern of electron transfer aligns with the positive findings of the relieved symptoms of a patient suffering from the mitochondrial myopathy Kearns-Sayre syndrome (Table 2.1) after receiving CoQ₁₀ supplementation (Ogasahara *et al.* 1989). From then on, numerous studies assessed the outcome of using CoQ₁₀ supplementation in the treatment of patients with various mitochondrial respiratory chain disorders (Hargreaves, 2014) with strikingly positive results (Linnane *et al.*, 2002; Quinzii & Hirano, 2010). The most consistent result induced by CoQ₁₀ treatment in the clinical studies of patients with mitochondrial disorders, was a progressive reduction in lactate and pyruvate levels in serum after exercise (Hargreaves, 2014). The therapeutic potential of CoQ₁₀ to ameliorate clinical/biochemical defects, even in patients with mitochondrial disorders not associated with decreased levels of CoQ₁₀ (Sacconi *et al.*, 2010), is an indication of an underlying involvement of CoQ₁₀ within these mitochondrial disorders (Rötig *et al.*, 2000; Hargreaves, 2014). This involvement is however, not restrictively linked to decreased levels of CoQ₁₀ in the endogenous Q-pool (Sacconi *et al.*, 2010; Hargreaves, 2014).

2.2 Coenzyme Q₁₀

2.2.1 What is Coenzyme Q₁₀?

In 1955, Coenzyme Q₁₀ (CoQ₁₀) was discovered by Fentenstein *et al.* and later given the name ubiquinone (a ubiquitous quinone) by Frederick Crane (1957) referring to its widespread synthesis in various animal tissues, and its quinonoid nature (Ernster & Dallner, 1995). The chemical structure of CoQ₁₀ was recognized by Wolf *et al.* (1958) as 2,3-dimethoxy-5,6-dimethylbenzoquinone (Figure 2.5) and its name was officially established as 'ubiquinone' by the IUPAC-IUB Commission on Biochemical Nomenclature (1975). Isolated CoQ₁₀ takes the form of a distinct yellow/orange lipophile powder, and when hydrogenated, CoQ₁₀ absorbs hydrogen and becomes colorless (Wolf *et al.*, 1958).

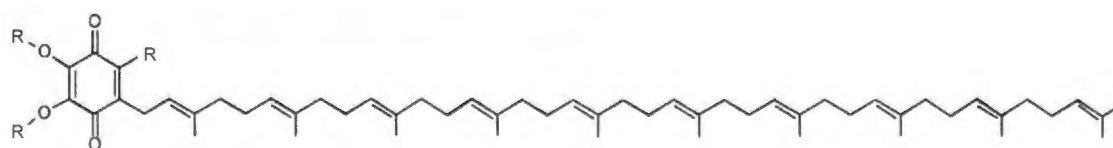


Figure 2.5: The chemical structure of CoQ₁₀. Ubiquinone (2,3-dimethoxy-5-methyl-6-ten-isoprene parabenzoquinone) with its redox active benzoquinone ring and long isoprenoid side chain consisting of 10 isoprene units of hydrocarbons as adapted from Hansen *et al.* (2004). R= CH₃.

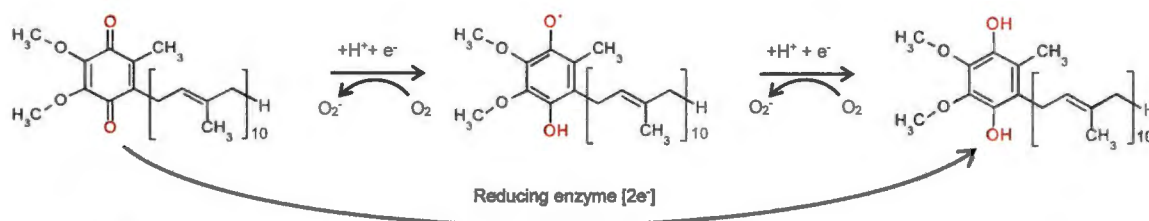


Figure 2.6: Illustration of CoQ₁₀ as a redox cycling molecule. From the ubiquinone molecule (left), the free radical intermediate, semiquinone (middle) is formed through a one electron reduction and a second electron reduction of the semiquinone radical forms ubiquinol (hydroquinone) (right). The semiquinone radical is oxidized back to the initial ubiquinone molecule in aerobic conditions in a reverse reaction with the generation of superoxide radicals. These reversible one electron reduction reactions enable the regeneration of its active form, ubiquinol. These reactions are catalyzed by numerous enzymes such as NADH ubiquinone oxidoreductase in the mitochondrion (Complex I: CI), microsomal NADH cytochrome b5 reductase (b5R), and NADPH cytochrome P450 reductase (El-Najjar *et al.*, 2011).

CoQ₁₀ belongs to a series of molecules (quinones) with similar chemical structure comprising of a common benzoquinone ring and differing lengths of a long isoprenoid side chain (El-Najjar *et al.*, 2011). More specifically, CoQ₁₀ is a benzoquinone derivative with a benzoquinone ring and a 10 isoprene unit side chain, hence the denotation CoQ₁₀ or ubiquinone₁₀. CoQ₁₀ is an amphipathic compound because of the hydrophilic benzoquinone ring and the long hydrophobic isoprenoid side chain moiety that conjugates the aromatic benzoquinone ring to a hydrophobic state (El-Najjar *et al.*, 2011). CoQ₁₀ can readily undergo redox cycling (Figure 2.6) owing to the electrophilic character of its redox active benzoquinone ring. This makes CoQ₁₀ prone to nucleophilic attack (El-Najjar *et al.*, 2011). The redox cycling creates an oxidized form, ubiquinone (CoQ₁₀), a free radical intermediate known as ubisemiquinone (CoQH•), and a reduced form, ubiquinol (CoQ₁₀H₂).

2.2.2 Synthesis and metabolism of Coenzyme Q₁₀

CoQ₁₀ is widely distributed in mammalian cells, and is present in most if not every cellular membrane in the body, particularly in the endomembranes of the Golgi apparatus and lysosomes and the inner membrane of the mitochondrion (Thelin *et al.*, 1992; reviewed by Linnane *et al.*, 2007). In addition to these three organelles, ubiquinone biosynthesis occurs in the endoplasmic reticulum, the plasma membrane and in peroxisomes (Ernster & Dallner, 1995). Ernster & Dallner (1995) reported varying levels of CoQ₁₀ (0.02 – 2.62 µg/g protein) in the subcellular liver fractions of rats. CoQ₁₀ is the main ubiquinone homologue in all tissues of the human body with 1-3% of the total ubiquinone content being CoenzymeQ₉ (ubiquinone 9) (Dallner & Sindelar, 2000). In normal, physiological conditions, all cells often rely on *de novo* synthesis of CoQ₁₀ (Tran & Clarke, 2007) which is dependent on the functional requirements of the cell (Dallner & Sindelar, 2000). On the contrary, plasma concentrations are influenced by dietary intake of CoQ₁₀ (Qunzii & Hirano, 2010).

With ageing (>35 years), all humans gradually lose the ability to synthesize CoQ₁₀, which causes deficiency thereof given that endogenous synthesis is the greatest source of this lipid soluble antioxidant (Beal *et al.*, 2002). In healthy patients, this decreased ability of CoQ₁₀ biosynthesis may be attributed to ageing, infection, stress and to a lesser extent because of poor eating habits (Kamzalov *et al.*, 2003; Boreková *et al.*, 2008). Synthesis of CoQ₁₀ has been said to initiate in the ER and terminate in the Golgi apparatus where CoQ₁₀ is redistributed to other cellular sites and to a small degree into circulation to be bound to lipoproteins (Ernster & Dallner, 1995).

Synthesis as well as rapid breakdown of CoQ₁₀ occurs in different animal cells with a half-life of 49-125h (Thelin *et al.*, 1992). Lenaz *et al.* (2007) stated that more than 84% of the CoQ₁₀ is free in living cells in the lipid bilayer while 10 – 32% can be bound to protein (Lass and Sohal, 1999). The extreme hydrophobicity of CoQ₁₀ restricts its existence into three possible states; forming aggregates, distributed in lipid bilayers, and protein bound (Lass & Sohal, 1999) of which the first two are apparent in living cells and the latter in cell free systems/solutions.

The biosynthesis of CoQ₁₀ results from the condensation of a benzoquinone ring with a 10-decaprenyl side chain (Figure 2.7). In mammals, the precursor of the benzoquinone ring is aromatic 4-hydroxybenzoic acid (4-HB) arising from the non-essential amino acid tyrosine which can also be synthesized from phenylalanine, and the ring structure is easily derived from the diet (Dallner & Sindelar, 2000). Acetyl-CoA is modified during a series of enzymatic reactions comprising the mevalonate pathway to produce the precursor of the 10-decaprenyl side which is farnesyl-pyrophosphate (FPP) (Bentinger *et al.*, 2010).

The initial reactions of the mevalonate pathway comprise condensing three acetyl-CoA molecules to form 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA). This occurs in two steps mediated by enzymes acetoacetyl-CoA synthetase and HMG-CoA synthase, respectively. Then the HMG-CoA molecule is converted to mevalonate through the enzyme HMG-CoA reductase, the extensively studied regulatory enzyme of cholesterol biosynthesis. Mevalonate is then successively phosphorylated consequently creating mevalonate phosphates (5P-mevalonate, 5-PP mevalonate). The mevalonate phosphates are then decarboxylated to yield isopentenyl pyrophosphate (IPP), the precursor of farnesyl-pyrophosphate (FPP), which is the end-product of the mevalonate pathway (Turunen *et al.*, 2004).

Isomerization of IPP yields dimethylallyl pyrophosphate. The IPP molecule and dimethylallyl pyrophosphate are the building blocks of FPP (Tran & Clarke, 2007). IPP and dimethylallyl pyrophosphate are utilized by farnesyl pyrophosphate synthase to form FPP, and subsequently form geranyl pyrophosphate (GPP) (Turunen *et al.*, 2004).

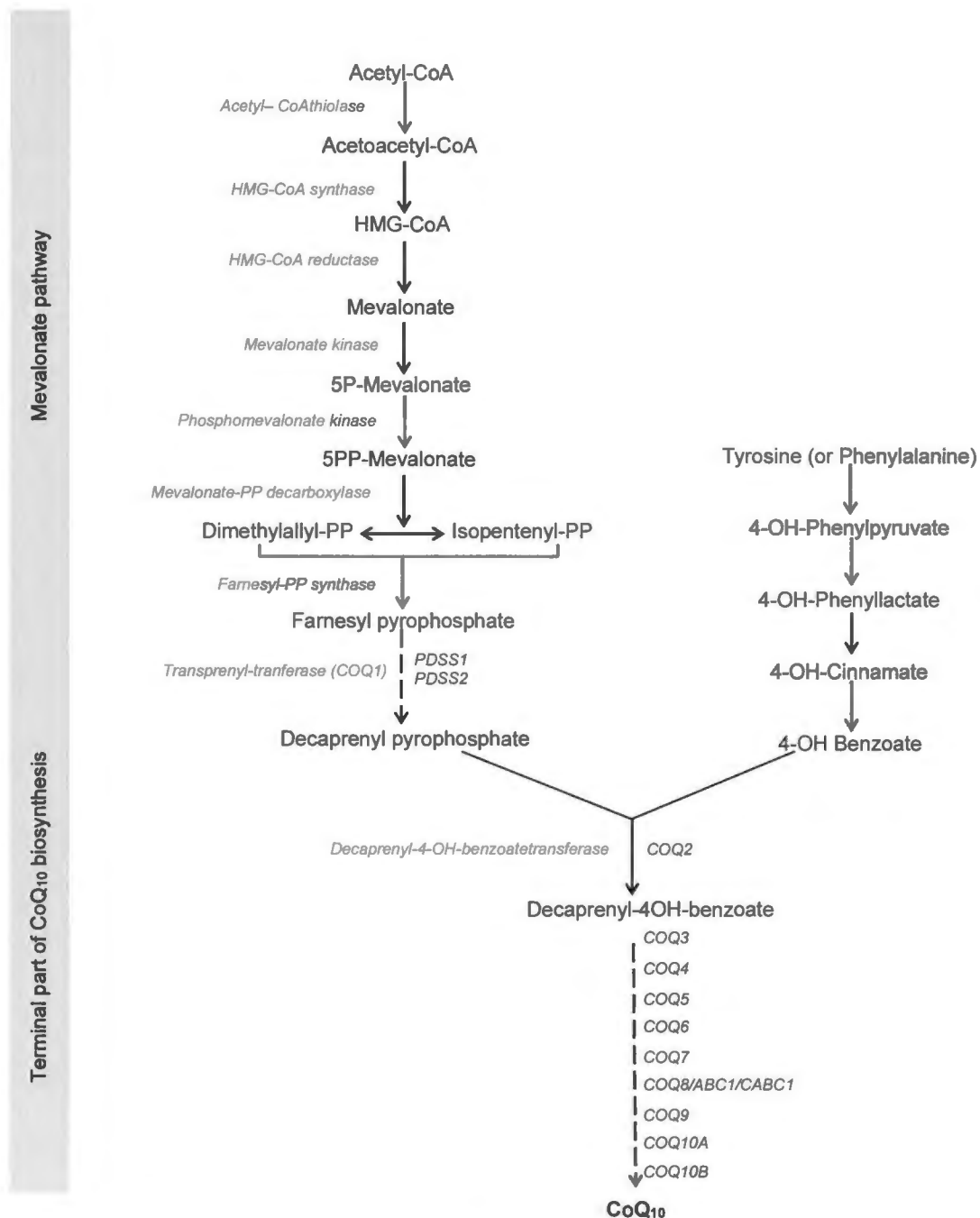


Figure 2.7: Coenzyme Q10 biosynthesis pathway. The simplified scheme of the enzymatic reaction pathway starting with the mevalonate pathway, with acetyl-coenzyme A (Acetyl-CoA) as its first substrate, and ends with farnesylpyrophosphate after a series of reactions in between. Terminal reactions of the CoQ₁₀ biosynthesis involve condensation of 4-OH-benzoate derived from the amino acids tyrosine or phenylalanine, and decaprenyl pyrophosphate derived from the mevalonate pathway. After 4-OH-Benzoate and decaprenyl pyrophosphate are condensed, several enzymes (encoded by CoQ₂₋₁₀ and other possible genes) catalyze the methylation, decarboxylation, and C-hydroxylation of the benzoquinone ring prior to formation of the functional CoQ₁₀ product. PPHB: polyprenyl-4-hydroxybenzoate. Enzymes are shown in grey italicized text to the left of the arrows and genes involved in the biosynthesis are indicated to the right of the reaction arrows. HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A. P: phosphate. PP: pyrophosphate. OH: hydroxy. Adapted from Dallner & Sindelar (2000).

After formation of the FPP, *i.e.* during the terminal part of CoQ₁₀ synthesis, FPP undergoes a series of enzymatic modifications where the two protein subunits PDSS1 and PDSS2 of the heterotetramer trans-prenyl-transferase (COQ1) catalyze the condensation of several IPP molecules with FPP during which the involvement of GPP is as an intermediate bound to the enzyme (Turunen *et al.*, 2004; Tran & Clarke, 2007). In humans, these two protein subunits of COQ1 are encoded by the prenyldiphosphate synthase, subunit 1 (PDSS1), and prenyldiphosphate synthase, subunit 2 (PDSS2) genes, respectively (Macinga *et al.*, 1998). Then, after the modification reactions, condensation of the decaprenyl pyrophosphate chain unto the parahydroxy-benzoquinone ring (also known as 4-OH-benzoate) is catalyzed by decaprenyl-4OH-benzoate transferase (COQ2) to produce decaprenyl-4OH-benzoate. COQ4 encodes a protein that is responsible for the organization of a multienzyme complex that is involved in the terminal part of CoQ₁₀ biosynthesis (Marbois *et al.*, 2009). Several substitutions and modifications of the aromatic benzoquinone ring structure occur which involve at least seven enzymes (COQ3-COQ10A and COQ10B) catalyzing C-hydroxylation, decarboxylation, and carbon and oxygen methylations to ultimately yield functional CoQ₁₀ (Duncan *et al.*, 2009; Bentinger *et al.*, 2010).

Though this biosynthesis pathway is generally accepted, it is not absolute, because the validity of the detail of the biosynthesis for mammals can still be elucidated as it is only well-known in yeast and bacteria (Bentinger *et al.*, 2010).

2.2.3 The role of Coenzyme Q₁₀ in the ETC

Together with the redox cycling character of CoQ₁₀, movement of its redox centre and bending of its long isoprenoid side chain allows a folded conformation to mediate inter- and intra-complex electron transport. The amphipathic nature of this highly hydrophilic CoQ₁₀ allows its interaction with the N2 iron cluster in CI which is located at the border between the hydrophobic and hydrophilic moieties of this enzyme complex. Ubiquinol (CoQ₁₀H₂) is more hydrophobic than ubiquinone, though its redox centre is more polar so ubiquinol is comfortably located at the polar side of the inner membrane. This explains how CoQ₁₀H₂ can transfer electrons to the Rieske iron-sulfur protein in CIII located near the hydrophilic phospholipid heads of the membrane lipid bilayer (Lenaz *et al.*, 2007). The proton motive Q-cycle enables the extrusion of protons from the mitochondrial matrix into CIII to reduce CoQ₁₀ in the second stage of the Q-cycle, and allows the redistribution of electrons to the other complexes of the ETC in cases where for example, CIII is inhibited by antimycin (Mitchell, 1975b). CoQ₁₀ attracts electrons from NADH within CI (Ernster *et al.*, 1969), from FADH₂ in CII (Lancaster, 2002), and from intermediate metabolites of β -oxidation (DiMauro & Rustin, 2009).

It has been established that protein bound CoQ₁₀ exist in the inner mitochondrial membrane, and co-exists with a mobile pool of CoQ₁₀ (Q-pool) (reviewed by Lenaz & Genova, 2009). During flux control analysis using succinate as substrate to induce ATP-producing conditions, Benard *et al.* (2008) described the existence of three different pools of CoQ₁₀: one utilized during normal respiration and the other serving as a mobile reserve pool utilized in a perturbed state of respiration to maintain normal energy fluxes as in the case of a mitochondrial disease of mitochondrial enzyme complex inhibition. A third mobile pool was present but was not able to participate in respiration dependant on succinate as a substrate. The percentages of the total CoQ₁₀ within these different pools differ between tissue types. The freely diffusing CoQ₁₀ within the Q-pool initiates binding between adjacent complexes I and III referred to as CI-III supramolecular aggregates or supercomplexes. As CoQ₁₀ binds to the binding sites formed by supercomplex CI-III, it ensures correct and efficient electron channeling between these complexes (reviewed by Lenaz & Genova, 2009). Other examples of documented supercomplexes are a combination of complex I, dimeric complex III, and complex IV (I₁III₂IV₁) or dimeric complexes III, and IV (III₂IV₂) and dimeric complex III, and IV (III₂IV₁) or dimeric complexes III (III₂) (Shagger & Pfeiffer, 2000). Any abnormal decrease in Q-pool concentration of CoQ₁₀ would result in a decrease of the protein-bound CoQ₁₀ within the supercomplex and thus bring about a collapse of electron transfer within the supercomplex.

Of course, the Q-pool also serves as a platform for the reverse electron transfer involving collisional interaction between CI and CII when NAD⁺ is reduced to NADH by electron transfer from succinate. There has been no report of a supercomplex formed through aggregation of CII with any of the other ETC complexes or ATP-synthase. Thus, the Q-pool serves as the dominating intermediate molecules over other dehydrogenases (dihydro-orotate dehydrogenase; EFT dehydrogenase, glycerol 3-phosphate dehydrogenase) for the mechanism of electron transfer between CI and CII to CIII (reviewed by Lenaz & Genova, 2009).

2.2.4 Other functions of Coenzyme Q₁₀

The Q-pool serves as a reservoir for other functions involving CoQ₁₀ such as binding to the mitochondrial permeability transition pore and uncoupling proteins (reviewed by Lenaz & Genova, 2009). CoQ₁₀ assists efficient ATP production in many energy requiring sub-cellular membrane systems enabling essential processes such as nerve conduction, and metabolite translocation (Linnane *et al.*, 2007). Within these subcellular organelles and membrane systems (including the inner mitochondrial membrane), CoQ₁₀ is the only endogenously synthesized hydrophobic antioxidant and acts as a very effective antioxidant in its reduced form (Ernster & Dallner, 1995; Turunen *et al.*, 2004).

Ubiquinol (CoQ₁₀H₂) is regenerated within cells by α -tocopherol, while ubiquinol regenerates the intracellular antioxidant ascorbate, and in the presence of a lipid soluble radical, CoQ₁₀H₂ has a sparing effect on both ascorbate and the antioxidant α -tocopherol (Shi *et al.*, 1999; Turunen *et al.*, 2004). Ubiquinol promotes the uptake of Vitamin E, and regenerates Vitamin E in low concentrations of ascorbate via the α -tocopheroxyl radical (Ernster & Dallner, 1995).

During oxidative stress, hydrogen peroxide (H₂O₂) inevitably reacts with the metal ions bound to DNA (nDNA and mtDNA) which generate hydroxyl radicals (OH[•]) which may cause nucleotide base oxidation, but CoQ₁₀H₂ neutralizes these radicals to prevent DNA oxidation (Bentinger *et al.*, 2010). In addition, CoQ₁₀ is also involved in DNA replication and repair through its role in pyrimidine synthesis (López-Martín *et al.*, 2007) and the regulation of the physiochemical properties of cellular membranes (Turunen *et al.*, 2004).

In circulation, CoQ₁₀ is an adversary for vascular superoxide by scavenging it and simultaneously preventing its formation by scavenging other radicals pertaining to the synthesis of superoxide (McCarty, 1999). CoQ₁₀ is mostly bound to low density lipoproteins (LDLs) and thus binds to reactive oxygen species present in circulation and simultaneously protects LDLs from oxidative damage (Gómez-Díaz *et al.*, 1997; Morré, D.J. & Morré, D.M., 2011).

CoQ₁₀ has been said to be responsible for the intonation of the β 2-integrin count on monocyte surfaces. Apparently, the uptake of CoQ₁₀ by monocytes and lymphocytes causes the release of signal substances and mediators into circulation which influences the expression of the NF κ B1-dependant genes. Therefore CoQ₁₀ exerts various anti-inflammatory effects. This prevents the extravasation of monocytes to damaged endothelial or intimal cells and oxidized LDLs and in this way prevents the progression of atherosclerosis and simultaneously protects LDL particles from oxidative damage (Turunen *et al.*, 2004). CoQ₁₀ also regulates the penetrability of the mitochondrial transition pores (Fontaine *et al.*, 1998) which, if opened, would permit the entry and escape of very large molecules. This would overwork the mitochondrial functions and lead to ATP depletion and consequential apoptosis because the membrane gradient potential would depolarize, cytochrome *c* would be translocated outside the mitochondria, and caspase-9 would be activated (Papucci *et al.*, 2003). The uncoupling proteins (UCPs) transport H⁺ ions from the mitochondrial intermembrane space to the matrix, and uncouple the OXPHOS system so heat is generated rather than energy. The uncoupling at UCP1 cannot commence in the absence of the obligatory cofactor of the reaction, namely CoQ₁₀ (Echtay *et al.*, 2001).

CoQ₁₀ serves as a mediator for electron transport in the plasma membrane during its interaction with the CoQ-dependent NADH oxidase (NOX) protein which is transversed over the external side of the plasma membrane (DeHahn *et al.*, 1997). NOX enables cell development and differentiation as it regulates the NAD⁺/NADH ratio and the amount of reduced ascorbate in circulation. Thus, in aiding development and differentiation, CoQ₁₀ plays an indirect role in differentiation and endothelial redox status regulation. It has been suggested that CoQ₁₀ contributes to endothelial function (vasodilatation) (Watts *et al.*, 2002) possibly through its stimulation of nitrogen oxide (NO) production (McCarty, 1999).

Furthermore, the formation of ubisemiquinone in CI, II and CIII at the oxidoreduction sites for CoQ₁₀ during electron transport is a major source of superoxide. Superoxide formation results in cellular H₂O₂ production which assists in cell signaling through the second messenger system. Linnane *et al.*, 2007 explains how the involvement of CoQ₁₀ as pro-oxidant in sub-cellular membrane ATP production systems is a major contribution to the H₂O₂ second messenger system. The second messenger system aids in the control of gene expression/transcription, modulation of protein activation and turnover, regulation of redox potential levels across sub-cellular membranes, and this messenger system mediates extracellular signaling of hormones and growth factors (Linnane *et al.*, 2007; Rhee, 2006).

2.3 Coenzyme Q₁₀ deficiency

CoQ₁₀ deficiency is a clinically and genetically heterogeneous mitochondrial disorder (Emmanuele *et al.*, 2012; Quinzii & Hirano, 2010). Patients affected by CoQ₁₀ deficiency have varying degrees of CoQ₁₀ shortage (Montero *et al.*, 2005). The resultant clinical phenotype of the CoQ₁₀ deficiency greatly depends on the location and the extent of the depletion in the affected tissue (Bentinger *et al.*, 2010) because the rate of synthesis and utilization of CoQ₁₀ are tissue specific (Dallner & Sindelar, 2000). Decreased muscle CoQ₁₀ levels are said to be an indication of a myopathic form of CoQ₁₀ deficiency (Lalani *et al.*, 2005), whereas a decrease in muscle and fibroblasts are an indication of an encephalomyopathic form (with predominant brain involvement) and more severe forms of CoQ₁₀ deficiency (Rötig *et al.*, 2000; Rahman *et al.*, 2001; Musumeci *et al.*, 2001). Decreased levels of CoQ₁₀ have been frequently found in patients with mitochondrial encephalomyopathies such as Kearns-Sayre syndrome (Ogasahara *et al.*, 1989; Sobreira *et al.*, 1997; Boitier *et al.*, 1998; Montero *et al.* 2005). Since the first case report of a CoQ₁₀ deficiency associated with a mitochondrial encephalomyopathy (Ogasahara *et al.*, 1989), CoQ₁₀ deficiency has been identified in more than 149 patients of all ages (Emmanuele *et al.*, 2012).

Because the activities of combined CI+III and CII+III enzyme complexes are dependent on ubiquinone availability, and the isolated ETC complexes are not, a deficiency of CoQ₁₀ causes decreased activity of the combined CI+III and CII+III (reviewed by Montero *et al.*, 2007). Therefore, in association with normal levels of the other isolated ETC enzyme activities, a CI+III and CII+III ETC deficiency is especially suggestive of a CoQ₁₀ deficiency (Rahman & Hanna, 2009). Concerning the etiology of CoQ₁₀ deficiencies, there is little conformity. The molecular genetic bases involved as well as the pathogenesis and pathophysiological consequences of this deficiency are still essentially speculative (Quinzii *et al.*, 2010).

CoQ₁₀ deficiency is a rare example of a Mendelian defect in the ETC (Rötig *et al.*, 2000; Rahman & Hanna, 2009). In most case reports, children are affected while parents are typically unaffected, which suggests an autosomal recessive pattern of inheritance considering that all the enzymes involved in the biosynthesis of CoQ₁₀ are nuclear encoded (Rötig *et al.*, 2007; Quinzii & Hirano, 2010). Most of the patients with CoQ₁₀ deficiency lack genetic diagnosis (Trevissan *et al.*, 2011), though causative genetic defects of CoQ₁₀ deficiency have been confirmed in some case reports (López *et al.*, 2006; Quinzii *et al.*, 2006; Mollet *et al.*, 2007, 2008; Duncan *et al.*, 2009; Quinzii & Hirano, 2011; Ashraf *et al.*, 2013). The genetic definition of patients with CoQ₁₀ deficiency has allowed distinguishing between primary CoQ₁₀ and secondary CoQ₁₀ deficiencies (Quinzii & Hirano, 2011). Primary CoQ₁₀ deficiencies are caused by mutations in COQ genes (Figure 2.7) directly involved in its biosynthetic pathways. Secondary CoQ₁₀ deficiencies are caused by mutations in genes not directly involved in the biosynthesis of CoQ₁₀ (Potgieter *et al.*, 2013).

There have been four main phenotypes identified that are commonly associated with CoQ₁₀ deficiency, namely i) a severe multisystemic infantile disease, ii) a widespread variant encompassing encephalomyopathy and nephropathy, iii) a pure myopathic, and iv) a cerebellar ataxic phenotype (Rötig *et al.*, 2007). Rötig *et al.* (2007) investigated the clinical spectrum for CoQ₁₀ deficiency in infants and pediatric patients, which was much the same as the spectrum recognized in adult patients. In adults there are five main clinical phenotypes, with two distinct phenotypes of encephalomyopathy and nephropathy as opposed to the widespread infantile form (Quinzii & Hirano, 2010). However, primary CoQ₁₀ deficiencies are expanding in clinical phenotypes, and therefore these traditional clinical phenotypes have become somewhat outdated. In addition to the nephrotic phenotype, steroid resistant nephrotic syndrome has been identified as a manifestation rarely associated with cytopathies, but typically associated with CoQ₁₀ deficiency. Other clinical features such as cardiomyopathy, deafness, optic atrophy, and various other combinations of symptoms are also found in patients with CoQ₁₀ deficiency (Desbats *et al.*, 2014).

Interestingly, the pure myopathic form has not been reported in association with a primary CoQ₁₀ deficiency (Trevisson *et al.*, 2011). Reduced CI+II and CII+III activities consistently presented in all of the clinical phenotypes of primary CoQ₁₀ deficiency, except for the cerebellar ataxia form (Musumeci *et al.*, 2001; Artuch *et al.*, 2006). All four phenotypes described in infants and pediatric patients presented with decreased muscle CoQ₁₀ concentrations (Rötig *et al.*, 2007). The clinical variability observed in CoQ₁₀ deficiency concerns the wide range in the age onset, varying from days to 7 decades. The severity of the disease also varies from the severe multisystemic form to the milder tissue specific manifestations (Desbats *et al.*, 2014). The clinical phenotype depends on the severity and the location of the deficiency (Bentinger *et al.*, 2010), thus a varying pattern of the tissue involvement is also observed, even between patients with the same gene mutation. Patients also respond differently to supplementation (Desbats *et al.*, 2014).

The lack of CoQ₁₀ may cause a human disease through several processes including a respiratory chain deficiency (RCD) (Quinzii *et al.*, 2008b), impaired pyrimidine biosynthesis (López-Martín *et al.*, 2007), increased ROS production or susceptibility, or both a RCD and increased production (or susceptibility) of ROS (Quinzii *et al.*, 2008b). Primary CoQ₁₀ deficiencies are caused by defects in the genes of the biosynthetic pathway of CoQ₁₀. Primary CoQ₁₀ deficiencies seem to correlate with the production of ROS. Severe (< 20% of normal CoQ₁₀) CoQ₁₀ deficiency shows little or no increase in ROS production leading to oxidative stress and markedly decreased ATP production, but the cell seems to deliberately resist the mitochondrial stress-response and induced apoptosis. This conferred resistance may be explained by the fact that the severe RCD causes a lack of electron flux along the ETC which would have heightened the sensitivity to the pathways leading to apoptosis. Partial CoQ₁₀ deficiency (30 - 40% of normal CoQ₁₀) on the other hand, has significantly increased ROS production while ATP synthesis is only partially decreased and there is oxidation of cell constituents leading to cell death. Similar response to that of severe CoQ₁₀ deficiency is expected within a cell with > 60% of normal CoQ₁₀. These results therefore indicate that a partial CoQ₁₀ deficiency leading to a mild RCD would be more deleterious than a severe RCD for some cellular functions (aerobic respiration with preference to glycolytic substrates) (Quinzii *et al.*, 2010).

The underlying mechanisms of secondary CoQ₁₀ deficiency are widely unknown. There are many functions of CoQ₁₀. It has a pivotal function as electron transport molecule in the ETC and is a potent lipophilic antioxidant in its reduced form (Ernster & Dallner, 1995; Turunen *et al.*, 2004).

The Q-pool serves as a reservoir for CoQ₁₀'s functions such as binding to the mitochondrial permeability transition pore and activating uncoupling proteins (reviewed by Lenaz & Genova, 2009). CoQ₁₀ also assists efficient ATP production in sub-cellular membrane systems to enable nerve conduction, and metabolite translocation (Linnane *et al.*, 2007). Depletion of CoQ₁₀ therefore may cause a variety of biochemical effects. The secondary CoQ₁₀ deficiencies are hypothetically caused by decreased electron transport and/or increased degradation of CoQ₁₀ through largely unknown mechanisms (Bujàn *et al.*, 2014). This results in mitochondrial dysfunction and impaired ATP production (Artuch *et al.*, 2006; reviewed by Montero *et al.*, 2007). The presence of increased ROS levels may be an indication of involved oxidative stress in the disease associated with CoQ₁₀ deficiency (Sacconi *et al.*, 2010). The critical role of CoQ₁₀ concerning electron transport in the ETC explains, to a certain degree, why CoQ₁₀ deficiency is consistently associated with mitochondrial ETC chain disorders. The frequent association of CoQ₁₀ deficiency in patients with mitochondrial disorders suggests that CoQ₁₀ may also contribute to some extent to the symptoms in these patients with secondary CoQ₁₀ deficiency. Being a potent lipophilic antioxidant itself, CoQ₁₀ scavenges ROS in the IMM and is responsible for the reactivation of other antioxidants (Sacconi *et al.*, 2010). Oxidative stress due to abnormal ROS generation in the mitochondrion has been said to decrease endogenous levels of CoQ₁₀ in humans (Beal *et al.*, 2002). Decreased CoQ₁₀ will cause a collapse of inter- and intra-complex electron transfer within supercomplexes, and between CI and CII to CIII during ATP production (reviewed by Lenaz & Genova, 2009). This often causes remarkably decreased activity of the ubiquinone dependent CI+III and CII+III enzyme complexes (Artuch *et al.*, 2006; reviewed by Lenaz & Genova, 2009). Therefore, looking at some of the pathological mechanisms underlying primary and secondary causes of CoQ₁₀ it is evident that an increase in either the generation or susceptibility of ROS and variable degrees of ETC deficiency (Mollet *et al.*, 2008), and apoptosis enhance the pathogenesis of CoQ₁₀ deficiency (Artuch *et al.*, 2006; Quinzii *et al.*, 2010).

The genes reported in patients with a secondary (acquired) CoQ₁₀ deficiency include the electron-transferring-flavoprotein dehydrogenase (ETFDH) (Gempel *et al.*, 2007), the APTX (aprataxin) gene (Quinzii *et al.*, 2005), and the BRAF gene (Aeby *et al.*, 2007). The ETFDH gene mutation is associated with a myopathic form of secondary CoQ₁₀ deficiency (Gempel *et al.*, 2007). Late onset GAll (type III) is said to be an allelic disease to the pure myopathic phenotype of secondary CoQ₁₀ caused by an ETFDH gene defect (Gempel *et al.*, 2007; Quinzii & Hirano, 2010). Other case reports of this same phenotype reported patients harboring the same MADD and ETFDH mutations but with normal muscle CoQ₁₀ levels (Quinzii & Hirano, 2010).

In GA II, incomplete metabolism of amino acids or fatty acids is a result of a faulty electron transfer from the FAD-containing coenzyme-A dehydrogenases, from amino acid metabolism, or fatty acid metabolism to CoQ₁₀ within the ETC. Electron-transferring-flavoprotein dehydrogenase (ETF dehydrogenase), also known as electron transfer flavoprotein-ubiquinone oxidoreductase, together with the electron transfer flavoprotein (ETF), form a component of the ETC, namely electron transfer flavoprotein:ubiquinone oxidoreductase (ETF-QO).

This ETF-QO component provides a short pathway for electron transfer between numerous mitochondrial flavoprotein dehydrogenases and the Q-pool in the inner mitochondrial membrane (reviewed by Watmough & Frerman, 2010). A possible explanation for this secondary CoQ₁₀ deficiency may be that, because CoQ₁₀ is said to accept electrons directly from the ETF, a dysfunction or decrease in the ETF's activity may cause a downregulation of CoQ₁₀ synthesis by some feedback mechanism (Gempel *et al.*, 2007), causing a CoQ₁₀ deficiency. CoQ₁₀ attracts electrons from NADH within CI (Ernster *et al.*, 1969), from FADH₂ in CII (Lancaster, 2002), and from intermediate metabolites of β -oxidation (DiMauro & Rustin, 2009) and the Q-pool serves as the main intermediate for electron transfer. An abnormal decrease in the Q-pool concentration would cause a decrease of the protein-bound CoQ₁₀ within the inner mitochondrial membrane so electrons are not optimally transferred (Kamzalov *et al.*, 2003; reviewed by Lenaz & Genova, 2009).

An alternative explanation for the CoQ₁₀ deficiency may be that the faulty binding of CoQ₁₀ to the ETF may cause degradation of the acceptor molecule which is CoQ₁₀ (Gempel *et al.*, 2007). Gempel *et al.* (2007) reported that a missense mutation in the EFTDH gene, encoding the EFT ubiquinone oxidoreductase, influenced the C-terminal of the protein and possibly caused impairment of the catalytic activity by changing the folding or stability of the EFT protein so electrons are no longer efficiently transferred via ETF-QO to the Q-pool. Gempel *et al.* (2007) reported that the patients with EFTDH mutations had combined CII+III deficiency accompanied by a rigorously decreased activity of isolated CI, and moderately decreased CIV activity in vastus lateralis muscle homogenates. Muscle CoQ₁₀ levels were also reduced below normal in all patients. Though the genetic basis differ and are in some cases still unknown, most cases of secondary myopathic CoQ₁₀ deficiency seem to be secondary to multiple acyl-CoA dehydrogenase deficiency (MADD), including mild Leigh syndrome (Rahman *et al.*, 2012), and very long chain acyl-CoA dehydrogenase deficiency (Bujàn *et al.*, 2014). The β -oxidation metabolic pathway is essential for formation of long-chain fatty acids that meet the energy needs of both skeletal and heart muscle. A missense mutation in the EFTDH gene inhibits fatty acid metabolism via β -oxidation which also prevents ketone body synthesis, a source of energy for the heart and brain.

Inhibition of β -oxidation may also cause cardiomyopathic hypertrophy associated with lipid deposit in cardiac muscle tissue, and secondary carnitine deficiency (reviewed by Watmough & Frerman, 2010). The cerebellar ataxia phenotype is the most common phenotype among patients with primary CoQ₁₀ deficiency in muscle (Rötig *et al.*, 2007). The APTX gene encodes for the protein aprataxin, involved in the repair of double stranded DNA. The lack of functional aprataxin is known to cause ataxia-oculomotor-apraxia 1 (AOA1) which is accompanied with cerebellar ataxia, decreased CoQ₁₀ levels in fibroblasts and muscle, along with pyramidal signs and seizures (Quinzii *et al.*, 2008a). The BRAF gene mutation was involved in cardiofaciocutaneous syndrome marked by a congenital defect of the heart, developmental delay and odd facial constriction. Further knowledge on the pathogenic mechanisms of these defects are unknown. However, toxicity caused by excessive ROS production has been said to be a communal mechanism for the development of at least three forms of inherited ataxia: abetalipoproteinemia, Friedreich's ataxia, and ataxia related to isolated vitamin E deficiency. Therefore, CoQ₁₀ deficiency is possibly another form of hereditary ataxia caused by oxidative damage (Musumeci *et al.*, 2001).

Secondary CoQ₁₀ deficiencies have also been identified in patients harboring mitochondrial DNA (mtDNA) point mutations or deletions (Rahman *et al.*, 2012; Sacconi *et al.*, 2010). A mtDNA depletion syndrome has also been identified as a cause of a secondary CoQ₁₀ deficiency as a result of the reduction of the mutation important nDNA gene. However, because the CoQ₁₀ biosynthetic genes are nuclear encoded, a mtDNA depletion syndrome may also give rise to primary CoQ₁₀ deficiency. Certain genetic factors which are able to bestow susceptibility to develop secondary CoQ₁₀ deficiency have also been described in patients with secondary CoQ₁₀ deficiency (Sacconi *et al.*, 2010). Therefore mutagenic changes in additional genes may also be causative/contributing factors in the pathogenesis of this deficiency (Quinzii *et al.*, 2008a; Sacconi *et al.*, 2010). Secondary CoQ₁₀ deficiencies may also be caused by insufficient dietary intake of CoQ₁₀, drug induced biosynthetic inhibition, or excessive cellular utilization (Potgieter *et al.*, 2013). Drug induced inhibition of CoQ₁₀ biosynthesis by pharmaceuticals *e.g.* statins frequently result in a secondary CoQ₁₀ deficiency. Statins are prescribed to patients with hypercholesterolemia for the non-selective inhibition of HMG-CoA, the first enzyme in the mevalonate biosynthetic pathway of cholesterol and therefore possibly resulting in a secondary CoQ₁₀ deficiency as it affects the synthesis of molecules such as dilichol and CoQ₁₀ which also share mevalonate as a precursor (Deichmann *et al.*, 2010). Secondary CoQ₁₀ deficiency has also been characterized by combined CI+III and/or CII+III ETC enzyme deficiency (Gempel *et al.*, 2007). The encephalopathic form of CoQ₁₀ deficiency is characterized by a triad of encephalopathy (mental retardation, seizures or both), ragged-red fibres (RRF), and myopathy with recurrent myoglobinuria.

The pure myopathic form of CoQ₁₀ deficiency is characterized by a combination of markedly increased creatinine kinase, myopathy and histological results indicating increased lipid storage in association with the CoQ₁₀ deficiency in muscle. The considerable similarity of CoQ₁₀ deficiency with other mitochondrial disorders makes the clinical identification of CoQ₁₀ deficiency a near impossible task (Rahman *et al.*, 2012).

Additionally, the diverging and complex genotype and phenotype of CoQ₁₀ deficiencies along with the frequent occurrence of secondary CoQ₁₀ deficiencies complicate the diagnosis of these deficiencies (Buján *et al.*, 2014). Though these features are helpful in the identification of a suspected CoQ₁₀ deficiency, they are insufficient for definite diagnosis of a deficiency, and also insufficient to distinguish between primary and secondary CoQ₁₀ deficiency. Therefore, diagnosis of a CoQ₁₀ deficiency will rely on biochemical evaluation, and diagnosis of this is confirmed by direct quantification of CoQ₁₀ levels in muscle (Rahman *et al.*, 2012). Decreased levels of CoQ₁₀ in muscle or fibroblasts have been denoted as the “hallmark” for CoQ₁₀ deficiency, because other markers (pyruvate, lactate, organic acids, alanine, and CoQ₁₀ concentrations) of mitochondrial dysfunction in body fluid are normal in practically every case report (Montero *et al.*, 2005, reviewed by Montero *et al.*, 2007). Thus, for evaluation of CoQ₁₀ levels, skeletal muscle is the tissue of choice (Quinzii & Hirano, 2010; Buján *et al.*, 2014). Histological results showing RRF and prominent lipid storage in muscle also aid in the diagnosis of a CoQ₁₀ deficiency, though they are not universal features (Trevissan *et al.*, 2011).

Patients with primary CoQ₁₀ deficiency present with varying clinical phenotypes associated with decreased CoQ₁₀ levels, and decreased CI+III and/or CII+III enzyme activity in muscle or fibroblasts (Rötig *et al.*, 2007; Emmanuele *et al.*, 2012). Besides the decreased CI+III and/or CII+III enzyme activity in muscle, primary CoQ₁₀ deficiencies are characterized by early-onset and greater severity of clinical symptoms, and a profound depletion of CoQ₁₀ within the affected tissue. Patients also respond remarkably well to CoQ₁₀ supplementation (Miles *et al.*, 2008; Quinzii *et al.*, 2008a; Potgieter *et al.* 2013). Secondary CoQ₁₀ deficiencies are usually not characterized by constant duration or severity of the disease nor progression, but rather a later onset, and a milder depletion of CoQ₁₀ (Miles *et al.*, 2008; Quinzii *et al.*, 2008a). These differences between primary & secondary CoQ₁₀ deficiency have diagnostic relevance (Miles *et al.*, 2008).

Decreased CoQ₁₀ levels in skin fibroblasts are also a good indication of a multisystemic CoQ₁₀ deficiency because of a defect in endogenous CoQ₁₀ biosynthesis (Quinzii & Hirano, 2010).

Other differences between these two forms of CoQ₁₀ deficiency (myopathic form and form with predominant brain involvement) besides the levels of CoQ₁₀ in tissue are the degree of CoQ₁₀ depletion. The myopathic form presents a profound decrease in the levels of CoQ₁₀ in muscle homogenates and a concomitant decrease in CII+III and CI+III enzyme activity. The encephalopathic form or ataxic form (with predominant brain involvement) present with a partial decrease in the levels of CoQ₁₀ in muscle. A greater depletion of cerebellar CoQ₁₀ levels is expected for the ataxic form (reviewed by Montero *et al.*, 2007). CoQ₁₀ deficiency predominantly affects children, who most commonly present with infantile onset cerebellar ataxia associated with seizures (Rahman *et al.*, 2012).

All the phenotypes presented in the CoQ₁₀ deficiency respond well to CoQ₁₀ supplementation (DiMauro *et al.*, 2007), especially patients with predominant muscle symptoms (Gempel *et al.*, 2007). Neurological symptoms associated with CoQ₁₀ deficiency show less improvement (Quinzii & Hirano, 2010) which may be attributed to the impaired distribution of CoQ₁₀ to tissues; in particular, CoQ₁₀'s restricted ability to distribute across the blood brain barrier (Bentinger *et al.*, 2003). However, publications have reported that prolonged treatment resulted in improved cerebellar symptoms (Artuch *et al.*, 2006). Extra-neurological signs such as cardiomyopathy and nephropathy are also treatable with CoQ₁₀ supplementation (Abey *et al.*, 2007). Therefore, early recognition of CoQ₁₀ deficiency with appropriate and timely treatment may avoid irreversible tissue damage in these infants. Additionally, early treatment commenced after early diagnosis shows maximal efficacy (Buján *et al.*, 2014).

Even partial CoQ₁₀ deficiency with normal respiratory chain CI+III and/or CII+III enzyme activities benefit from exogenous CoQ₁₀ supplementation. CoQ₁₀ treatment showed more drastic and rapid improvement of the clinical features in younger patients than in older patients with the cerebellar ataxic phenotype (Musumeci *et al.*, 2001), and patients with a primary CoQ₁₀ deficiency respond better to supplementation than patients with a secondary CoQ₁₀ deficiency (Detbats *et al.*, 2014).

2.3.1 Levels of CoQ₁₀ in ETC deficiency

CoQ₁₀ levels are influenced by ETC deficiencies in that their decrease can be secondary to the ETC deficiency (secondary CoQ₁₀ deficiency) or the ETC deficiency may be secondary to a defect in CoQ₁₀ biosynthesis (primary CoQ₁₀ deficiency). On the contrary, all the ETC enzyme complex activities may also be normal while the patient has mild/partial (30 - 40% of normal CoQ₁₀) CoQ₁₀ deficiency (Sacconni *et al.*, 2010; Quinzii *et al.*, 2008b).

Though CoQ₁₀ levels in ETC deficiencies are described with variability in the degree of CoQ₁₀ shortage, age of onset, and severity of the clinical manifestation (Rötig *et al.*, 2007; Mollet *et al.*, 2008), decreased levels of CoQ₁₀ have consistently been associated with combined ETC complex (CI+III and/or CII+III) deficiencies (Emmanuele *et al.*, 2012). In fact, Montero *et al.* (2005) observed significant positive correlation between the enzyme activities of CI+III and CII+III and the respective CoQ₁₀ content in the muscle homogenates of 34 pediatric patients and 2 adult patients. Several studies on CoQ₁₀ deficiency in pediatric patients with ETC deficiencies had isolated ETC complexes with normal levels of activity while the quinone dependent enzyme activities CI+III and CII+III were markedly decreased in the affected muscle (Ogasahara *et al.*, 1989; Sobreira *et al.*, 1997; Boitier *et al.*, 1998; Rötig *et al.*, 2000; Rahman *et al.*, 2001; Lalani *et al.*, 2005; Montero *et al.*, 2005; Salviati *et al.*, 2005; Artuch *et al.*, 2006; López *et al.*, 2006; Gempel *et al.*, 2007; Miles *et al.*, 2008; Montero *et al.*, 2009; Sacconi *et al.*, 2010).

In general, when reduced CI+III and CII+III activity are accompanied by normal activities for the other isolated (CI and CIII) ETC enzymes, it indicates severe CoQ₁₀ depletion (< 20% of normal CoQ₁₀) (Quinzii & Hirano, 2010; Quinzii *et al.*, 2008b). Yet there are the exceptions, where a patient has lower levels of CII+III activity (along with an isolated CIII deficiency), but with normal levels of CoQ₁₀ (> 60% of normal CoQ₁₀) (Mollet *et al.*, 2007; Quinzii *et al.*, 2008b), or where the muscle CoQ₁₀ content of patients with isolated ETC deficiencies (Musumeci *et al.*, 2001) have been reported to be relatively higher (Montero *et al.*, 2005), substantially higher (Ogasahara *et al.*, 1989), similar to (Salviati *et al.*, 2005), or even lower than (Lalani *et al.*, 2005) the muscle CoQ₁₀ content of patients with combined CI+III and CII+III deficiencies. A male with a combined complex deficiency of complexes CI+III+IV reported by Musumeci *et al.* (2001) also had higher muscle CoQ₁₀ content than the patients with CI+III and CII+III deficiencies reported by Montero *et al.* (2005). Primary CoQ₁₀ deficiencies are marked by increased severity, early onset and a profound depletion of CoQ₁₀ within the affected tissue (Quinzii *et al.*, 2008a). A study on the biochemical alterations in bioenergetics, anti-oxidant defenses, and oxidative stress of different gene mutations of primary CoQ₁₀ deficiencies indicated that CoQ₁₀ levels are in direct relationship with the decreased ATP synthesis through OXPHOS (Quinzii *et al.*, 2008b). Therefore, the lowest levels of CoQ₁₀ are expected to be associated with CI+III and CII+III deficiencies in infants and pediatric patient groups in comparison to isolated RCDs.

The measurement of CoQ₁₀ in muscle biopsy remains the most reliable test for CoQ₁₀ deficiency diagnosis (Rahman *et al.*, 2012). Thus, even when the exception of mild or normal (Mollet *et al.*, 2007) CI+III and CII+III activities were found, yet associated with a CoQ₁₀ deficiency, direct measurement of CoQ₁₀ in frozen skeletal muscle would still confirm diagnosis of a CoQ₁₀ deficiency.

It is even recommended that CoQ₁₀ be measured in all patient muscle samples suspected of a mitochondrial disorder, and not exclusively patients with CI+III and/or CII+III deficiencies (Rahman *et al.*, 2012). Total muscle CoQ₁₀ content (sum of the oxidized and reduced levels of CoQ₁₀) best represented the available CoQ₁₀ for respiratory activity (Miles *et al.*, 2008; Itkonen *et al.*, 2013), and is considered the best predictor of an ETC enzymatic complex abnormality in pediatric participants with supposed mitochondrial myopathy (Miles *et al.*, 2008). As mentioned in Section 2.2.3, the redox cycling character of CoQ₁₀ allows it to mediate inter- and intra-complex electron transport between CI and CII to CIII in the ETC (Lenaz *et al.*, 2007). This electron transfer is conducted either via protein bound or free flowing CoQ₁₀ within the Q-pools (reviewed by Lenaz & Genova, 2009). Bearing in mind protein bound CoQ₁₀ co-exists with the mobile pool of CoQ₁₀, and that an abnormal decrease in the Q-pool concentration would cause a decrease of the protein-bound CoQ₁₀ within the inner mitochondrial membrane (Kamzalov *et al.*, 2003), it seems reasonable that total muscle CoQ₁₀ content was identified as the best predictor of an ETC enzymatic complex abnormality (Miles *et al.*, 2008).

Because the percentages of the total CoQ₁₀ within the Q-pools differ between tissue types (reviewed by Lenaz & Genova, 2009), the total CoQ₁₀ determination ought to be carried out using the same tissue type (and preferably the same tissue sample) used for the ETC deficiency diagnoses. A suggested cut-off value of 55% of the calculated normal reference mean may serve as an indication of primary CoQ₁₀ deficiency (Lalani *et al.*, 2005). Established reference values of CoQ₁₀ content in skeletal muscle can contribute to the accurate diagnosis of CoQ₁₀ deficiency (Boitier *et al.*, 1998; Lalani *et al.*, 2005; Montero *et al.*, 2008).

2.4 Experimental approach of the study

A cohort of South African patients with suspected ETC deficiencies underwent muscle biopsy for respiratory chain analyses. The cohort consisted of patients diagnosed with respiratory chain deficiency and others in which no respiratory chain deficiency was detected. As mentioned in Chapter 1, the aim of this study was to quantify the levels of total CoQ₁₀ in a cohort of South African pediatric patients with suspected ETC deficiencies. For this purpose, an analytical research approach with high performance liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) with electrospray ionization in positive ionization mode (ESI(+)) was used to meet the listed objectives: (i) optimize the separation and quantification of CoQ₁₀ using LC-MS/MS, and (ii) investigate the levels of CoQ₁₀ in ETC deficiencies by quantifying total CoQ₁₀ in South African patient samples using a standardized LC-MS/MS method. To accomplish these objectives, an experimental approach was laid out as depicted in Figure 2.8 with the first step being the optimization of the LC-MS/MS method.

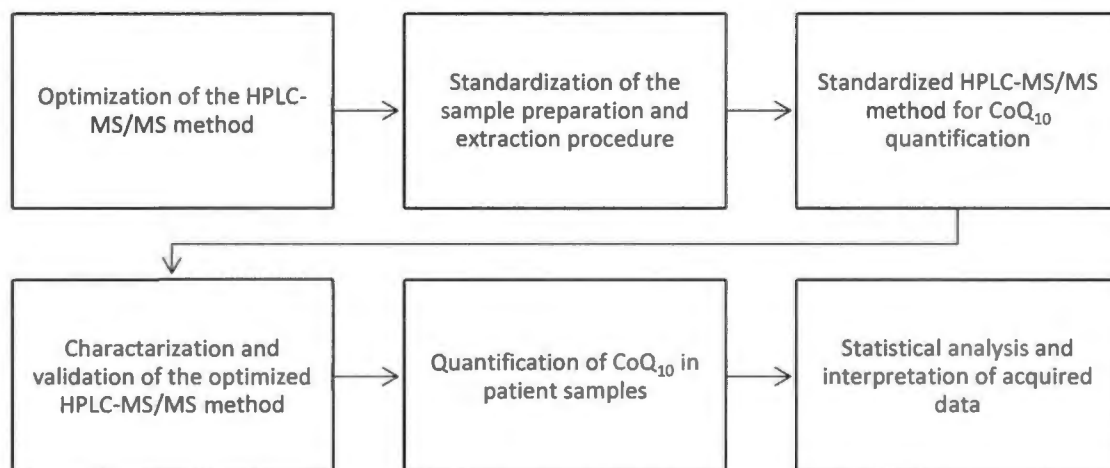


Figure 2.8: Diagram of the experimental approach of this study. The first step taken in this approach was the optimization of the analytical separation (LC) and detection (MS/MS) techniques. This optimized LC-MS/MS method was then used in the standardization of the sample preparation procedure. This produced a standardized LC-MS/MS method for the quantification of CoQ₁₀ in muscle homogenate supernatants. Validation of the linearity of the standardized LC-MS/MS method permitted the submission of patient samples to the standardized LC-MS/MS method. The levels of CoQ₁₀ were then quantified and the sample data was statistically analyzed, interpreted, and discussed.

In terms of this study, optimization involved experimental determination of the optimal (as efficient as possible) performance of the influential parameters and variables of the LC-MS/MS method. Standard CoQ₁₀ samples were utilized to optimize the detection technique of mass spectrometry with electrospray ionization in positive ionization mode (ESI(+)-MS/MS), as well as the LC separation technique. These optimized parameters were then applied to standardize the sample preparation and extraction procedure. The preparation procedure involved a cell lysis and protein precipitation step, and a consecutive centrifugation step prior to organic extraction that prepared the samples for analysis with LC-MS/MS. Validation of the standardized LC-MS/MS method involved obtaining a calibration curve to determine the linearity of the detection method using Microsoft® Excel®¹ to construct a graph from the LC-MS/MS data obtained through Agilent®² qualitative analysis software. For the validation of the standardized method's performance in biological samples, the precision of the method was determined through calculation of the intra- (within run) and interday (between runs) coefficient of variance. After validation of the analytical research approach with LC-MS/MS, the patient samples were submitted to the standardized sample preparation procedure and LC-MS/MS method to quantify their levels of CoQ₁₀ followed by interpretation of the statistical inferences on all the sample measurements.

¹Microsoft Office® and Microsoft Excel® are registered trademarks of Microsoft Corporation in the United States and other countries.

²Agilent Technologies, Inc. (Agilent) is a leading measurement company providing services and products for chemical analysis and life sciences (Santa Clara, California, United States)

CHAPTER 3: STANDARDIZATION OF THE ANALYTICAL METHOD FOR THE QUANTIFICATION OF TOTAL CoQ₁₀ IN SKELETAL MUSCLE

3.1 Introduction

Few studies have reported on the quantification of CoQ₁₀ in human skeletal muscle (Duncan *et al.*, 2005; Montero *et al.*, 2005; Mollet *et al.*, 2008; Montero *et al.*, 2009; Duberley *et al.*, 2013a) and before 2013, there were no reports on the quantification of CoQ₁₀ with LC-MS/MS using a stable isotope as internal standard (Duberley *et al.*, 2013a; Itkonen *et al.*, 2013). Analytical techniques reported for CoQ₁₀ quantification include HPLC coupled to ultraviolet (UV) (Rötig *et al.*, 2000; Li *et al.*, 2006; Sacconi *et al.*, 2010), electrochemical detection (ECD) (Tang *et al.*, 2004; Montero *et al.*, 2005) or mass spectrometry (MS) (Teshima & Kondo, 2005; Lekli *et al.*, 2008; Li *et al.*, 2007; Ruiz-Jiménez *et al.*, 2007; Li *et al.*, 2008).

High performance liquid chromatography coupled to triple-quadropole tandem mass spectrometry (LC-MS/MS) ensures a rapid, selective and highly sensitive detection technique, and also provides rapid quantitative (mass/concentration) and qualitative (structural) information about the analyte, unlike the comparable ECD method (Kishikawa & Kuroda, 2013). Nevertheless, even successfully applied and established methods should be standardized and validated before being applied to clinical samples (Barshop & Gangoiti, 2007). The quantification of CoQ₁₀ has certain drawbacks concerning its ionization and fragmentation that limit the utility and sensitivity of these techniques (Hansen *et al.*, 2004; Duberley *et al.*, 2013a). CoQ₁₀ is a very large (863.34 g/mol) and highly hydrophobic molecule, and its amphipathic nature makes it prone to nucleophilic attack which complicates the ease of elucidation during the separation technique, and narrows down the scope of potential mobile phases (Tang *et al.*, 2001).

The analytical method in this study consisted of a sample preparation procedure in which the muscle CoQ₁₀ would be extracted prior to analysis with LC-MS/MS. The mass spectrometry and chromatography conditions had to be optimized before these parameters could secure a standardized analytical method for quantification of total CoQ₁₀ in skeletal muscle using a non-physiological internal quantification standard.

3.2 Reagents and standards

3.2.1 Stock solutions

Stock solutions of CoQ₁₀ (1 mg/mL) and the stable isotope (1 mg/mL), deuterium labeled CoQ₁₀ (CoQ₁₀[²H₆]) were prepared in *n*-hexane and stored in glass KIMAX[®] tubes with Teflon-lined caps at -80°C. Serial dilutions of the CoQ₁₀ and the stable isotope stock solutions yielded working standard samples of CoQ₁₀ (1 µg/mL) and the stable isotope (0.5 µg/mL). All dilutions were made with acetonitrile (MeCN) and the standard samples of CoQ₁₀ and the stable isotope were kept at -20°C. All stock solutions and standard dilutions were protected from light exposure by tin foil and kept in glass KIMAX[®] tubes. CoQ₁₀ is a thermolabile (Wolf *et al.*, 1958) and a light sensitive molecule (Kaikkonen *et al.*, 1999), thus all samples were handled at 4°C and in light protection, unless indicated otherwise.

All the reagents used in this study and the suppliers where they were purchased are specified in Appendix A.

3.2.2 Preparation of reduced CoQ₁₀

The preparation of ubiquinol (reduced CoQ₁₀ or CoQ₁₀H₂) through the reduction of ubiquinone (oxidized CoQ₁₀) was conducted essentially as described by Ruiz-Jiménez *et al.* (2007). A 20 µL ubiquinone sample (1 mg/mL) was transferred to a 1.5 mL Eppendorf[®] tube containing 180 µL of *n*-hexane. Another 50 µL methanol (MeOH) and 2 mg sodium borohydride (NaBH₄) were added, and the mixture was stirred for 3 minutes and allowed to stand at room temperature for 5 minutes. Then, 100 µL (100 µM) aqueous ethylenediaminetetraacetic acid (EDTA), prepared using deionized Milli-Q water (18Ω), was added. The tube was shaken for 1 minute and centrifuged for 3 minutes at 2220 x *g*. Ubiquinone is almost insoluble in water, whereas ubiquinol is thoroughly insoluble in water, or even an aqueous organic solution (Ruiz-Jiménez *et al.* 2007). Therefore, since the highly hydrophobic ubiquinol would not partition into the aqueous methanol phase, the concentration of the ubiquinol in *n*-hexane would be a 10 x dilution of the starting ubiquinone concentration (1 mg/mL), thus being 100 µg/mL. From the upper organic phase containing the extracted ubiquinol, 100 µL was carefully transferred to a 1.5 mL Eppendorf[®] tube and serial dilutions of ubiquinol were made with MeCN to a final concentration of 1 µg/mL.

³KIMAX[®] is a registered trademark of Gerresheimer Glass, Inc. (Vineland, New Jersey, United States)

⁴Eppendorf[®] is a registered trademark of Eppendorf AG and a life science company aimed at providing products for academic and commercial research laboratories in pharmaceutical, biotechnological, chemical and food industries that perform production, process analysis, and quality assurance (Hamburg, Germany, Europe)

The sample was then transferred to a pulled point glass vial insert in an Agilent⁵ 2 mL clear screw cap MS vial using a ± 150 mm Volac[®] disposable glass Pasteur pipette and placed in the MS autosampler tray to be immediately analyzed by LC-MS/MS. The complete reduction of high ubiquinone concentrations is evident by the disappearance of its distinct yellow color (Ruiz-Jiménez *et al.* 2007), and full conversion to ubiquinol can be confirmed by LC-MS/MS analysis.

3.3 Skeletal muscle sample preparation

Smuts *et al.* (2010) previously performed human skeletal muscle biopsies to eventually prepare 600 x g homogenate supernatants for biochemical analyses. These samples were utilized in this study. Sample workup is discussed in Section 4.2 of Chapter 4. On collection, Smuts *et al.* (2010) placed the muscle tissues in sterile containers and without any prior preparation, the whole tissue samples (± 300 mg) were immediately wrapped in tin foil, and placed in Eppendorf[®] or similar tubes to be frozen at -80°C . This procedure had been completed within 3 hours after sample collection and transport was done on dry ice.

3.3.1 Preparation of 600 x g muscle homogenate supernatants

To prepare a skeletal muscle homogenate, and consecutive 600 x g supernatant, a frozen muscle sample was thawed on ice. Tin foil was then removed. After preparing a cold, sterile metal plate (sterilized with 70% ethanol (EtOH)) it was used as a platform to cut off fat tissue from the muscle sample using a sterile blade to attain a clean portion of tissue sample (± 100 mg). Sterile tweezers was used, and washed with 70% EtOH after handling the sample. The sample was weighed, and a sterile blade was used to cut the sample as finely as possible. Then the sample was suspended in an isosmotic buffer [containing mannitol (210 mM), 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES) (5 mM), sucrose (70 mM), and ethylene glycol tetraacetic acid (EGTA) with the pH set to 7.2 using potassium hydroxide (KOH, 0.1 mM)] to a 10% (w/v) solution to be homogenized in a 30 mL tightly fitting, glass, motor-driven Potter-Elvehjem homogenizer. The 30 ml glass homogenizer was immersed in ice water while the sample was homogenized using 15 strokes, or rather until the homogenate appeared to have even dispersion and no solid parts. Next, the homogenate was centrifuged at 600 x g at 4°C for 10 minutes, and lastly, the supernatant was transferred to a clean Eppendorf[®] or micro-centrifuge tube to be frozen at -80°C until further analysis.

⁵Agilent Technologies, Inc. (Agilent) is a leading measurement company providing services and products for chemical analysis and life sciences (Santa Clara, California, United States)

[®] Volac is a registered trademark of Poulten & Graf Ltd, an international enterprise specializing in the manufacture and distribution of high quality volumetric glassware, medical syringes, and dispensers (Germany, Wertheim and England, London)

3.3.2 Sample preparation prior to LC-MS/MS analysis

For the purposes of this study, a skeletal muscle supernatant sample (prepared as described in Section 3.3.1) was thawed on ice and a volume of 30 μL from was then transferred to a marked novel KIMAX[®] tube along with 30 μL (0.5 $\mu\text{g/mL}$) of the stable isotope, deuterium labeled CoQ₁₀ (CoQ₁₀[²H₆]) (0.5 $\mu\text{g/mL}$) using a micropipette. All marked KIMAX[®] tubes were protected from light exposure by tin foil to avoid photochemical decomposition of CoQ₁₀ (Kaikkonen *et al.*, 1999) and stored at -80°C until further analysis. For preparation prior to LC-MS/MS analysis, a KIMAX[®] tube containing the supernatant and stable isotope was thawed at room temperature. After the sample had been thawed, a volume of 1 mL of a sodium dodecyl sulphate (SDS) solution (0.1 M) was then added with a micropipette. After thoroughly mixing and then vortexing the glass tube for 30 sec, 1 mL of a freezer kept (-20°C) ethanol:isopropanol (95:5, v/v) solution was added as a protein precipitation step (Lang *et al.*, 1986; Benoist *et al.*, 2003). The tube was then mixed and briefly vortexed for 10 seconds prior to the liquid-liquid extraction, which was conducted similar to the extraction by Ruiz-Jiménez *et al.* (2007) in a fume cabinet.

The sample mixture was extracted 2 x with 2 mL *n*-hexane. The micropipette tip was replaced between every addition of *n*-hexane to the sample mixture. After the first addition of 2 mL *n*-hexane to the aqueous sample mixture, the organic extraction commenced as follows: The mixture was then whirl mixed in a rotor for 5 minutes, and afterwards briefly and thoroughly mixed before centrifugation at 4°C for 1 minute at 1833 x *g*. After centrifugation, the upper organic phase was transferred to a second KIMAX[®] tube using a disposable ± 150 mm Volac[®] glass Pasteur pipette and kept momentarily at 4°C. The second volume of 2 mL *n*-hexane was then added to the aqueous sample mixture for the second extraction conducted as with the first addition of 2 mL *n*-hexane.

Meanwhile, *i.e.* while the second extraction mixture had been whirl mixed, 30 μL of an ethanolic 1,4-Benzoquinone (0.4 mg/mL) was added to the first organic extract to oxidize any present ubiquinol (CoQ₁₀H₂) to ubiquinone (CoQ₁₀) essentially as described by Hansen *et al.* (2004). This extract was then vortexed for 5 seconds to enhance the oxidation of ubiquinol. After centrifugation, the second organic extract was added to the first. The extract pool was then very briefly vortexed for even dispersion of 1,4-Benzoquinone and dried under nitrogen gas (N₂) in light protection for approximately 20 minutes at room temperature. After this, the sample residue was reconstituted in 60 μL of 100% MeCN which would yield a 50% CoQ₁₀ concentration of the original 30 μL sample and a final concentration of 0.25 $\mu\text{g/mL}$ for the stable isotope. The reconstituted sample was vortexed for 20 seconds and carefully transferred using a glass Pasteur pipette to a pulled point glass vial insert in a clear 2 mL Agilent screw cap MS vial to be analyzed by LC-MS/MS.

3.4 Analytical equipment and apparatus

Sample MS vials were placed in a MS autosampler tray which was constantly held at 4 °C. For sample handling and mobile phase delivery, an Agilent 1290 Infinity LC (1290-Bin Pump) was used. The LC also consisted of an autosampler, a vacuum degasser, a column compartment with thermostat control, and an injection needle flush port. An Agilent ZORBAX StableBond SB-C18 narrow-bore (2.1 x 30 mm, 3.5 µm) column-cartridge was used in reversed phase chromatography. A Zorbax SB-Aq guard column (2.1 x 150 mm, 5 µm) was used to guard the column-cartridge.

For detection of the analytes, an Agilent 6460 Triple Quadrupole mass spectrometer (MS) was used. The eluted sample molecules entered the MS via an electrospray ionization (ESI) interphase in positive ionization mode. Nitrogen gas was used as nebulizing gas. The 6460 MS uses Agilent Jetstream Technology for thermal gradient focusing to collimate the ion spray/nebulizer spray through an extremely hot sheath gas (Mordehai & Fjeldsted, 2009). The LC and the mass spectrometer were interfaced by a computer running MassHunter data acquisition software. This software supported the autosampler (Anon., 2008) that enabled programmed sample injection onto the LC column. MassHunter software provided extracted and integrated chromatograms of a detected analyte's mass spectrum, and enabled comparison of different data sets. MassHunter also enabled the set-up of the acquisition method and the sample work list, on-screen tuning of the mass spectrometer, as well as programmed management and monitoring of instrument parameters (Anon., 2008).

The LC-MS/MS system performed detection when the ionized molecules in the electrospray were eventually transferred into a gaseous phase in the ESI source and accelerated through three separate sets of parallel rods of the MS system, often called 'triple-quad'. The triple-quad system is organized in a linear fashion, and can emit an electrical field that either deflects ions, or conveys ions to arrive at the MS detector, all according to their mass/charge (m/z) ratio (reviewed by Ho *et al.*, 2003). The first (Q1) and third (Q3) set of parallel rods are mass analyzers, and the second middle hexapole (Q2) is a collision cell (reviewed by Ho *et al.*, 2003). The influence of an electric or magnetic field on the movement of the ions based on their m/z ratios is the main principle on which molecules are separated within the MS. During mass spectrometry Q1 mass selects the analyte of interest (precursor ion) which then reaches Q2 where the precursor ion is activated by collision with a collision gas to undergo further fragmentation (a process known as collision induced dissociation, CID) and form a unique product ion/fragment ion as a result.

The product ion is structurally related to the precursor ion and can be monitored by Q3 to provide qualitative (structural) information about the analyte ion before arriving at the detector of the MS. The moment the ions impact the detector, it generates a signal which is recorded by the computer running the data acquisition software, *i.e.* MassHunter in this study. These signals are graphically displayed as a mass spectrum that shows the relative abundance of the recorded signals according to the m/z ratios of the detected analyte ions (reviewed by Ho *et al.*, 2003).

3.5 Standardization of the mass spectrometry conditions for the detection of CoQ₁₀

Optimal mass spectrometry conditions for the detection of the target analytes were determined using MassHunter optimizer software. The optimizer software provided automatic optimization of data acquisition parameters for each compound analyzed. Specifically, it automated the selection of the best precursor ion, along with the optimization of the fragmentor voltage, and automated selection of the best product ion and the optimal collision energy for each compound analyzed (Anon., 2010). The compounds analyzed were ubiquinone (CoQ₁₀), ubiquinol (CoQ₁₀H₂) and deuterium labeled ubiquinone (CoQ₁₀[²H₆]). With ammonium formate as ionization agent, the theoretical precursor and product ion ratio transitions for ubiquinone and ubiquinol were 880.7 m/z - 197.2 m/z and 882.7 m/z - 197.2 m/z , respectively (Ruiz-Jiménez *et al.*, 2007; Hansen *et al.*, 2004), and the tentative precursor and product ion ratio transition for the stable isotope was 886.7 m/z - 203.2 m/z , derived from the similarity of its structure to CoQ₁₀ (see Figure 3.2, Section 3.5.1.1). The product ion scan of the protonated adducts ($[M + H]^+$) and ammonium formate adducts ($[M + NH_4HCO_2]^+$) at 880.7 m/z and 863.7 m/z , respectively, both gave a product ion of 197.2 m/z because of the elimination of the long isoprenoid side chain. Likewise, CoQ₁₀H₂ also gave a 197.2 m/z product ion for its precursor ion at 882.7 m/z (Figure 3.1).

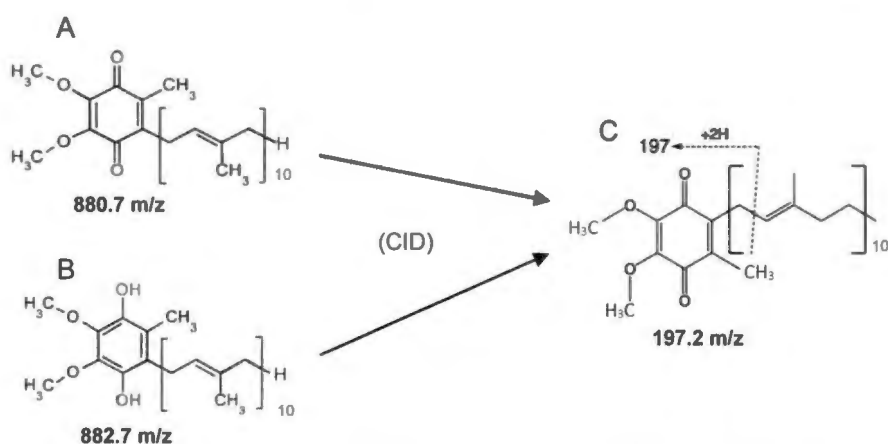


Figure 3.1: Selected transitions for the detection and quantification of the target analytes. Product ions of CoQ₁₀ (A) and CoQ₁₀H₂ (B) with the resulting product ion (C) derived from elimination of their isoprenoid side chains. Figure adapted from Teshima & Kondo, 2005.

3.5.1 Analytical conditions

A ubiquinone (0.5 µg/mL), ubiquinol (0.5 µg/mL), and a stable isotope (0.5 µg/mL) standard was prepared in a 100% MeCN in 2 mL Agilent screw cap MS vials. The mobile phase consisted of 100% methanol, 5 mM ammonium formate solution. 0.1 µL of each standard was separately infused into the MS via ESI in positive mode. Ramp parameters for the fragmentor voltage and the collision energy were set in the range of 80 V – 200 V, and 0 V – 50 V, respectively. The flow and temperature of the drying gas (N₂) were 6 L/min and 300°C, respectively. The nebulizer pressure was 30 psi. The capillary voltage was set at 3500 V and the nozzle voltage at 500 V. The dwell time was 50 ms for each ion species.

3.5.1.1 Results and discussion

The optimal mass spectrometry conditions for the detection and quantification of CoQ₁₀ and CoQ₁₀H₂ with LC-MS/MS are summarized in Table 3.1. MassHunter automated the selection of the best (most abundant) precursor and product ions for all three the compounds namely, CoQ₁₀, CoQ₁₀H₂, and CoQ₁₀[²H₆], as well as each precursor ion's optimal fragmentor voltage, and selected each compound's optimal collision energy for each transition. The optimal and collision energy fragmentor voltages were identified as such because they gave the greatest abundance of detected product ions, *i.e.* the best ratio transitions for each compound analyzed. With ammonium formate as ionization agent, the optimal precursor and product ion ratio transitions for the detection and quantification of ubiquinone (CoQ₁₀) and ubiquinol (CoQ₁₀H₂) were 880.73 m/z - 197.2 m/z and 882.73 m/z - 197.2 m/z, respectively. The optimal ratio transitions for the protonated adducts ([M + H]⁺) of ubiquinone (CoQ₁₀) and ubiquinol (CoQ₁₀H₂) were 863.73 m/z - 197.2 m/z and 865.73 m/z - 197.2 m/z, respectively. These results (Table 3.1) were used throughout the rest of the study as the optimal mass spectrometry conditions for the detection of CoQ₁₀. The optimal ratio transition for ammonium formate adduct of the stable isotope was 886.73 m/z – 203.1 m/z, and the optimal ratio transition for the protonated ion adduct of the stable isotope was not determined. Figure 3.2 illustrates the chemical structure of the stable isotope (CoQ₁₀[²H₆]) for which optimal mass spectrometry conditions was also obtained for its detection (Table 3.1).

Table 3.1: Multiple reaction monitoring conditions for the quantification of CoQ₁₀, CoQ₁₀H₂, and the internal standard (CoQ₁₀[²H₆]).

Compound	Ionization adduct	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision energy (V)
CoQ ₁₀	[M+NH ₄ HCO ₂] ⁺	880.73	197.2	137	12
CoQ ₁₀ H ₂	[M+NH ₄ HCO ₂] ⁺	882.73	197.2	146	24
CoQ ₁₀	[M + H] ⁺	863.73	197.2	185	38
CoQ ₁₀ H ₂	[M + H] ⁺	865.73	197.2	200	34
CoQ ₁₀ [² H ₆]	[M+NH ₄ HCO ₂] ⁺	886.73	203.1	132	20

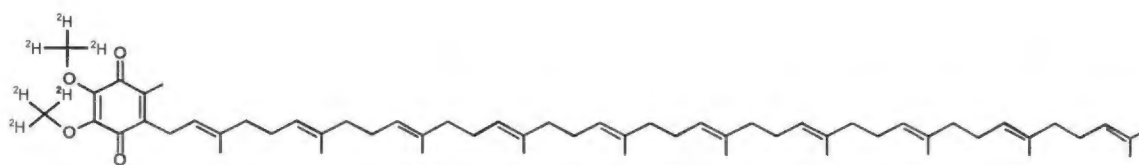


Figure 3.2: The chemical structure of deuterium labeled (CoQ₁₀[²H₈]) (adapted from Hansen *et al.*, 2004).

3.6 Standardization of the chromatographic separation conditions for CoQ₁₀

3.6.1 Introduction

Because CoQ₁₀ is such a large hydrophobic molecule, its elution from the C18 column is inevitably complicated by its stern affinity to the C18 stationary phase. It is also an unstable and thermo labile molecule (Wolf *et al.*, 1958). The standardization of the chromatographic separation of CoQ₁₀ focused on the mobile phase composition; the ionization agent dissolved in the mobile phase; and the flow rate.

3.6.2 The effect of mobile phase composition on chromatographic separation of CoQ₁₀

Most studies of CoQ₁₀ measurement use isocratic separation with a methanol:ethanol composition (El-Najjar *et al.*, 2011). Ruiz-Jiménez *et al.* (2007) used the same column as in this study and investigated the efficacy of isocratic separation of CoQ₁₀ with different mobile phase compositions, namely ethanol, acetonitrile, methanol, and 2-propanol. By doing so, they established that methanol gave optimal chromatographic performance. However, the effect of adding water to the semi-non polar methanol during separation of CoQ₁₀ was first investigated in this study. Three mobile phase compositions were utilized in the investigation of the effect of mobile phase composition on the isocratic chromatographic separation of CoQ₁₀. The proposed mobile phase, namely 100% methanol containing 5mM ammonium formate was prepared. A second mobile phase composition of 1% water and 99% methanol was prepared along with a third mobile phase composition of 5% water and 95% methanol, both with 5mM ammonium formate.

3.6.2.1 Analytical conditions

A CoQ₁₀ standard (1 µg/mL) was utilized for this experiment. The constant flow rate of the mobile phase was 0.2 ml/min and 5 µL was injected onto the LC column. The column temperature was set at 45°C and the run time was 20 minutes. The injection needle was washed in a flush port containing a methanol:isopropanol:water (70:10:20, v/v/v) solution for 10 seconds. The eluent from the column entered the MS via electrospray ionization (ESI) in positive mode and MRM was used as data acquisition mode. The flow and temperature of the drying gas (N₂) were 6 L/min and 300°C, respectively.

The nebulizer pressure was 30 psi. The capillary voltage was set at 3500V and the nozzle voltage at 500V and the dwell time was 50ms for each ion species. The MS conditions obtained during the optimization of the MS conditions for the quantification of CoQ₁₀ (Section 3.5.1.1, Table 3.1) was used.

3.6.2.2 Results and discussion

Table 3.2 lists the retention times of CoQ₁₀ during separation with the three different mobile phase compositions. The 100% methanol mobile phase gave a retention time (RT) of 9.770 minutes for CoQ₁₀, while addition of 1% water markedly increased the RT to 12.304 minutes. After increasing the water percentage to 5% (95% methanol, 5% H₂O) there was no peak detected for CoQ₁₀, and it seemed the standard CoQ₁₀ had not eluted from the column even after a 25.000 minute runtime (data not shown). It is therefore evident that the addition of water had drastically extended the RT of the analyte. These results proved the extreme hydrophobic nature of the CoQ₁₀ molecule. Because the analyte only eluted after $\pm$ 10 minutes using the 100% organic methanol mobile phase, and because the total run time of a complete sample batch had to be considered, it was decided to use a 100% methanol mobile phase to shorten the total run time. From this point onward, 100% methanol with 5mM ammonium formate was used for separation of CoQ₁₀ samples in the standardization of the chromatographic conditions, though the ionization agent was yet to be optimized.

Table 3.2: The retention time of CoQ₁₀ in different mobile phase compositions.

Mobile phase composition	Retention time (minutes)
100% MeOH	9.770
99% MeOH, 1% H ₂ O	12.304
95% MeOH, 5% H ₂ O	>25.000

3.6.3 The effect of ionization agents on the quantification of CoQ₁₀

Considering the structure of CoQ₁₀, it is a lipid soluble molecule (Edlund, 1988) which is not readily vaporized nor easily ionized. However, Teshima & Kondo (2005) stated that the addition of a 1-alkylamine ionization agent to the mobile phase has the potential of increasing the sensitivity and the selectivity up to 5 times compared to a quantitative analysis without an analyte ammonium-adduct. Barshop & Gangoit (2007) also stated that addition of 1-alkylamine ionization agents has improved ionization to such an extent that it overcame the suppression of analyte ionization. A study by Ruiz-Jiménez *et al.* (2007) supported this claim after showing that ammonium formate was the optimal ionization agent between four others.

However, based on the claim made by Teshima & Kondo, improvement in the ionization of CoQ₁₀ to increase sensitivity of the LC-MS/MS method relied on optimization of the ionization agent, more than on the ionization mode. Therefore, ammonium formate and a non-alkylamine, formic acid, were examined as ionization agents.

3.6.3.1 Analytical conditions

For this experiment a CoQ₁₀ standard (1 µg/mL) was utilized (Section 3.2.1). For the isocratic separation of the standard sample mixture, 99.9% methanol with 0.1% formic acid (dissolved in 1 mL Milli-Q water (18Ω)), and 100% methanol with 5mM ammonium formate were prepared. Except for an injection volume of 1 µL, the same parameters as described in Section 3.6.2.1 were applied.

3.6.3.2 Results and discussion

Figure 3.3 shows the MRM chromatograms obtained from the separation of CoQ₁₀ with methanol containing 0.1% formic acid. Figure 3.4 illustrates the MRM chromatogram of CoQ₁₀ separation with methanol containing 5mM ammonium formate. Chromatographic separation of both mobile phases produced a stable baseline and good compound retention. As can be seen from the results in Table 3.3, the signal intensity or abundance of both the protonated form ([M + H]⁺) and the ammonium salt ([M+NH₄HCO₂]⁺) of CoQ₁₀ was greatly increased with the use of ammonium formate as ionization agent. The abundance of the ammonium salt of CoQ₁₀ at transition 880.7 m/z – 197.2 m/z was 45.8 fold higher, and the protonated form was 19.4 fold higher than when formic acid was used as ionization agent. This difference in abundance was most likely due to the ionization efficiency and affinity of the adduct ions to the analyte (Teshima & Kondo, 2005). The alkyl group of the ammonium adduct (NH₄HCO₂⁺) of ammonium formate would have a greater affinity to the long, amphipathic CoQ₁₀ molecule than the hydrogen adduct (H⁺) of formic acid.

Table 3.3: The detected abundance of the protonated form CoQ₁₀ and the ammonium salt of CoQ₁₀. These abundances were determined after isocratic separation with formic acid and ammonium formate as ionization agent, respectively.

Ionization agent	Abundance	
	Protonated CoQ ₁₀ (863.7 – 197.2 m/z)	CoQ ₁₀ Ammonium salt (880.7 – 197.2 m/z)
Formic acid (Figure 3.3)	89134	32632
Ammonium formate (Figure 3.4)	1 735 954	1 495 880

Also, due to the greater affinity and thus greater stability of the ammonium formate adduct ions to CoQ₁₀, it was decided to monitor the ammonium salt of CoQ₁₀ ([M+NH₄HCO₂]⁺) at transition 880.7 m/z – 197.2 m/z as opposed to the protonated ([M + H]⁺) analyte adduct of CoQ₁₀ at transition 863.7 m/z – 197.2 m/z.

The results summarized in Table 3.3 is supported by other studies (Barshop & Gangoiti, 2007; Ruiz-Jiménez *et al.*, 2007) which found that the use of a 1-alkylamine ionization agent such as ammonium formate improves the ionization of the hydrophobic CoQ₁₀ molecule in comparison to ionization with a non-alkylamine such as formic acid. Therefore, it was decided to use ammonium formate as ionization agent with methanol as mobile phase in further analyses of CoQ₁₀.

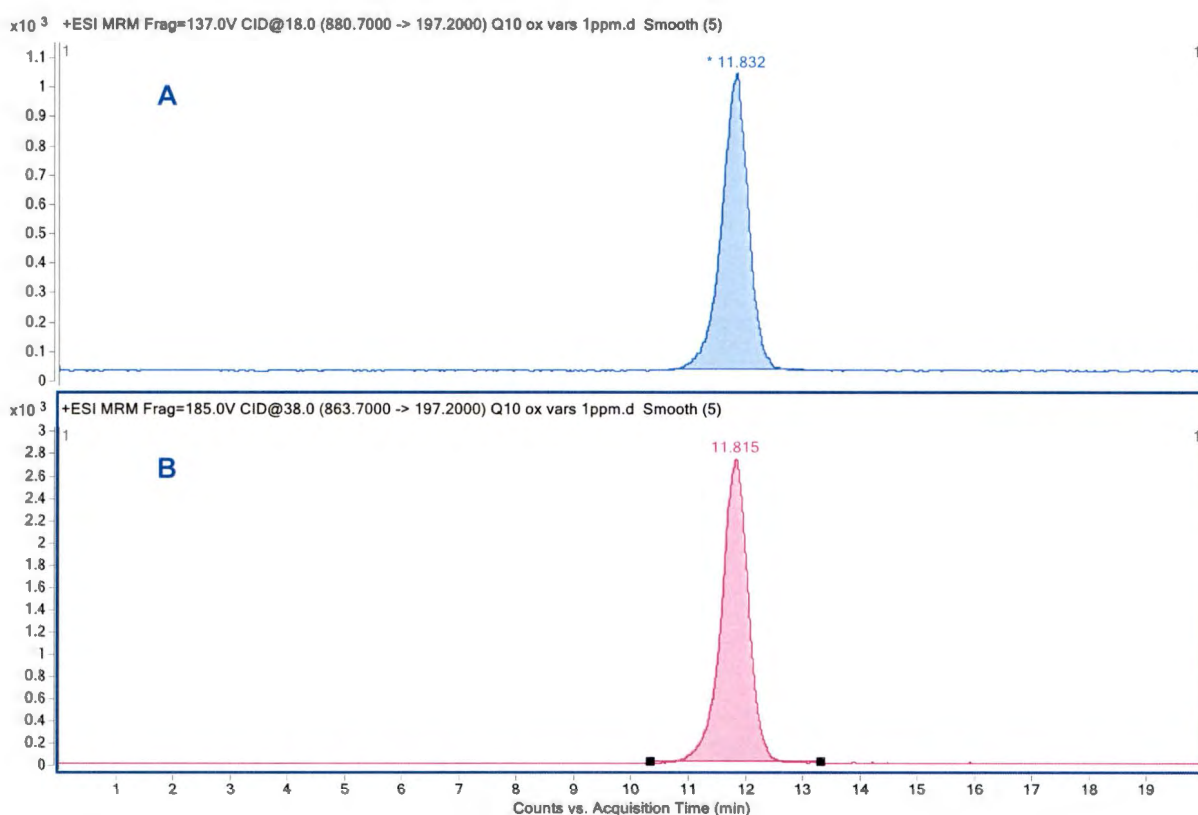


Figure 3.3: Chromatographic separation of a standard CoQ₁₀ sample (1 µg/mL) with methanol and formic acid. The MRM chromatogram illustrates the chromatographic separation of CoQ₁₀ and its relative abundance when using formic acid as ionization agent. The top chromatogram (A) shows the abundance of the ammonium salt ([M+NH₄HCO₂]⁺) of CoQ₁₀ at transition 880.7 m/z – 197.2 m/z, and the bottom chromatogram (B) shows the abundance of the protonated ([M + H]⁺) analyte adduct of CoQ₁₀ at transition 863.7 m/z – 197.2 m/z.

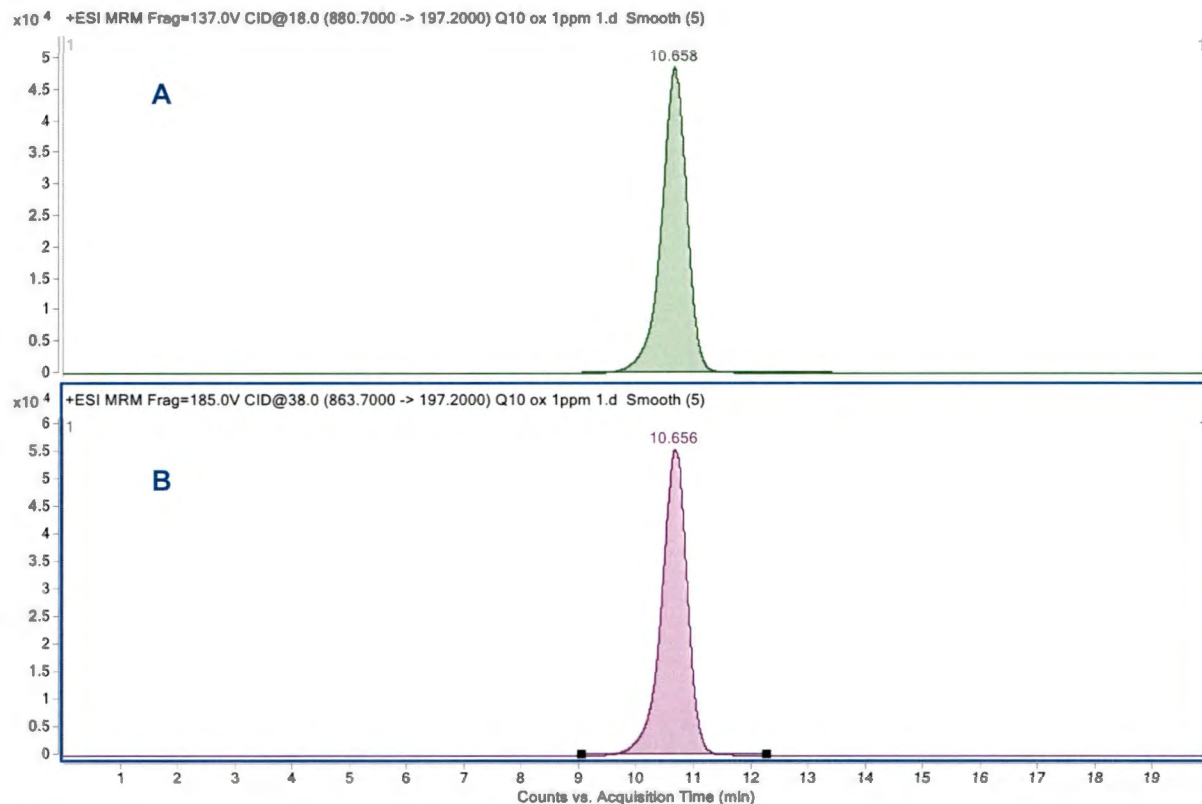


Figure 3.4: Chromatographic separation of a standard CoQ₁₀ sample (1 µg/mL) with methanol and 5mM ammonium formate. The MRM chromatogram illustrates the chromatographic separation of CoQ₁₀ and its relative abundance when using ammonium formate $[M+NH_4HCO_2]^+$ as ionization agent. The top MRM chromatogram (A) shows the abundance of the ammonium salt $([M+NH_4HCO_2]^+)$ of CoQ₁₀ at transition 880.7 m/z – 197.2 m/z, and the bottom MRM chromatogram (B) shows the abundance of the protonated $([M + H]^+)$ analyte adduct of CoQ₁₀ at transition 863.7 m/z – 197.2 m/z.

3.6.4 The effect of HPLC flow rate on the chromatography of CoQ₁₀

The flow rate is usually adjusted according to a change in the column internal diameter (Gritti & Guiochon, 2011), or to obtain the ideal Gaussian peak. Though modern day columns are designed to decrease the amount of tailing on analyte peaks, peak tailing remains a problem (Dolan, 2003). Isocratic separation tends to cause peak tailing, which is why optimization of the flow rate was attempted to better the peak resolution, peak elucidation, and symmetrical shape of the analyte peak.

3.6.4.1 Analytical conditions

The same analytical conditions as in Section 3.6.3.1 were applied for the LC-MS/MS analysis of a CoQ₁₀ standard (0.5 µg/mL), with exception that the constant flow rate of the 100% MeOH, 5mM ammonium formate mobile phase was increased from 0.2 ml/min to 0.3 ml/min during a second analysis of the same CoQ₁₀ standard, and the run time was adjusted to 15 minutes.

3.6.4.2 Results and discussion

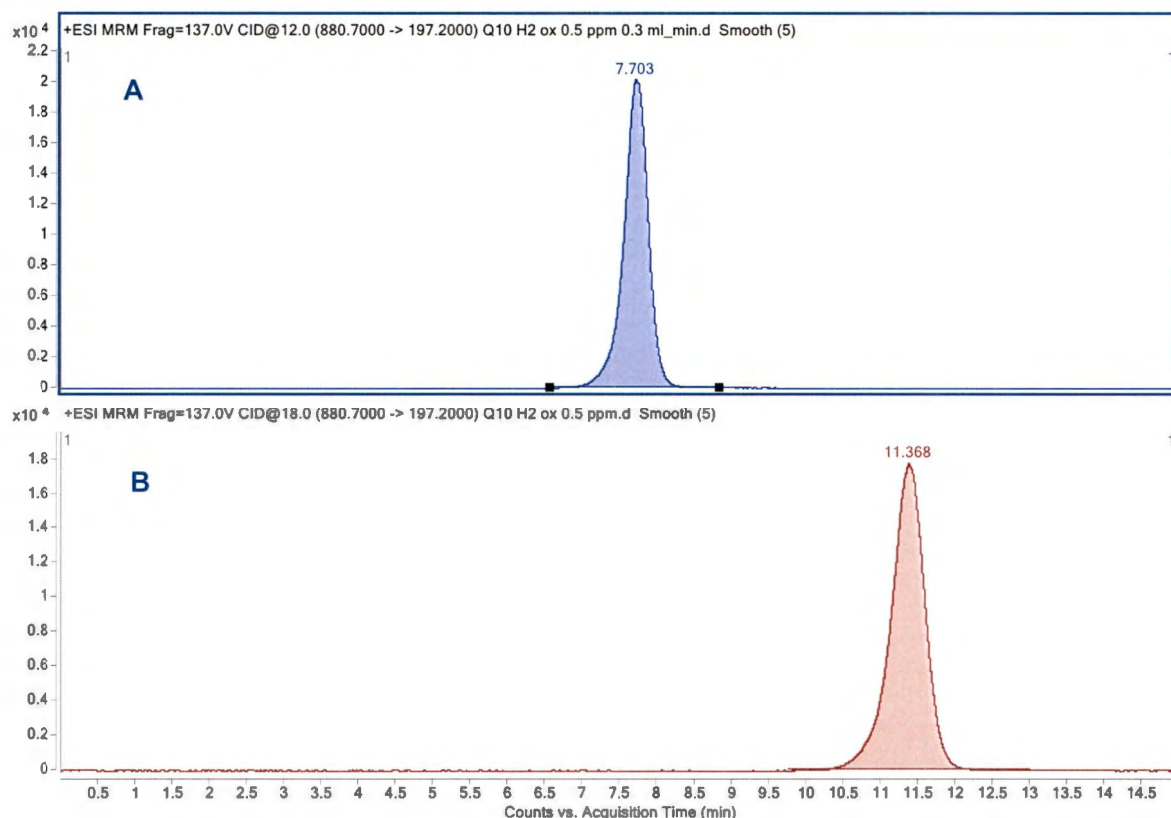


Figure 3.5: Chromatographic separation of a CoQ₁₀ standard sample (0.5 µg/mL) at different flow rates. These chromatograms illustrate the isocratic separation of a standard CoQ₁₀ at flow rate 0.3 ml/min (A) and 0.2 ml/min (B) with a RT of 7.703 min (A) and 11.368 min (B), respectively.

Figure 3.5 illustrates the MRM chromatograms of a CoQ₁₀ standard separated at a flow rate of 0.2 ml/min (B), and again at 0.3 ml/min (A). The analysis at 0.2 ml/min had a RT of 11.368 minutes for CoQ₁₀. When separated at a flow rate of 0.3 ml/min, the RT decreased with ± 3.6 minutes from 11.368 minutes to 7.703 minutes. This increased flow rate to 0.3 ml/min also improved the CoQ₁₀ peak resolution, and thus the peak elucidation time by narrowing the peak width. The peak separated at a 0.2 ml/min flow rate (B) had a peak elucidation time of ± 1.75 minutes, and when the sample was separated at a flow rate of 0.3 ml/min (A), the peak elucidation time decreased to ± 1.25 minutes.

The increase in flow rate also slightly improved peak tailing. This was beneficial because the isocratic separation of the analyte peak with the 100% methanol mobile phase was prone to cause peak tailing, as opposed to gradient separation (Dolan, 2003). With a 0.3 ml/min flow rate, a sharper peak was obtained (Figure 3.5, A) which would be beneficial during integration of the chromatograms, and would reduce the total run time. Subsequently, the flow rate was adjusted from 0.2 ml/min to 0.3 ml/min and the run time was kept at 15 minutes.

3.7 Oxidized and reduced forms of CoQ₁₀

3.7.1 Introduction

Numerous procedures report the simultaneous analysis of the oxidized and reduced form of CoQ₁₀ and their ratio (CoQ₁₀:CoQ₁₀H₂), *i.e.* the redox status of CoQ₁₀ rather than the total CoQ₁₀ content (Menke *et al.*, 2000; Tang *et al.*, 2004; Ruiz-Jiménez *et al.*, 2007). Studies reported the total oxidation of the sample CoQ₁₀H₂ already during the sample preparation procedure (Duberley *et al.*, 2013a), within the MS interface (Hansen *et al.*, 2004), or on-column oxidation (Menke *et al.*, 2000) and that the measurement of CoQ₁₀ is more reliable than CoQ₁₀H₂ (Menke *et al.*, 2000; Hansen *et al.*, 2004).

A major cause of CoQ₁₀H₂ formation during sample preparation is the extraction and drying of the samples (Menke *et al.*, 2000) as well as the exposure to atmospheric oxygen (Tang *et al.*, 2001). This may explain why Hansen *et al.* (2004) recommended the quantification of total CoQ₁₀ (sum of CoQ₁₀ and CoQ₁₀H₂) rather than the redox status for the purposes of repetitive clinical determination of CoQ₁₀. However, though the simultaneous analysis of CoQ₁₀ and CoQ₁₀H₂ is complicated, it is still feasible with the proper resources (Hansen *et al.*, 2004; Tang *et al.*, 2004). The redox status of CoQ₁₀ has significant meaning when looking at health related issues concerning CoQ₁₀, as it can be an indicator of oxidative stress (reviewed by Garomel *et al.*, 2002). Patients have been reported with a decreased ratio of CoQ₁₀ along with increased levels of oxidative damage (Artuch *et al.*, 2006), but such results of different studies have been controversial (Miles *et al.*, 2008).

The use of butylated hydroxytoluene (BHT) to prevent auto-oxidation of CoQ₁₀H₂ is essential for the quantification of the redox status in a sample (Milaeva *et al.*, 2010). However, in view of how the patient sample homogenates used in this study were formerly collected, stored and prepared (see Section 3.3.1), as well as the time elapsed since collection of the muscle biopsies in a study initiated by Smuts *et al.* (2010), it was decided not to attempt the quantification of both the oxidized and reduced form, but rather total CoQ₁₀ content (sum of the oxidized and reduced form). The purpose of the preparation method carried out by Smuts *et al.* (2010) was to prepare the samples for analyses of mitochondrial respiratory chain enzyme activity and protein content (See Chapter 4, Section 4.2.3). Therefore, their preparation method was exempt of the necessary precautions to prevent (auto-) oxidation of CoQ₁₀H₂ within the biological samples; a preventive measure which would be essential for the envisioned measurement of the redox status of the analyte within the biological samples. Consequently, the sample preparation procedure was modified to quantify the total CoQ₁₀ content by oxidizing the CoQ₁₀H₂ content which was extracted from the biological samples to the more stable CoQ₁₀ prior to LC-MS/MS analysis (See Section 3.3.2).

3.7.2 The auto-oxidation of CoQ₁₀

Hansen *et al.* (2004) recommended, for the purposes of repetitive clinical determination of CoQ₁₀, that the total CoQ₁₀ ought to be quantified of rather than their redox status (CoQ₁₀: CoQ₁₀H₂) based on the instability of the reduced form, CoQ₁₀H₂. The MRM of the chromatographic separation of CoQ₁₀H₂ presented certain difficulties in this study. It was presumed that the CoQ₁₀H₂ analyte auto-oxidized during chromatographic separation. To investigate this matter, an LC-MS analysis of a freshly prepared CoQ₁₀H₂ sample (as described in Section 3.2.2) was carried out.

3.7.2.1 Analytical conditions

The CoQ₁₀H₂ sample (1 µg/mL) was analyzed with the same analytical conditions as in Section 3.6.4.1, with the exception of a constant flow rate of 0.3 ml/min and a run time of 15 minutes.

3.7.2.2 Results and discussion

Figure 3.6 illustrates the chromatographic separation of CoQ₁₀H₂. The total ion chromatogram (TIC) in Figure 3.6 indicates that the majority of the sample was reduced (1), and is also indicated by the MRM chromatogram at transition 882.7 m/z – 197.2 m/z (3), and rightly so because the CoQ₁₀H₂ sample was freshly prepared and immediately analyzed with LC-MS/MS. But the TIC also indicated that a percentage of the injected ubiquinol (CoQ₁₀H₂, 1) was oxidized to ubiquinone (CoQ₁₀, 2) during chromatographic separation. This oxidation of CoQ₁₀H₂ was better indicated by the bottom MRM chromatogram at transition 880.7 m/z – 197.2 m/z. The CoQ₁₀ spectrum indicated that a percentage of the initial ubiquinol injected (3) was instantly oxidized within the ion source to ubiquinone, as can be seen at transition 880.7 m/z – 197.2 m/z (4), and then increasingly so during column separation (5), because peak number 4 in the CoQ₁₀ MRM chromatogram has the same RT as the actual CoQ₁₀H₂ sample at transition 882.7 m/z – 197.2 m/z (3). In other words, the MS detected a percentage of the CoQ₁₀H₂ molecules as CoQ₁₀ within the CoQ₁₀ spectrum (880.7 m/z – 197.2 m/z) even though they eluted at the expected RT of CoQ₁₀H₂. Therefore, it is evident that the CoQ₁₀H₂ molecules oxidized spontaneously within the ESI source as they eluted from the column. The MRM chromatogram at transition 880.7 m/z – 197.2 m/z (4-5) indicates the chromatographic separation CoQ₁₀. However, where the single CoQ₁₀ peak is expected at its normal RT of ± 7.7 minutes (5), another peak (4) is seen at the same RT as the actual CoQ₁₀H₂ sample (3). Therefore, the peak 4 is where the injected CoQ₁₀H₂ sample was instantly oxidized in the ESI source. The MRM chromatogram at transition 880.7 m/z – 197.2 m/z (4-5) also indicates that the CoQ₁₀H₂ sample is then also increasingly oxidized (to a more hydrophilic oxidized form) within the column to gradually elute from the column approaching the actual retention time of CoQ₁₀ (5). This on-column and in-source oxidation has also been observed in other studies (Menke *et al.*, 2000; Hansen *et al.*, 2004).

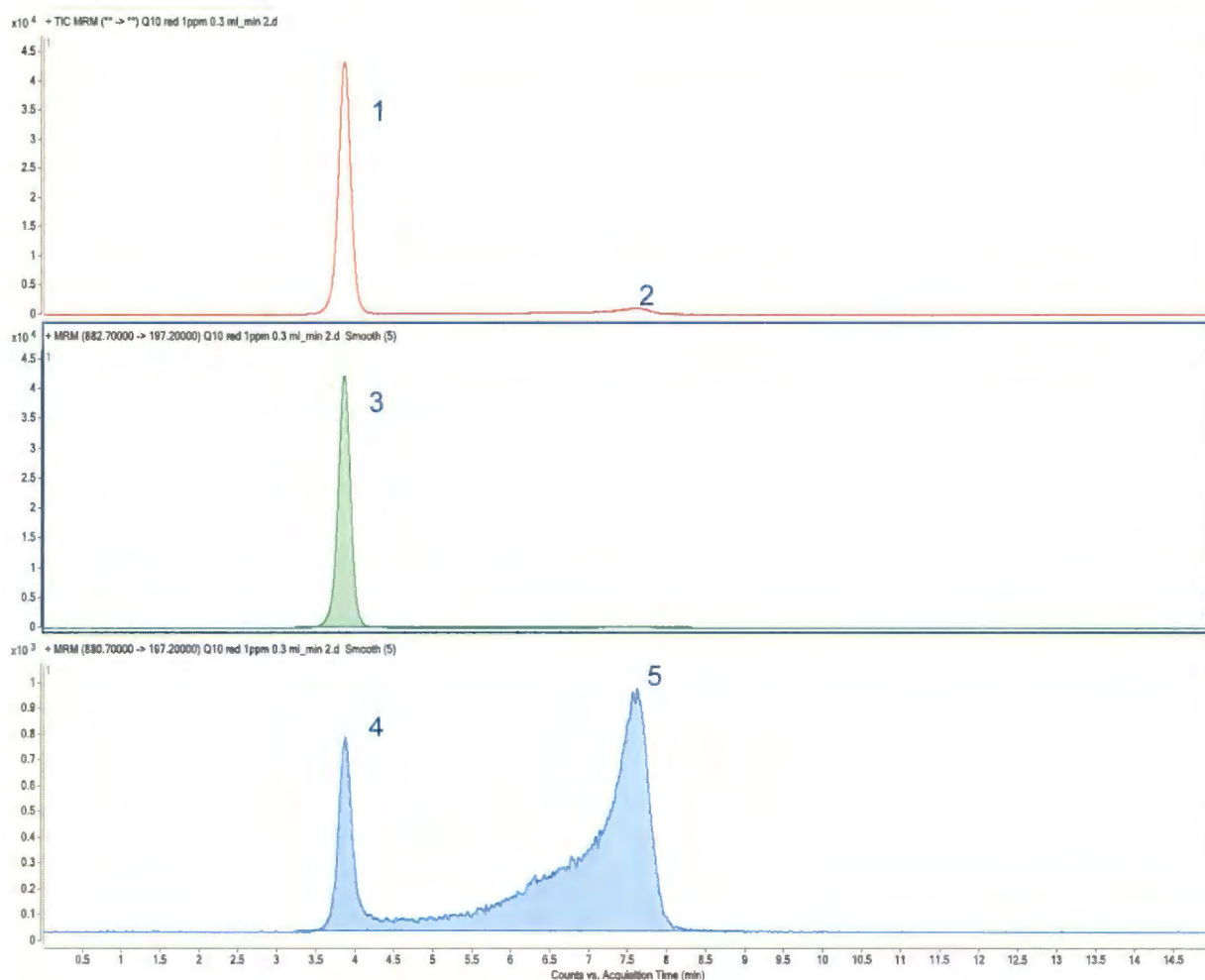


Figure 3.6: Chromatograms of the separation of a standard CoQ₁₀H₂ sample. The chromatograms illustrate the chromatographic separation of a 1 µg/mL CoQ₁₀H₂ standard during LC-MS analysis. The bottom MRM chromatogram (4,5) indicating the initial ubiquinol injected (3) which was instantly oxidized to ubiquinone within the ion source (4) and then increasingly so during column separation (5).

In summary, Figure 3.6 demonstrated that the prepared CoQ₁₀H₂ sample was instantly oxidized within the ESI source and auto-oxidized during column separation. In the light of these setbacks, it would not be sensible to attempt to analyze the redox state of CoQ₁₀. Tang *et al.* (2001) stated that artifactual oxidation of CoQ₁₀H₂ can also occur as a result of chemical reduction of CoQ₁₀ to produce CoQ₁₀H₂, which was the case in this study (Section 3.2.2). As mentioned earlier, the oxidation of CoQ₁₀H₂ could be because of the amphipathic nature of its structure, making it prone to nucleophilic attack and activating its redox cycling via a free-radical intermediate, ubisemiquinone (CoQH•) (Hansen *et al.*, 2004).

All things considered, it was decided that the CoQ₁₀H₂ content would be chemically oxidized to CoQ₁₀ during the sample preparation procedure (Section 3.3.2) to avoid the drawback of the extreme instability of CoQ₁₀H₂.

3.7.3 Chemical oxidation of CoQ₁₀

Concerning the redox state of ubiquinone in human muscle tissue, 65% is reduced (Ernster & Dallner, 1995). Therefore, a 600 x g human muscle homogenate supernatant was utilized to determine the efficiency of the proposed oxidation agent, namely ethanolic 1,4-Benzoquinone (0.4 mg/mL). The preparation of the human homogenate and consecutive 600 x g supernatant is discussed in Section 3.3.1, and the preparation prior to LC-MS/MS analysis is described in Section 3.3.2. Two volumes of the same muscle homogenate supernatant was simultaneously submitted to the sample preparation procedure (Section 3.3.2), but one of the two the steps whereby 1,4-Benzoquinone was added, had been omitted for this experiment. After preparation, the muscle extracts were then analyzed by LC-MS/MS, firstly, to observe the total CoQ₁₀ content within the sample without 1,4-Benzoquinone and secondly, to investigate whether the CoQ₁₀H₂ content of the sample had been totally oxidized after oxidation with 1,4-Benzoquinone.

3.7.3.1 Analytical conditions

The same parameters as in Section 3.7.2.1 were applied, with the exception that the run time was adjusted to 12 minutes, which was divided into three specific time segments as shown in Table 3.4 below. These programmed time segments prevented contamination to the ion source as it purposefully directed salts and polypeptides, most likely found in the biological sample extracts, to waste. By diverting these damaging salts and polypeptides, the programmed valve switch could improve peak resolution of the relative analyte compound.

Table 3.4: Programmed valve switch time segments for the mobile phase delivery to MS or to waste.

Time (minutes)	Valve switch
0	To waste
3	To MS
10.5	To waste

3.7.3.2 Results and discussion

Figure 3.7 illustrates the MRM chromatograms of the LC-MS/MS analysis of a human muscle homogenate extract without addition of the oxidation agent, 1,4-Benzoquinone. Figure 3.8 illustrates the MRM chromatograms of the same human muscle homogenate extract treated with 1,4-Benzoquinone. The total ion chromatogram in Figure 3.8 and the MRM chromatogram at transition 882.7 – 197.2 m/z (2) illustrated that there was a small trace of CoQ₁₀H₂ present (RT = 4.417 minutes) in the sample not treated with 1,4-Benzoquinone. However, there was no peak detected in Figure 3.8 at the actual RT of CoQ₁₀H₂ (RT = ± 4.400 minutes) in the MRM chromatogram at the transition 882.7 – 197.2 m/z of CoQ₁₀H₂ (2).

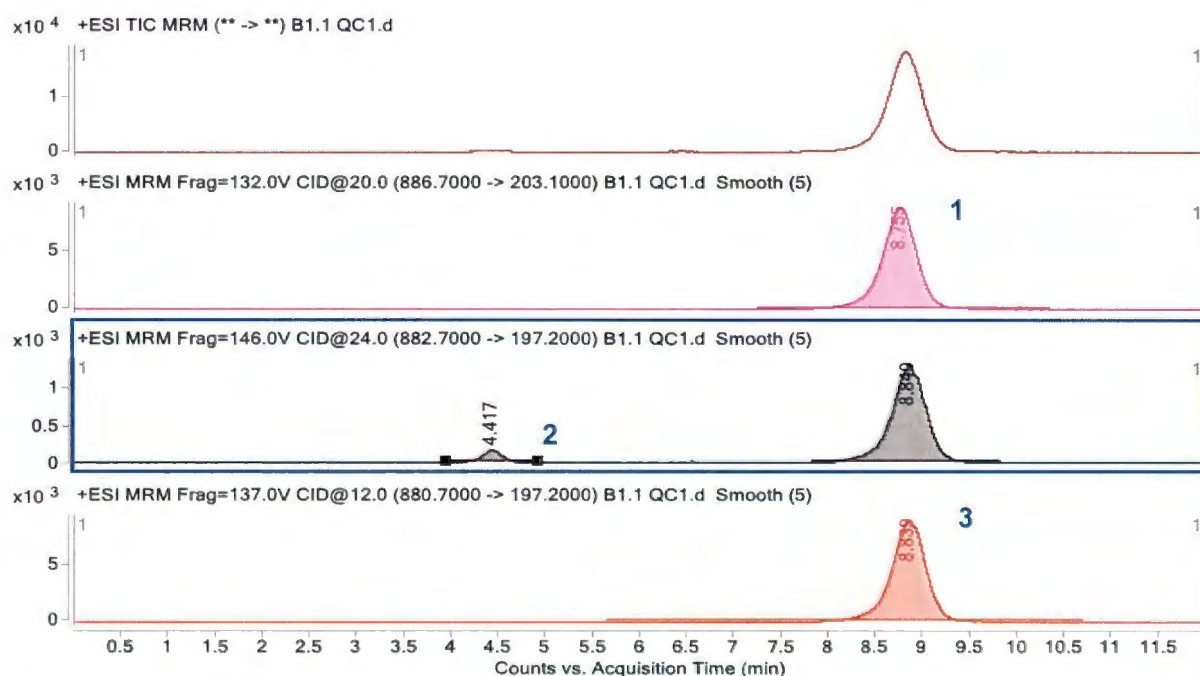


Figure 3.7: LC-MS/MS analysis of a human muscle homogenate extract. The MRM chromatograms were extracted from the total ion chromatogram and illustrated that a small trace of CoQ₁₀H₂ (2) was present, as well as a much greater amount of CoQ₁₀ (3). Peak 1 depicts the MRM chromatogram of the stable isotope, CoQ₁₀[²H₆]. The MRM chromatogram to the right of peak 2 in transition 882.7 – 197.2 m/z is due to spontaneous reduction of CoQ₁₀ within the ESI source.

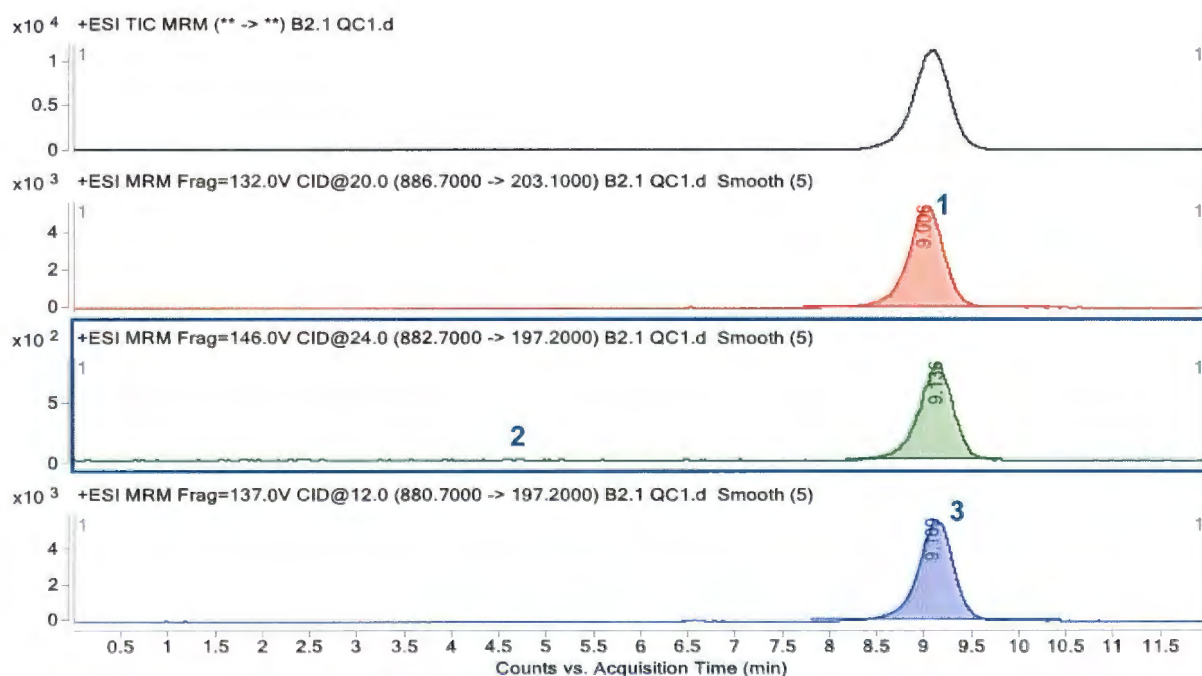


Figure 3.8: LC-MS/MS analysis of a human muscle homogenate extract after 1,4-Benzoquinone treatment. The total ion chromatogram and the MRM chromatogram at transition 882.7 – 197.2 m/z (2) illustrating that there was no trace of CoQ₁₀H₂ present in the sample after treatment with 1,4-Benzoquinone. Peak 3 and 1 illustrate the MRM chromatograms of CoQ₁₀ and CoQ₁₀[²H₆], respectively.

Therefore, when comparing the MRM chromatograms in Figure 3.7 and Figure 3.8 of the same muscle sample at transition 882.7 – 197.2 m/z (2 of Figure 3.7 and 2 of Figure 3.8), it is evident that the total oxidation of CoQ₁₀H₂ by 1,4-Benzoquinone prior to LC-MS analysis was successful.

The MRM chromatograms at transition 880.7 – 197.2 m/z (3) of Figure 3.7 and in peak 3 of Figure 3.8 illustrate the MRM chromatogram of CoQ₁₀. In Figure 3.7 and Figure 3.8, the CoQ₁₀H₂ ammonium salt at transitions 882.7 – 197.2 m/z had a RT identical to the actual retention time of CoQ₁₀ (RT = ± 9.00 minutes) and an MRM spectrum identical to CoQ₁₀ at transition 880.7 – 197.2 m/z. Thus it seemed that a percentage of CoQ₁₀ had undergone reduction within the ion source during the ESI interface in Figure 3.7 and Figure 3.8. The addition of the stable isotope as an internal standard for quantification of the total CoQ₁₀ content within the samples, would improve both the precision and the accuracy of the method (Yamashita & Yamamoto, 1997). Any loss during ionization would have been compensated for by equal loss of the stable isotope during quantification because of its similar fragmentation caused by collision induced dissociation within the MS (reviewed by Ho *et al.*, 2003; Duberley *et al.*, 2013a).

Both Figures 3.7 and 3.8 indicate the MRM chromatograms from the chromatographic separation of the stable isotope at ratio transition 886.7 - 203.1 m/z (A1 and B1). The purchased stable isotope, deuterated CoQ₁₀, had a purity >98.0% and its RT differed only slightly from the RT of CoQ₁₀, but a lot more from the RT of CoQ₁₀H₂. This isotope had the same chemical composition as CoQ₁₀ and was very similar in molecular mass, differing only in the mass of the stable isotopes (see Figures 2.1 in Chapter 2, and Figure 3.1 in Section 3.5.1.1), making it an ideal internal standard for the quantification of CoQ₁₀ with LC-MS/MS.

3.8 The standardized analytical assay for the quantification of total CoQ₁₀ in muscle

The analytical method used in this study for the quantification of CoQ₁₀ consisted of a sample preparation procedure in which the CoQ₁₀ content would be oxidized during extraction followed by analysis with LC-MS/MS. A volume of 30 µL of a muscle homogenate supernatant was transferred to a marked KIMAX[®] tube and 30 µL of the stable isotope, labeled CoQ₁₀ (CoQ₁₀[²H₆]) (0.5 µg/mL) was added to the supernatant using a micropipette. The marked KIMAX[®] tube was protected from light exposure by tin foil to avoid photochemical decomposition of CoQ₁₀ within the samples (Kaikkonen *et al.*, 1999). Then, 1 mL (0.1M) sodium dodecyl sulphate (SDS) was mixed with the 30 µL supernatant and 30 µL internal standard in the marked KIMAX[®] glass tube.

After mixing and vortexing for 30 seconds, 1 mL of a freezer kept (-20°C) ethanol:isopropanol (95:5, v/v) solution was added as a protein precipitation step. The tube was then mixed and briefly vortexed for 10 seconds prior to the first extraction. The extraction was conducted as follows: the sample mixture was extracted 2x with 2 mL *n*-hexane. After adding 2 mL *n*-hexane, the sample mixture was whirl mixed in a rotor for 5 minutes and then centrifuged at 4°C for 1 minute at 1833 x *g*. The upper organic phase was transferred to an identically marked KIMAX® tube using a ±150 mm Volac® disposable glass Pasteur pipette. The first extract was momentarily stored at 4°C while the extraction process was repeated to complete a second extraction of the sample mixture. While the sample mixture of the second extraction was whirl mixed, 30 µL of ethanolic 1,4-Benzoquinone (0.4 mg/mL) was added to the first extract and briefly vortexed for 5 seconds to enhance the total oxidation of ubiquinol. After centrifugation of the sample mixture, the second organic extract was transferred to the first. The extract pool was then very briefly vortexed for even dispersion of 1,4-Benzoquinone and dried under nitrogen gas (N₂) in light protection for approximately 20 minutes at room temperature. After this, the residue was reconstituted in 60 µL of 100% MeCN to yield a final concentration of 0.25 µg/mL for the stable isotope. The sample was vortexed for 20 seconds and carefully transferred to a pulled point glass vial insert in a certified Agilent 2 mL clear screw cap MS vial using a glass Pasteur pipette and immediately analyzed by LC-MS/MS.

For sample handling and mobile phase delivery, an Agilent 1290 Infinity LC (1290-Bin Pump) was used. The injection volume for each sample 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. The mobile phase used for the isocratic separation of the target analyte was 100% methanol containing 5mM ammonium formate pumped at a constant flow rate of 0.3 ml/min. The run time for each sample analysis totaled 12 minutes, which was divided into three specific time segments (see Table 3.4 in Section 3.7.3.1). Chromatographic separation was performed by an Agilent Zorbax C18 column-cartridge (2.1 x 30 mm, 3.5 µm), and a Zorbax SB-Aq guard column (2.1 x 150 mm, 5 µm) was used. The column temperature was kept at 45°C.

For detection of the analytes, an Agilent 6460 Triple Quadropole mass spectrometer (MS) was used. Samples were delivered to the MS via electrospray ionization (ESI) in positive polarity. Multiple reaction monitoring (MRM) was used for the monitoring of transitions between the precursor and product ions of the target analytes (CoQ₁₀, CoQ₁₀H₂, and the stable isotope, CoQ₁₀[²H₆]). The optimal ESI-MS/MS conditions for the detection and quantification of CoQ₁₀ with HPLC-MS/MS are summarized in Table 3.1. Nitrogen gas was used as nebulizing agent.

Table 3.5: The mobile phase gradient timetable for the column wash method. This gradient was applied as a wash step after multiple separations of CoQ₁₀ in biological samples.

Time (minutes)	%B1
0	100
1	100
31	5
41	5
61	100
81	100

Sheath gas and nebulizing/drying gas (N₂) temperature and flow rate were set at 300°C and 6 L/min, respectively for both these parameters. The nebulizer pressure was set at 30 psi, the capillary voltage at 3500 V, and the nozzle voltage at 500 V. The dwell time for the product ion of each of the target analyte ion species was set at 50 ms. For the detailed description of the quantification/calculation of unknown CoQ₁₀ concentration within the prepared biological samples, see Section 3.9, Equation 3.2. A column wash method was established and applied after every 4th sample batch, *i.e.* after analysis of 60 samples analyzed in batches of 15 samples each (see Section 4.2.1 in Chapter 4 for details sample batch composition).

The same ESI-MS/MS parameters for the column wash method were applied as described above, though the scan segments were restricted to a limited mass range of 100m/z – 1000m/z with a scan time of 500 ms. The fragmentor and cell accelerator voltages were set at 135 V and 7 V, respectively. The injection needle was washed in the flush port for 1 second in a methanol:isopropanol:water (70:10:20, v/v/v) solution for the column wash method, and the mobile phase gradient timetable is listed in Table 3.5. Run time was estimated at 92 minutes with a constant flow rate of 0.2 ml/min. Stop time was set at 61 minutes with a post run time of 30 minutes. Column temperature was set at 45°C. 100% Methanol 5mM ammonium formate was used as mobile phase B1 and 100% MeCN, 0.1% formic acid as mobile phase B2.

For re-use of the KIMAX tubes, all caps and tubes were immersed in a solution of phosphate free soap (40 mL soap concentrate in 2L ddH₂O) and left to soak overnight. Using a narrow bottle brush, the tubes were individually scrubbed after which the tubes and caps were rinsed 5 times with ddH₂O water. Tubes and caps were then individually rinsed 5 x with methanol and left to dry in a closed environment prior to a 3 x rinse with *n*-hexane.

3.9 Validation of the standardized CoQ₁₀ assay

A calibration range for CoQ₁₀ was produced using a blank (100% MeCN) and 12 dilution standards separately prepared in triplicate with 100% MeCN to yield a concentration range of 0.001, 0.005, 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, 1, 1.5, 1.75, and 2 µg/mL, respectively, each in an Eppendorf® tube. Stable isotope standard (0.5 µg/mL) was added to each dilution to yield a fixed concentration of 0.25 µg/mL within the final volume, of which 30 µL was transferred to a pulled point glass vial insert in 2 mL clear screw cap vials and immediately analyzed with LC-MS according to the analytical conditions described in Section 3.7.3.1. The stable isotope (CoQ₁₀[²H₆]) used in this study as an internal quantification standard, was purchased from ¹³C Molecular⁷ (purity > 98.0%).

Linear regression attempts to explain the relationship between the paired observations (of the analyte and the stable isotope) using a straight line, and tries to minimize the residues (sum of squares of the distance of the observed data points from the straight line) by using the method of least squares. The coefficient of determination (R^2) then measures how well this least squares regression curve (depicted in Figure 3.9) describes the observed data points, *i.e.*, it gives an indication of how effective the curve is in predicting the abundance/area of the detected CoQ₁₀, based on a given concentration. If $R^2 = 1$, the least squares regression curve will give a perfect prediction of the area. With the R^2 for CoQ₁₀ to the stable isotope CoQ₁₀[²H₆] in the calibration results being > 0.999, the linearity of the CoQ₁₀ HPLC-MS/MS quantification method is affirmed, and therefore proves linear detection by the LC-MS/MS method.

The relative standard deviation (%RSD) was calculated for all three replicates of each concentration level quantified for the construction of the calibration curve. The %RSD expresses the standard deviation of each sample as a percentage of the average of the sample group (Swanepoel, J.H.W *et al.*, 2011). The %RSD is also termed the coefficient of variance (CV). The precision of the sample analyses during the calibration experiment ranged from 0.8 – 5.6% expressed as %RSD. The linear dynamic range of the quantification method was established as 0.01 – 2 µg/mL for CoQ₁₀, and would suit the expected range of CoQ₁₀ within the patient samples to be analyzed in this study (Duncan *et al.*, 2005). The data obtained from the calibration curve were also used in determining the response factor of CoQ₁₀ using Equation 3.1. The response of an analyte refers to its peak size/area on a chromatogram, which is proportional to the amount of the injected substance successfully reaching the MS detector, taking into account its injected volume and molecular weight.

⁷¹³C Molecular, Inc. is a company providing analytical services and products for pharmaceutical and bio-medical sciences, specializing in the synthesis of labelled reference standards and surrogate internal standards (Fayetteville, North Carolina, United States)

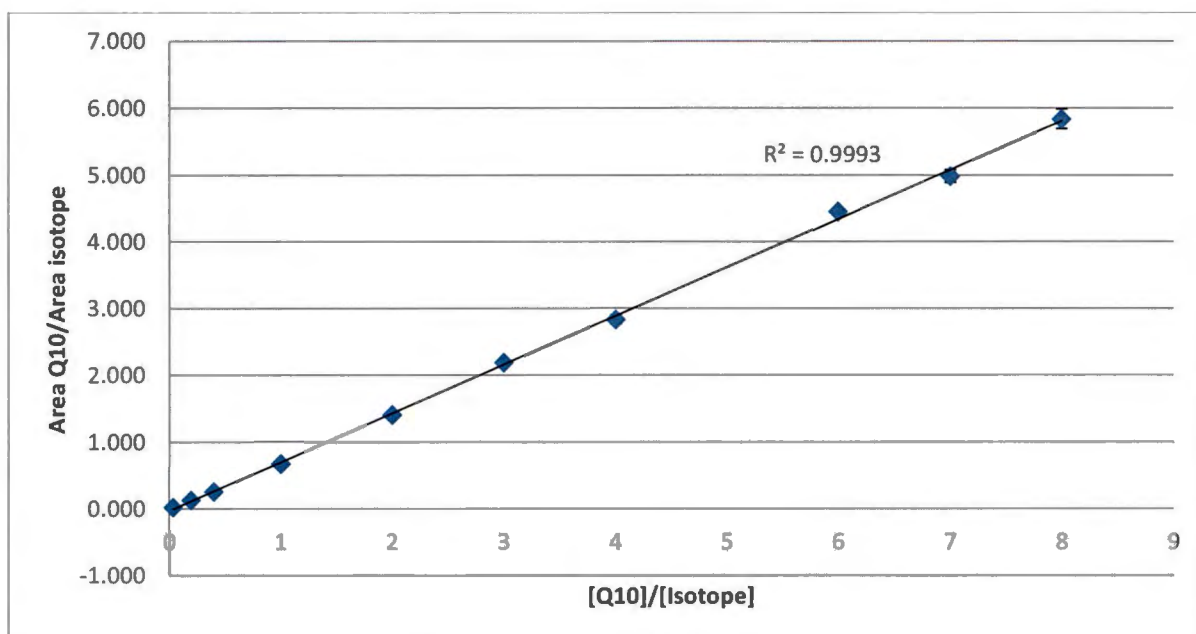


Figure 3.9: Calibration curve of CoQ₁₀ with the stable isotope CoQ₁₀[²H₆] as obtained by the CoQ₁₀ LC-MS/MS method. The concentrations ranged from 0.01 to 2 µg/mL of the CoQ₁₀ standard with an R² value > 0.999. Deuterated CoQ₁₀ (CoQ₁₀[²H₆]) was used as stable isotope to obtain the calibration curve to quantify the total CoQ₁₀ detected in each sample.

The response factor (RF) describes the ionization (during ESI) and detection of the molecule in relation to the effective ionization of its internal standard. A RF of 1.0 would therefore mean that the analyte's response is equal to the effective response of the internal standard. The mean RF for CoQ₁₀ in relation to the response of the internal standard (CoQ₁₀[²H₆]) within the linear range was calculated as 1.47. This RF value was included in the formula (Equation 3.1) used to quantify the total CoQ₁₀ within each sample analyzed. A more substantial description of the standardized method's performance in biological samples is given in Section 4.4 of Chapter 4. In short, the intraday (within-batch) CV was calculated as 1.96% after the sequential analysis of 5 quality control (QC) samples. The interday (between-batch) CV was calculated as 2.37% after analysis of 18 QC samples from 18 different batches.

Equation 3.1: Equation for the calculation of the response factor (RF) of the analyte.

$$RF = \frac{R(IS)}{R(A)} \times \frac{[A]}{[IS]}$$

Where:

- RF = Response factor
- $R(IS)$ = response/area of the internal standard (CoQ₁₀[²H₆])
- $[A]$ = concentration of the analyte (CoQ₁₀)
- $R(A)$ = response/area of the analyte
- $[IS]$ = concentration of the internal standard

Equation 3.2: Equation for the calculation of the concentration of CoQ10 within each sample.

$$[CoQ_{10}] = RF \times 0.25 \times 2$$

With $[CoQ_{10}]$ = the concentration of the analyte within the sample matrix
 RF = the mean response factor
0.25 = the fixed concentration (0.25 µg/mL) of the internal quantification standard ($CoQ_{10}[^2H_6]$)
2 = volume correction

3.10 Summary

After autosomal optimization by MassHunter software, optimal ESI-MS/MS conditions of the data acquisition parameters for an MRM mode for each of the target analytes (CoQ_{10} , $CoQ_{10}H_2$, and the stable isotope $CoQ_{10}[^2H_6]$) were produced. The unique product ion for $CoQ_{10}[^2H_6]$ had yet to be determined, as this stable isotope had not yet been utilized in previous reports prior to the compilation of the LC-MS/MS method used in this study. The drawbacks of CoQ_{10} quantification concerning its ionization and fragmentation (Hansen *et al.*, 2004; Duberley *et al.*, 2013a) was addressed by using ammonium formate as ionization agent and a stable isotope as internal standard (deuterium labeled CoQ_{10} ($CoQ_{10}[^2H_6]$), respectively. The $CoQ_{10}H_2$ content within the samples had been totally oxidized within the samples. Therefore, the MRM ratio transitions for $CoQ_{10}H_2$ were used only for monitoring purposes to examine the spontaneous reduction of CoQ_{10} within the ESI source. The poor ionization of CoQ_{10} had been improved by the addition of ammonium formate to the mobile phase as ionization agent (Teshima & Kondo, 2005), and by the use of an internal standard (Yamashita & Yamamoto, 1997) which greatly increased the sensitivity of the detection method.

The optimization of the influential variables of the LC separation of CoQ_{10} produced a faster and more economical method by decreasing the run time from 20 minutes to 12 minutes through adjusting the flow rate, also owing to the use of the StableBond SB-C18 (2.1 x 30 mm, 3.5 µm) cartridge column with narrow-bore (2.1mm diameter). The highly hydrophobic nature of the analyte almost entirely eliminated potential matrix effects, as it eluted from the column only after ± 9 minutes during isocratic separation of the biological sample. Accordingly, the mobile phase produced a stable baseline and good compound resolution and separation throughout the method. Optimization of the mobile phase to 100% methanol with 5mM ammonium formate allowed a sample injection volume of 1 µL. Though the analytes were isocratically separated, Figure 3.5 in Section 3.6.4.2 and Figure 3.7 in Section 3.7.3.2 illustrated good peak resolution for both a standard and biological sample, respectively. The addition of 1,4-Benzoquinone during extraction secured total oxidation of the $CoQ_{10}H_2$ to allow the quantification of the total CoQ_{10} content within the samples analysed, and to avoid the imprecision of $CoQ_{10}H_2$ quantification (Tang *et al.*, 2001).

The standardized LC-MS/MS method could now be validated for the quantification of the levels of total CoQ₁₀ in patient samples with ETC deficiencies. The stable isotope was used to determine the linearity of the standardized LC-MS/MS method. A calibration curve was constructed which gave an $R^2 > 0.999$ for the CoQ₁₀ quantification method over the linear range of 0.01 – 2 µg/mL. This R^2 value thoroughly affirmed linearity of detection by the standardized LC-MS/MS method. For the quantification of CoQ₁₀ in this study, the stable isotope CoQ₁₀[²H₆] was used, a deuterated CoQ₁₀. A more substantial description of the standardized method's performance in biological samples is given in Section 4.4 of Chapter 4. In short, the intraday CV was calculated as 1.96% and the interday CV was calculated as 2.37%.

It is important to mention that the method in this study had already been designed and standardized prior to the knowledge of the method used by Duberley *et al.* (2013a) that had been published in January 2013, and the practical part of the study had been completed before the study report by Itkonen *et al.* had been available online in November of 2013. Duberley *et al.* (2013a) quantified CoQ₁₀ using LC-MS/MS and synthesized a deuterated CoQ₁₀ internal standard. Itkonen *et al.* (2013) had a similar aim and objective to this study and therefore, both Duberley *et al.* and Itkonen *et al.* used the same stable isotope for the quantification of CoQ₁₀ in human muscle homogenates and muscle mitochondria, respectively. To illuminate the differences and similarities, the features of their methods and the current study's standardized method features were listed in Table 3.6 below.

Table 3.6: A summary of the method features of the current study and two other comparative LC-MS/MS methods for CoQ₁₀ quantification. The same stable isotope was used as internal standard, namely isotopically labeled CoQ₁₀ (CoQ₁₀[²H₆]).

Analyte	R ²	Linear dynamic range	Reference
CoQ ₁₀	0.9993	10 - 2320nM	Current study
CoQ ₁₀	0.9995	2 - 200nM	Duberley <i>et al.</i> , 2013a
CoQ ₁₀	-	0.5 - 1000nM	Itkonen <i>et al.</i> , 2013

CHAPTER 4: CoQ₁₀ CONCENTRATIONS IN SOUTH AFRICAN PATIENT SAMPLES

4.1 Introduction

The detection of CoQ₁₀ deficiency of the ETC or respiratory chain (RC) is the most important application of a CoQ₁₀ quantification method thus far (Itkonen *et al.*, 2013). Quantification of CoQ₁₀ in muscle samples is a definite and very reliable diagnosis of a CoQ₁₀ deficiency (Rahman *et al.*, 2012). Now that the LC-MS/MS method had been standardized, it was suitable for the quantification of total CoQ₁₀ in skeletal muscle.

The experimental group investigated in this current study was similar to the cohort of South African patients with neuromuscular disorders investigated in a study by Smuts *et al.* (2010). The most important findings described by Smuts *et al.* (2010) were centred on the differences between African patients and Caucasian patients in the cohort. Clinically, the African patients presented predominantly with a distinctive myopathic phenotype (62.5%) while the Caucasian patients largely presented with central nervous system involvement, *i.e.* encephalopathy (7.5%) and encephalomyopathy (30%). Most remarkably, 52.5% of the African patients with RCD were diagnosed with combined complex CI+III and especially CII+III RCDs in contrast to the 17.4% of the other ethnicities. Of all the patients with RCDs in the South African cohort of this study, 38% (Caucasian and African patients) had CII+III deficiencies. Therefore, owing to the key role of CoQ₁₀ in ATP production as an electron carrier from CI and CII to CIII, and because CoQ₁₀ deficiency is often associated with mitochondrial disorders (Hargreaves, 2014), the total muscle CoQ₁₀ concentrations in South African patients were quantified to statistically investigate the CoQ₁₀ concentrations in relation to the ETC activities within the patients. The influences of age, gender, and ethnicity on the CoQ₁₀ concentrations were investigated prior to the establishment of a reference interval, which was used to subsequently identify patients with decreased CoQ₁₀ concentrations (Quinzii & Hirano, 2010).

4.2 Biological samples

4.2.1 Ethical approval

Ethical approval from the Ethics Committees of the University of Pretoria (No. 91/98 and amendments) and the North-West University (No. 02M02) was obtained for the study. The parents of all the patients gave their informed consent for the use of the muscle biopsies of their children for research purposes.

4.2.2 Patients

The experimental group of this study was collected at the Steve Biko Academic hospital's Paediatric Neurology Unit in Pretoria, South-Africa where muscle biopsies were performed (from 2006 – 2013) on patients (mostly children) who had a Mitochondrial Disease Criteria score ≥ 6 , or when they presented with a classical phenotype related to a mitochondrial disorder and had two or more distinct physiological systems involved. On collection, the muscle biopsies were submitted to a preparation procedure (as described in Section 3.3.1 in Chapter 3) to prepare 600 x g muscle homogenate supernatants. The samples formerly investigated by Smuts *et al.* (2010) were also obtained from patients assessed at this Neurological Unit. The experimental group investigated in this study consisted of a total of one hundred and fifty five 600 x g quadriceps (vastus lateralis) muscle homogenate supernatant samples. All samples analyzed in this study had undergone enzyme analysis. The prepared homogenates were used for the enzyme analyses to ultimately diagnose mitochondrial RCDs in patients (n= 76), or classify the samples as clinically referred controls (n= 79). After submission of the samples to the standardized sample preparation method (Chapter 3, Section 3.3.2), total muscle CoQ₁₀ concentrations were quantified with the standardized LC-MS/MS method (Chapter 3, Section 3.8). The following patient data were systematically collected: age, gender, ethnicity, and respiratory chain enzyme assessments. Thus, these above mentioned procedures (sample collection, clinical and biochemical characterization, enzyme analyses, and diagnosis of RCDs) did not form part of this study, though the availability of the enzyme activity data allowed proper sample grouping. All the patient samples (n=155) were categorized in three sample groups. The first sample group was defined as the Clinically Referred Control (CRC) group, and consisted of patients who were suspected of having an ETC deficiency but where no RCD could be detected on enzymatic level (n=79). The second sample group compiled all patient biopsies diagnosed with a respiratory chain deficiency (excluding a CII+III defect), designated as the Other RCD group (n=47). The third group consisted of patients with combined CII+III deficiencies, designated as the CII+III defect group (n=29).

4.2.3 Mitochondrial respiratory chain analyses

Enzyme analyses were performed according to previously described standard operating procedures to determine the activities of mitochondrial ETC complex I, II, CII+III, III, IV, and citrate synthase (CS). The CS activity was used as a mitochondrial content marker. Total protein concentration of the 600 x g homogenate supernatants was determined using a Bicinchoninic Acid Kit (BCA-1). The criteria used to confirm a respiratory chain enzyme deficiency in this case cohort were more stringent than initially described by Smuts *et al.* (2010), and Reinecke *et al.* (2012).

The criteria invoked here was that the respective enzyme activity value of CII and CIV had to be normal (greater than the 5th percentile of the laboratory control value when expressed to CS) to be used as marker for a defect in other complexes. A diagnosis of an ETC deficiency was subject to the following requirements: i) the activity of the respective enzyme was lower than the 5th percentile of the control value when expressed to CS and lower than the 5th percentile of the control value when expressed against another marker (CII or CIV), or ii) the activity of the respective enzyme was lower than at least the 5th percentile of the control value when expressed to CS and one of the markers (e.g. CII) with the other being deficient (e.g. CIV), or iii) when expressed to CS with both CII and CIV being deficient.

4.3 Quantification of CoQ₁₀ using the standardized LC-MS/MS method

4.3.1 Batch composition for CoQ₁₀ analyses

A randomization function in Microsoft® Excel® was used to randomize a list of all the samples. Eleven consecutive samples were selected to compile a sample batch until all the samples in the randomized list were distributed into a sample batch/work list. A sample batch of 14 samples was analyzed with LC-MS/MS in a single run succession, of which 11 of them were patient samples. The standard order of a work list was as follows: QC_{blank}, QC₁, Blank, Control, P_a, P_b, P_c, P_d, P_e, P_f, P_g, P_h, P_i, P_j, P_k, QC₂. Though the Blank sample contained no muscle homogenate supernatant, it was submitted to the sample preparation procedure (Chapter 3, Section 3.3.1) just as the other biological samples. Addition of a Blank sample to the work list was purposed to investigate possible matrix effects within a batch by mimicking a biological sample without the suspended cell constituents and cell debris present. The QC and Control sample concentrations were determined after the first batch analysis, and were analyzed along with the patient samples. This gave an indication of the method performance during each batch analysis and enabled the monitoring of the method performance and any batch effect. Thus the QC and Control samples enabled the examination of the precision and repeatability of the method (Braggio *et al.*, 1996).

To compile a single quality control (QC) pool, a 130 µL sample from each of eight patient muscle homogenate supernatants (P1, P2, P4, P5, P60, P59, P57, P56) were added together. Aliquots from the QC and Control pools were transferred to separate glass KIMAX® tubes which were wrapped in tin foil and stored (at -80°C). Thus, the QC sample analyzed in every batch was an aliquot of a single QC pool. Likewise, a separate Control pool was prepared by combining 90 µL samples from each of seven patient muscle homogenate supernatant samples (P22, P23, P33, P34, P54, P53, P55).

Normally all the samples in the experimental group are included in the QC pool, however, some homogenates had barely enough sample volume necessary for a single LC-MS/MS analysis. Consequently, only homogenates with abundant sample volume were included in the QC and Control pools. Therefore, the manner in which the samples included in the QC and Control pools were selected, had no specific order. They were selected solely due to their larger sample volume. The same quality control (QC) sample would be injected three times per batch analysis. Firstly, to serve as an equilibratory run (denoted QC_{blank}), secondly, to serve as the actual QC sample (QC₁), and thirdly, so a difference in concentration of the QC sample before and after the batch run could be observed (QC₂) as an indication of a batch effect or “carry over” (Braggio *et al.*, 1996; Bresolle *et al.*, 1996).

4.3.2 Data quality

The intra- (within-batch) and interday (between-batch) coefficient of variance (CV) for the total CoQ₁₀ assay was 1.96% (determined after sequential analysis of 5 QC samples) and 2.37% (determined after analysis of 18 QC samples in 18 different batches over a period of 4 months). The intraday CV is considered to more accurately describe the precision of the optimized method's performance while the interday CV better represents the precision of routine conduct during preparation and analysis with the method, and is therefore commonly subject to greater variability (Bressolle *et al.*, 1996). The method precision was within the internationally accepted limits (< 10%). Therefore, there was no need for replicate analysis or batch corrections, and the CoQ₁₀ content of the biological samples could be determined after a single run (Shah *et al.*, 2000).

4.3.3 Normalization of CoQ₁₀ concentrations

Measured CoQ₁₀ content in skeletal muscle samples can be expressed against total protein content (nmolCoQ₁₀/mg protein) (Duberley *et al.*, 2013a), against volume (nmolCoQ₁₀/mL) (Duncan *et al.*, 2005), per wet mass of muscle tissue (nmolCoQ₁₀/g wet weight), per muscle mitochondria (nmolCoQ₁₀/mg protein) (Ogahashara *et al.*, 1998; Boitier *et al.*, 1998; Itkonen *et al.*, 2013), or normalized against citrate synthase activity (CoQ₁₀/UCS) (Itkonen *et al.*, 2013). Most studies on muscle CoQ₁₀ concentrations used homogenates (Boitier *et al.*, 1998; Duncan *et al.*, 2005; López *et al.*, 2006; Gempel *et al.*, 2007; Miles *et al.*, 2008; Montero *et al.*, 2008, 2009; Duberley *et al.*, 2013). As previously described (Chapter 3, Section 3.3.1), each muscle sample (±100 mg) was suspended in an isosmotic buffer to a 10% (w/v) solution during the homogenization procedure. However, due to the restricted sample size of some biopsies (< 50 mg), the solution volume had to be adjusted by adding adequate buffer solution (minimum 300 µl) to achieve sufficient homogenization. Consequently, some homogenates had not been an exact 10% (w/v) solution before its centrifugation at 600 x g. Also, the biopsies did not always consist purely of muscle tissue, but also of lipoid tissue.

For these reasons, normalization against wet weight (nmol/g), against volume (nmolCoQ₁₀/mL) and against total protein concentration (nmolCoQ₁₀/mg protein) was ruled out. Therefore only the CoQ₁₀/CS ratios were used to reflect the CoQ₁₀ concentrations in relation to mitochondrial content within the samples. In literature, the use of citrate synthase (CS) activity for normalization is labeled the most accurate indicator of the CoQ₁₀ content available for respiratory activity because CS activity reflects the mitochondrial mass of the sample analyzed (Montero *et al.*, 2008; Itkonen *et al.*, 2013). Since approximately 50% of CoQ₁₀ is located within the mitochondrion, and CoQ₁₀ biosynthesis mainly occurs within the IMM (Bentinger *et al.*, 2010), the expression of CoQ₁₀ concentration in relation to CS activity would accurately reveal a CoQ₁₀ deficiency (Hargreaves, 2014). Montero *et al.* (2008) stated that diagnostic effectiveness of CoQ₁₀ deficiency was improved by assessing the CoQ₁₀/CS ratios as opposed to assessing the CoQ₁₀ concentrations normalized to total protein concentrations in muscle. A patient with a severely decreased muscle CII+III ETC activity presenting with rhabdomyolysis (myopathy) had normal CoQ₁₀ concentrations when normalized to protein concentration, but when normalized to CS activity, the patient showed severely decreased concentrations of CoQ₁₀ (Montero *et al.*, 2008).

4.4 Statistical methods used

Before the sample data could be statistically analyzed, the distribution of the data points within the clinically referred controls had to be determined. The distribution was described as skewed to the left (Shapiro-Wilk, $p < 0.001$). Accordingly, non-parametric analysis was used. Tukey's method was used to identify outliers. Comparisons between groups were performed using the Mann–Whitney U test, the Kruskal-Wallis test, and a parametric ANOVA univariate test of significance. Statistical significance was recognized when the p -value was < 0.050 . For comparison of the CoQ₁₀ concentrations between the three sample groups, an ANOVA univariate test was applied with additional exploration of the differences using both a parametric post hoc test and a non-parametric Kruskal-Wallis test. The use of the parametric ANOVA univariate test was only applied during an overall statistical analysis of all three sample groups. This was allowed on account of the central limit theorem, as the three sample groups were of sufficient size (Le Cam, 1986; Rosenblatt, 1956).

4.5 CoQ₁₀ concentrations in samples of patients referred for RCD analyses

The potential influence of certain variables (ethnicity, gender, and age) on CoQ₁₀ concentrations were investigated to determine significant differences within the clinically referred control (CRC) group and whether the reference interval ought to be partitioned accordingly (Itkonen *et al.*, 2013).

Investigating the effect of gender on the biological variability of CoQ₁₀ was important because males and females have differing levels of disease susceptibility modulated by their gender-specific reactions to gene mutations and harmful environmental factors (Ordovas, 2007). These levels of disease susceptibility between genders may influence the concentrations of CoQ₁₀ within an individual (Borekova *et al.*, 2008). The overall ratio of males to females (1.03:1) was comparable with previous reports (Kaikkonen *et al.*, 2002; Miles *et al.*, 2003; Sklaldal *et al.*, 2003). The age groups of the clinically referred controls and the patients with RCDs were rather comparable which improved case-control comparison, and so gender or race was not likely to create confounding factors (Reinecke *et al.*, 2012), but was still investigated. To examine the effect of ethnicity, gender, and age on the concentrations of CoQ₁₀, all the samples in the CRC group were included in the statistical analyses (n= 79). To examine the effect of the ETC activity on the CoQ₁₀ concentrations, the samples in all three the sample groups were included in the statistical analyses (n= 155).

4.5.1 Effect of ethnicity on muscle CoQ₁₀ levels

4.5.1.1 Results and discussion

Comparisons were made between the muscle CoQ₁₀ levels of male (n= 40) and female (n= 39) subjects from three different races. The median CoQ₁₀/CS ratio (ratio expressed as nmol/U) of each group represented all the measured muscle CoQ₁₀/CS levels within each ethnic group. Figure 4.1 indicated that there was no significant difference between the CoQ₁₀/CS ratios in skeletal muscle of African (n=15), Indian (n=4), and Caucasian (n=21) male subjects ($p= 0.287$). The minimum and maximum of the Indian male subjects were 2.537 nmol/U and 4.501 nmol/U, respectively, and their 25th - 75th percentile interquartile box plot ranged from 2.685 – 4.225 nmol/U. This interquartile range was similar to the Caucasian male data set's range from 2.896 – 5.012 nmol/U. The Caucasian male subjects had a minimum and maximum of 1.540 nmol/U and 7.268 nmol/U, respectively. The African males had a wider interquartile range between 3.263 – 5.743 nmol/U, and their minimum and maximum limits of the data set was 2.902 and 7.492, respectively. The CoQ₁₀/CS ratios of Indian and Caucasian male subjects were extremely similar ($p > 0.999$), which was affirmed by their similar median CoQ₁₀/UCS ratios, *i.e.* 3.505 nmol/U for the Caucasian male subjects, and 3.391 nmol/U for the Indian male subjects. The median CoQ₁₀/CS ratio of the African male subjects was 4.028 nmol/U which did not differ significantly from the median ratios of the Caucasian ($p= 0.867$) or the Indian male subjects ($p= 0.425$). Therefore, the results from Figure 4.1 indicated that the CoQ₁₀/CS ratios of Indian and Caucasian male subjects were extremely similar, and also did not differ significantly from the CoQ₁₀/CS ratios of African male subjects. The Indian and Caucasian median ratios were lower and their interquartile ranges were narrower compared to the African male subjects.

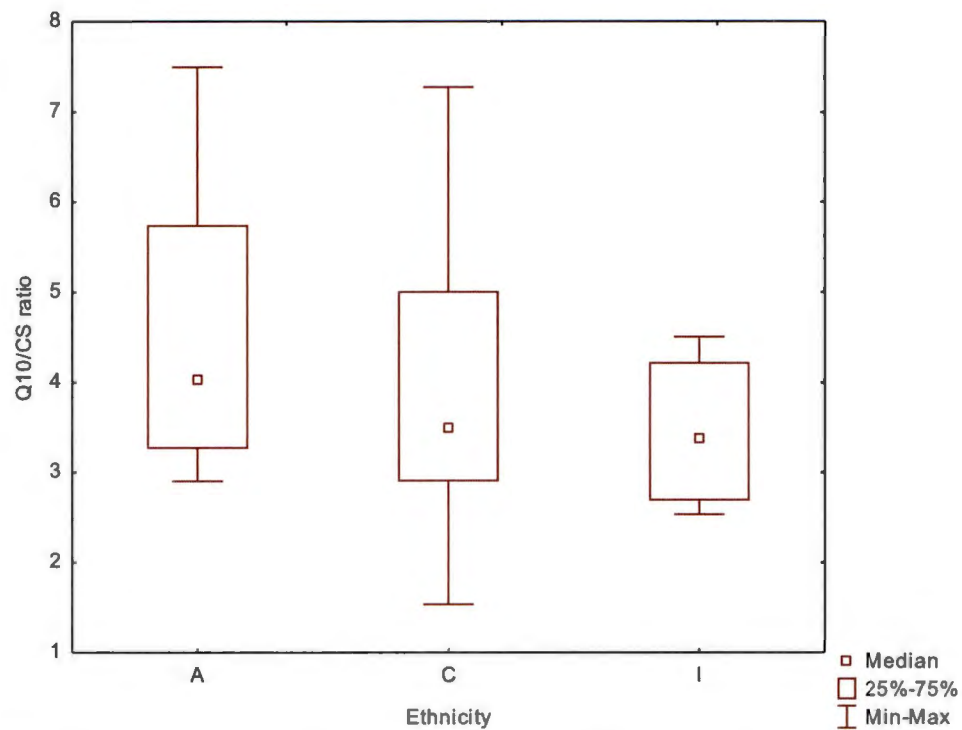


Figure 4.1: Interquartile box plots of male CoQ₁₀ concentrations in different ethnicities. The figure illustrates the CoQ₁₀/CS ratios (expressed as nmol/U) of African (A), Indian (I), and Caucasian (C) men. No statistical difference was indicated (Kruskal-Wallis, $p = 0.287$).

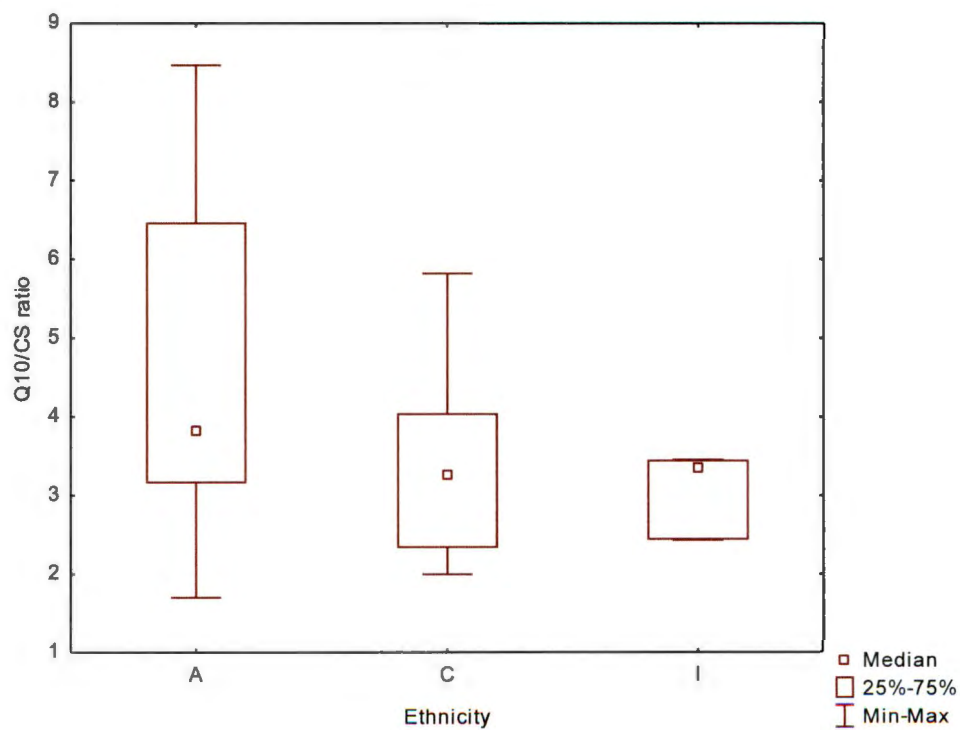


Figure 4.2: Interquartile box plots of female CoQ₁₀ concentrations in different ethnicities. No statistical difference was indicated ($p = 0.134$). A: African, I: Indian, C: Caucasian.

No significant difference between the CoQ₁₀/CS ratios in skeletal muscle of African (n=23), Indian (n=3), and Caucasian (n=13) female subjects ($p= 0.134$) was detected. The interquartile box plots for the female subjects are displayed in Figure 4.2. The African female subjects had an interquartile range between 3.161 – 6.465 nmol/U and a minimum and maximum of 1.710 nmol/U and 8.458 nmol/U, respectively. Comparison of the median CoQ₁₀/CS ratios between Caucasian and Indian female subjects showed extreme similarity ($p > 0.999$). The median CoQ₁₀/CS ratio for the Indian female subjects was 3.352 nmol/U, and 3.269 nmol/U for the Caucasian female subjects. These ratios were lower than the median CoQ₁₀/CS ratio of African female subjects, which was 3.837 nmol/U. Accordingly, the CoQ₁₀/CS ratio of the African females was less comparative to the ratios in Caucasian females ($p= 0.207$), and Indian females ($p= 0.694$). The interquartile range was widest for the African female subjects (Figure 4.2). A similar pattern was seen for the male subjects of these ethnic groups (Figure 4.1) concerning their interquartile ranges, and median CoQ₁₀/CS ratios. A wider interquartile range and higher median ratio were also found for the African male subjects compared to their male Caucasian and Indian counterparts. Also, the interquartile range for the African and Caucasian females were wider than those found in African and Caucasian males.

Because there were no significant effects observed for ethnicity on CoQ₁₀/CS ratios within the separate gender groups, further comparisons were made with the collective CoQ₁₀/CS ratios for both males and females within the three ethnic groups to examine the overall effect of ethnicity on the CoQ₁₀/CS ratios in this cohort (Figure 4.3). For the overall analysis, no significant effect was found, with no substantial differences between the CoQ₁₀/CS ratios of the three ethnic groups ($p= 0.074$). The interquartile range of the African ethnic group was 3.262 – 5.838 nmol/U, and the interquartile range of the Caucasian ethnic group was 2.691 – 4.853 nmol/U, and between 2.537 – 3.949 nmol/U for the Indian ethnic group. The median CoQ₁₀/CS ratios (of the male and female CoQ₁₀/CS ratios) in the African (n=38), Indian (n=7), and Caucasian (n=34) ethnicities, were 3.932 nmol/U, 3.352 nmol/U, and 3.386 nmol/U, respectively (Figure 4.3).

Reports on the influence of ethnicity on muscle CoQ₁₀ concentrations remain limited. Miles *et al.* (2008) observed that ethnicity had a significant effect on the enzyme activity between a probable ETC deficit patient group (with low levels of CoQ₁₀) and the disease control group. However, the significance was disregarded due to the study design, small sample size and therefore disproportionate ethnic representation. Statistically, the CoQ₁₀/U ratios of Indian and Caucasian ethnic groups were extremely similar ($p > 0.999$), and the CoQ₁₀/CS ratios of the African subjects did not differ significantly from either the Caucasian ($p= 0.191$) or the Indian group ($p= 0.224$). The African group seemed to have the widest interquartile range (Figure 4.3).

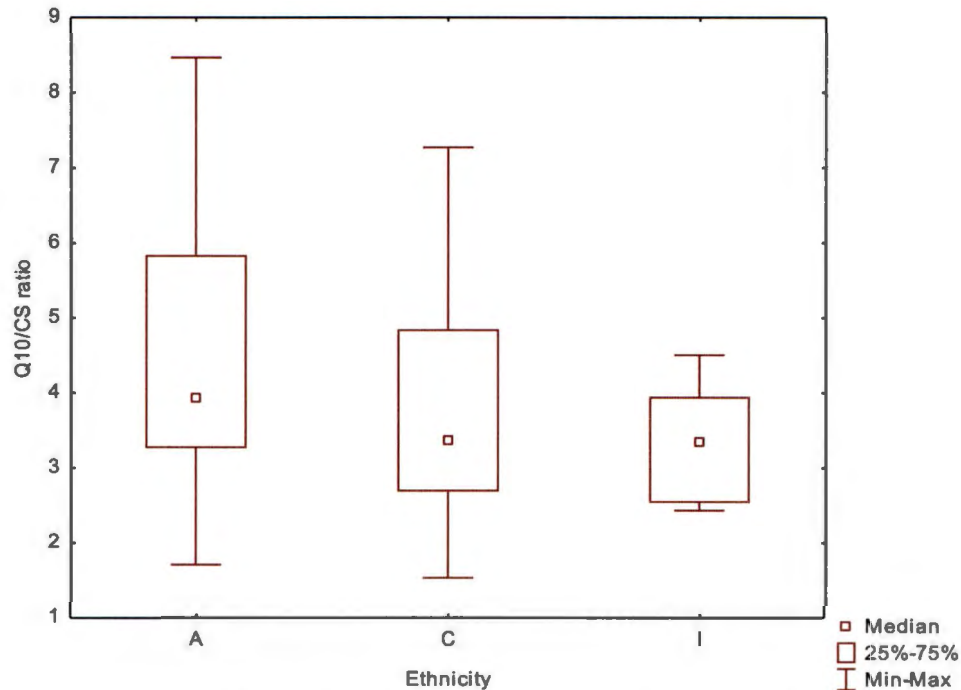


Figure 4.3: Interquartile box plots of patient (male and female) CoQ₁₀ concentrations according to ethnicity. No statistical difference was indicated between the races ($p=0.074$). A: African, I: Indian, C: Caucasian.

4.5.2 Effect of gender on muscle CoQ₁₀ levels

For the investigation of gender in the cohort of clinically referred controls ($n=79$), separate analyses of the CoQ₁₀/CS ratios between the male and female subjects were performed for each ethnic group followed by a similar analysis of the overall effect of gender on the CoQ₁₀/CS ratios in the three ethnic groups combined.

4.5.2.1 Results and discussion

Figure 4.4 illustrates the interquartile box plots of the CoQ₁₀/CS ratios in African male and female subjects. No statistically significant difference was found between the CoQ₁₀/CS ratios of the African male ($n=15$) and female subjects ($n=23$) as indicated by the p -value of 0.988. It may seem that the African female subjects have a wider distribution of CoQ₁₀/CS ratios, although their median CoQ₁₀/CS ratios were similar. The median CoQ₁₀/CS ratio of the male subjects was 4.028 nmol/U and 3.837 nmol/U for the female subjects. Figure 4.5 illustrates the box plots of the CoQ₁₀/CS ratios in Caucasian male and female subjects. For this ethnic group, no significant difference was found between the male and female subjects ($p=0.296$). From Figure 4.5, it may seem that the Caucasian male subjects ($n=21$) measured higher levels of CoQ₁₀ concentrations than the female subjects ($n=13$). However, the median CoQ₁₀/CS ratio of the Caucasian male subjects was 3.505 nmol/U and the female subjects had a slightly decreased median ratio of 3.269 nmol/U.

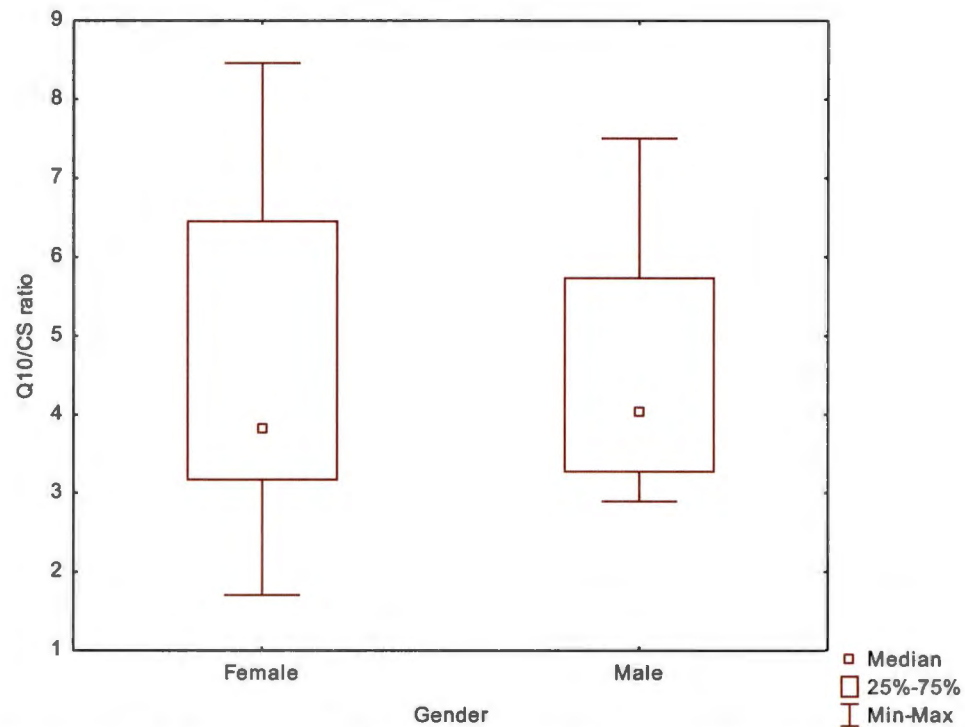


Figure 4.4: Interquartile box plots of CoQ₁₀ concentrations in African males and females. The figure indicates the data distribution and median of CoQ₁₀/CS ratios (nmol/UCS) of African male subjects compared to female subjects that had no statistically significant difference (Mann–Whitney U , $p = 0.988$).

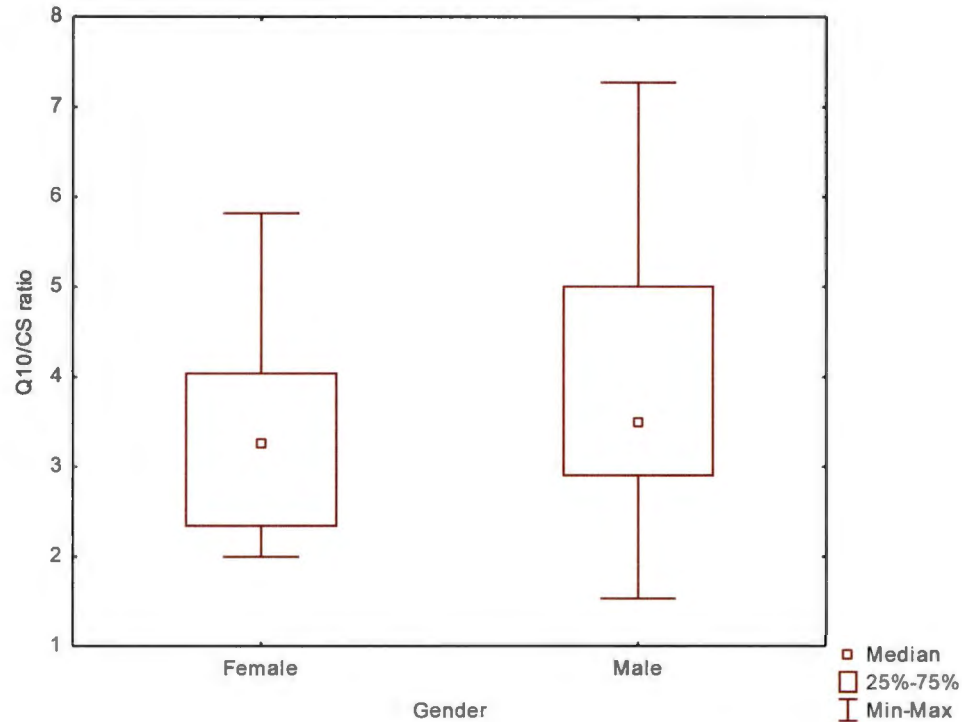


Figure 4.5: Interquartile box plots of CoQ₁₀ concentrations in Caucasian male and female subjects. The figure illustrates a comparison of the CoQ₁₀/CS ratios in Caucasian male and female subjects. No statistical difference was indicated ($p = 0.296$).

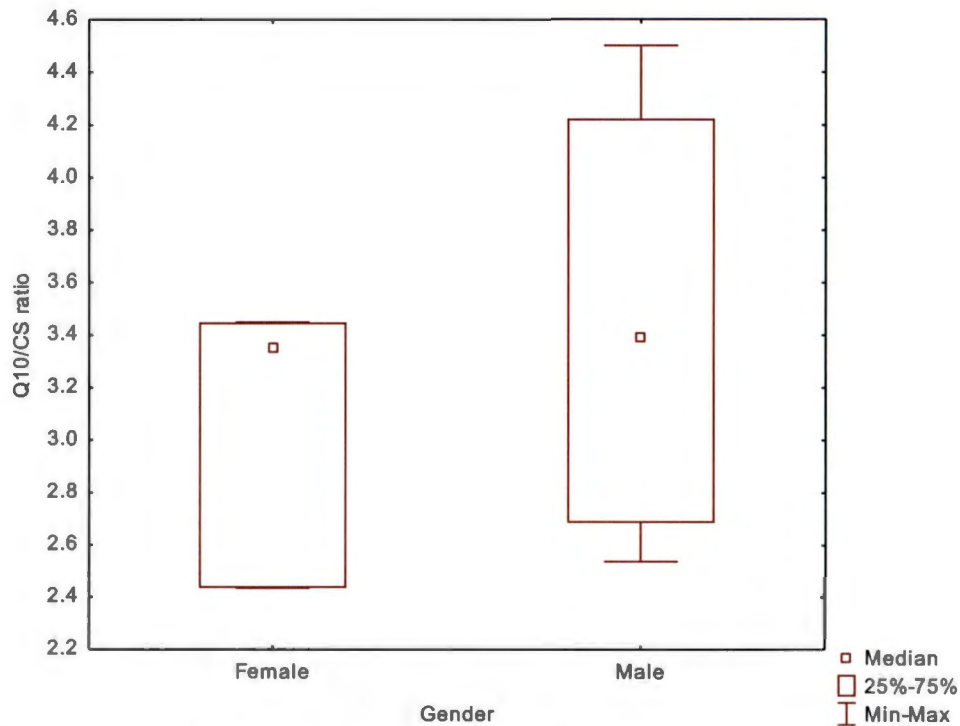


Figure 4.6: Interquartile box plots of CoQ₁₀ concentrations in Indian male and female subjects. The figure illustrates a comparison of the CoQ₁₀/CS ratios in Indian male and female subjects. No statistical difference was indicated ($p=0.480$).

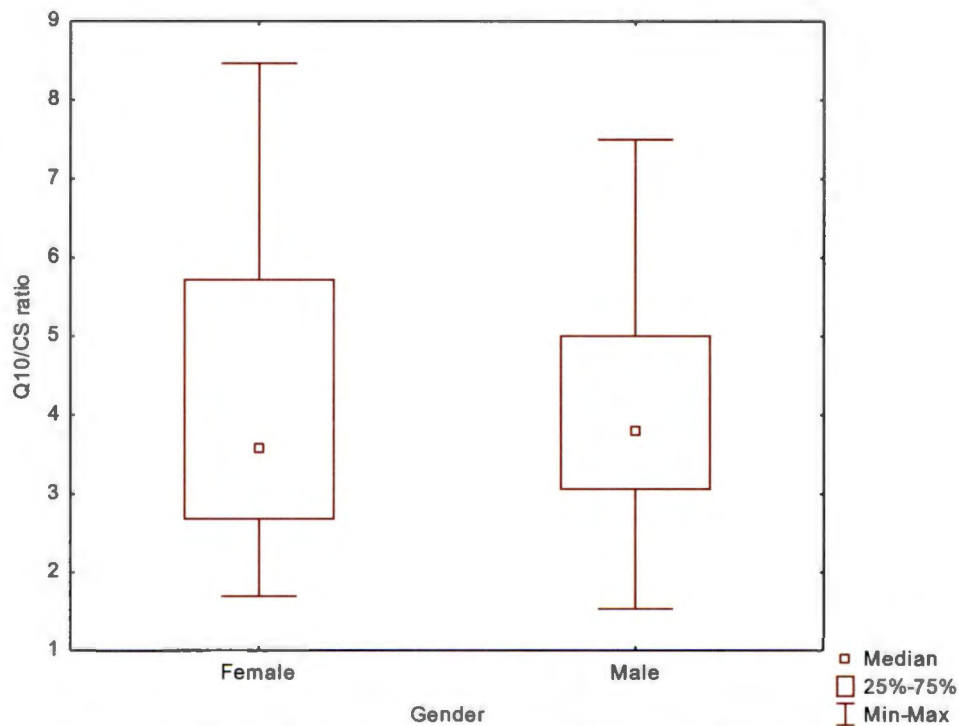


Figure 4.7: Interquartile box plots of CoQ₁₀ concentrations in male and female subjects of all three ethnicities. The figure indicates the difference in the collective CoQ₁₀/CS levels of males and females of all three ethnicities. No statistical difference was indicated ($p=0.631$).

The minimum and maximum limits of the data sets and the interquartile ranges illustrated in the Figures in this Section were similar to the measurements mentioned in Section 4.5.1.

Comparison between the Indian male ($n=4$) and female ($n=3$) subjects did not show significant difference in their CoQ₁₀/CS ratios either ($p=0.480$). The CoQ₁₀/CS ratios in Indian male and female subjects are illustrated by Figure 4.6. The median CoQ₁₀/CS ratio of the Indian male subjects was 3.391 nmol/U and the female subjects had a median ratio of 3.352 nmol/U. These findings are consistent with findings in other studies of muscle homogenate CoQ₁₀ content: no dependence with gender was found when using total protein concentration (Duncan *et al.*, 2005; Miles *et al.*, 2008) or tissue wet weight (Benoist *et al.*, 2003) for normalization. Itkonen *et al.* (2013) reported significantly higher levels of muscle mitochondrial CoQ₁₀ (nmol/mg wet weight) in men compared to women. However, when the CoQ₁₀ levels were normalized to CS, no significant influence of gender on muscle CoQ₁₀ levels were found. Kaikkonen *et al.* (2002) and Miles *et al.* (2003) found higher levels of plasma CoQ₁₀ ($\mu\text{g/L}$) in men compared to women. However, tissue levels of CoQ₁₀ concentration are mainly dependent on *de novo* synthesis (Quinzii & Hirano, 2010) whereas plasma CoQ₁₀ concentrations are influenced by dietary intake of CoQ₁₀ and do not reflect cellular levels of CoQ₁₀ accurately (Desbats *et al.*, 2014). Therefore the results in plasma cannot be used to interpret data found in skeletal muscle. Duncan *et al.* (2005) reported no correlation between the measured plasma and skeletal muscle CoQ₁₀ content.

Because gender had not shown a significant effect on CoQ₁₀/CS ratios within the separate ethnic groups, an overall comparison of CoQ₁₀/CS ratios between the male ($n=40$) and female ($n=39$) subjects was carried out. The data distribution of this analysis is illustrated by Figure 4.7. For the overall comparison no significant difference was inferred ($p=0.631$). Therefore, because neither the ethnicity nor the gender of the subjects had an influence on their CoQ₁₀/CS ratios, an overall analysis was performed to investigate the effect of age on the CoQ₁₀/CS ratios within the clinically referred control subjects.

4.5.3 Effect of age on CoQ₁₀ levels

Itkonen *et al.* (2013) categorized their patients according to specific age groups, and this method was imitated in this study during the statistical analysis of the effect of age on CoQ₁₀/CS ratios.

4.5.3.1 Results and discussion

The standard deviation of the data sets within the different age groups depicted in Figure 4.8 seem to increase from age group 2 – 4, *i.e.*, as age increases, yet the median CoQ₁₀/CS ratios of the groups do not differ significantly.

Therefore, it can be concluded that the muscle CoQ₁₀/CS ratios between the age groups had no significant changes between the younger (group 1) and older children (group 2) or between the (younger) children (group 1 and 2) and the adults (Group 3 and 4). A *p*-value of 0.601 inferred no significant effect of age on the CoQ₁₀/CS levels within the particular age groups defined in the subscript of Figure 4.8. Itkonen *et al.* (2013) reported significant age related changes in muscle mitochondrial CoQ₁₀ concentrations (nmol/g wet weight), but as soon as these CoQ₁₀ concentrations were normalized to CS activity [nmol/(min x g wet weight)], the correlation with age was invalidated (Itkonen *et al.*, 2013). This agreed with other studies who also reported no significant age-related changes in muscle CoQ₁₀ levels normalized to total protein concentrations (Duncan *et al.*, 2005; Miles *et al.*, 2008; Duberley *et al.*, 2013a), or wet weight tissue (Sacconi *et al.*, 2010).

The median age of the participants (*n*= 79) was 3 years and 4 months (range, 4 days to 50 years). The female subjects (*n*= 39) had a median age of 3 years and 5 months (range, 4 days to 50 years), and the male subjects (*n*= 38) had a median age of 3 years and 8 months (range, 9 days to 19 years). The mean CoQ₁₀/CS ratios for group 1 (< 1 year) was 3.533 nmol/U, and 4.157 nmol/U for age group 2 (1 – 10 years), 3.563 nmol/U for age group 3 (11 – 25 years), and 4.020 nmol/U for age group 4 (26 – 60 years).

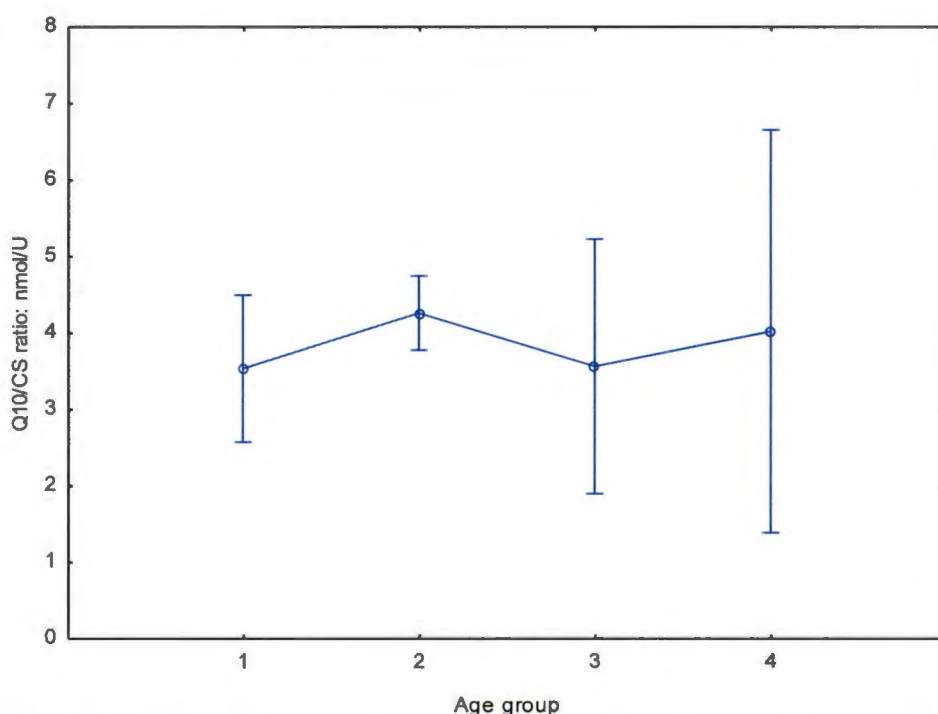


Figure 4.8: Illustration of the CoQ₁₀ concentrations in skeletal muscle homogenates according to age. The clinically referred controls were split into four age groups (as directed by Itkonen *et al.*, 2013) as follows: 1, < 1 years (*n*=15), 2, 1 – 10 years (*n*=57), 3, 11 – 25 years (*n*=5), and 4, 26 – 60 years (*n*=2). No significant difference was indicated for the CoQ₁₀/CS ratios between the different age groups (ANOVA, *p*= 0.601).

Few studies have examined age related differences in human muscle CoQ₁₀ content, and thus the effect of ageing on CoQ₁₀ remains unclear (Molyneux *et al.*, 2008). With no statistical difference between the different age groups (ranging from 4 days to 50 years), it can be concluded that ageing also did not have an effect on the CoQ₁₀/CS ratios found in this cohort of clinically referred controls.

4.5.4 ETC activity and CoQ₁₀ levels

Because there were no significant influence of ethnicity, gender, or age on total muscle CoQ₁₀/CS ratios in the CRC samples, an overall comparison was performed between the CoQ₁₀/CS ratios of all three the sample groups to obtain a global overview. The three groups are the Other RCD group (n= 47), which included patients diagnosed with respiratory chain deficiencies (excluding CII+III defects), the CII+III defect group (n= 29) which included patients with combined CII+III deficiencies, and the CRC group (n= 79), which included patients referred to the clinic after being expected of a mitochondrial disorder, but with no prevailing disorder detected. All the samples analyzed in this study (n= 155) were included in the statistical analysis of the ETC activity and the CoQ₁₀/CS ratios after removal of outliers (n= 2).

4.5.4.1 Results and discussion

Statistical analysis of the CoQ₁₀/CS ratios in the three sample groups inferred that muscle CoQ₁₀/CS ratios differed significantly between the sample groups ($p < 0.001$). Figure 4.9 illustrates the stratification of the mean muscle CoQ₁₀/CS ratios measured in the three sample groups. Table 4.1 summarizes the mean and median CoQ₁₀/CS ratios calculated for the respective sample groups, as well as their interquartile (25th % - 75th %) box plot ranges (Figure 4.10). The minimum and maximum limits of the CRC group were 1.540 nmol/U and 8.458 nmol/U, respectively. The median CoQ₁₀/CS ratio in this group was 3.606 nmol/U, and the mean CoQ₁₀/CS ratio was 4.133 nmol/U. The minimum and maximum limits of the Other RCD group were 1.914 nmol/U and 7.430 nmol/U, respectively. The median CoQ₁₀/CS ratio of the Other RCD group was 3.171 nmol/U, and the mean CoQ₁₀/CS ratio was 3.483 nmol/U. The minimum and maximum limits of the CII+III defect group were 0.861 nmol/U and 5.075 nmol/U, respectively. The median CoQ₁₀/CS ratio of the CII+III defect group was 2.474 nmol/U, and the mean CoQ₁₀/CS ratio was 2.552 nmol/U.

Table 4.1: The mean and median CoQ₁₀/CS ratios and interquartile ranges calculated for patient muscle CoQ₁₀ levels. The CoQ₁₀/CS ratios of the sample groups are expressed as nmol/U.

RCD group	Mean CoQ ₁₀ /CS ratios (nmol/U)	Median CoQ ₁₀ /CS ratios (nmol/U)	Interquartile range (25 th % - 75 th %)
CRC	4.133	3.606	2.902 – 5.048 nmol/U
Other RCD	3.483	3.171	2.460 – 4.259 nmol/U
CII+III defect	2.552	2.474	1.654 – 3.140 nmol/U

CRC: clinically referred controls; Other RCD: respiratory chain defects excluding CII+III defects.

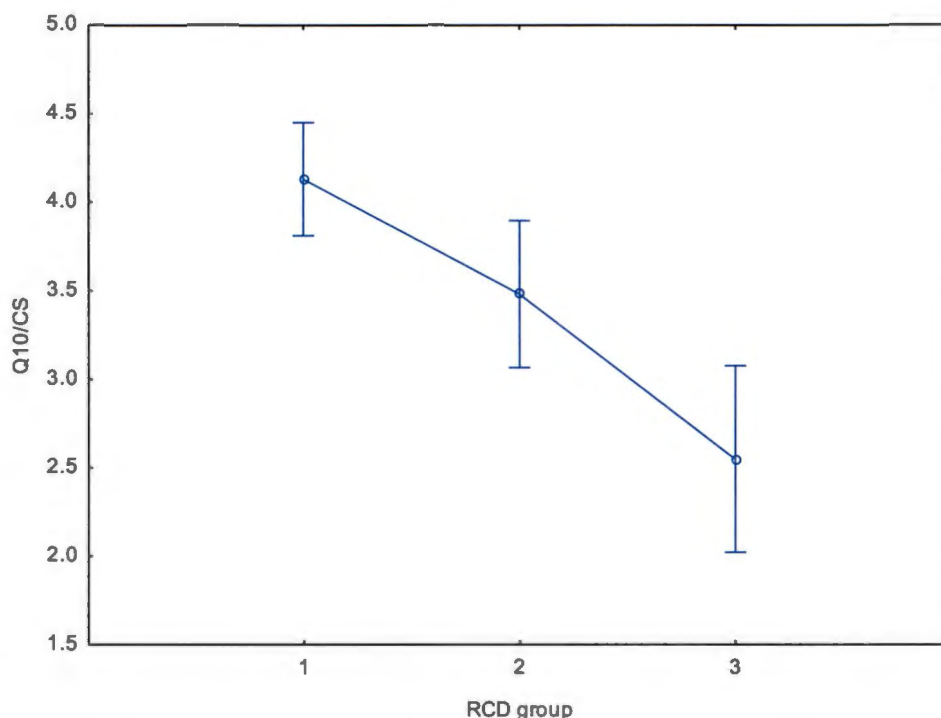


Figure 4.9: Illustration of mean CoQ₁₀ concentrations in all samples analyzed. Patient data were divided into three groups according to their RCDs as follows: 1, clinically referred controls (CRC group); 2, other respiratory chain deficiencies excluding CII+III deficiencies (Other RCD group); 3, CII+III deficiencies (CII+III defect group). A significant difference was found between the CRC group and the CII+III deficiencies (Post Hoc test (Tukey unequal N HSD), $p < 0.001$).

Further examination of the differences between the sample groups showed that both the median CoQ₁₀/CS ratios of the CRC group ($p < 0.001$) and the Other RCD group ($p = 0.036$) differed significantly from the CII+III defect group. No significant difference however, was found between the Other RCD group and the CRC group ($p = 0.072$), but the Other RCD group measured a lower median CoQ₁₀/CS concentration than the CRC group. Figure 4.10 illustrates the interquartile box plots of the median muscle CoQ₁₀/CS ratios in the three sample groups which differed as follows: 3.606 nmol/U for the CRC group, 3.171 nmol/U for the Other RCD group, and 2.474 nmol/U for the CII+III defect group.

Table 4.2: Summary of the statistical results on the muscle CoQ₁₀ levels in the different ETC activity groups. Comparisons of CoQ₁₀/CS levels between sample groups performed using parametric and non-parametric tests.

Sample group comparisons	Parametric (p -value)	Non-parametric (p -value)
CRC : Other RCD	0.072	0.095
CRC : CII+III defect	< 0.001	< 0.001
Other RCD : CII+III defect	0.036	0.019

CRC: clinically referred controls; Other RCD: multiple ETC chain defects (excluding CII+III defects). Statistically significant differences are shown in bold.

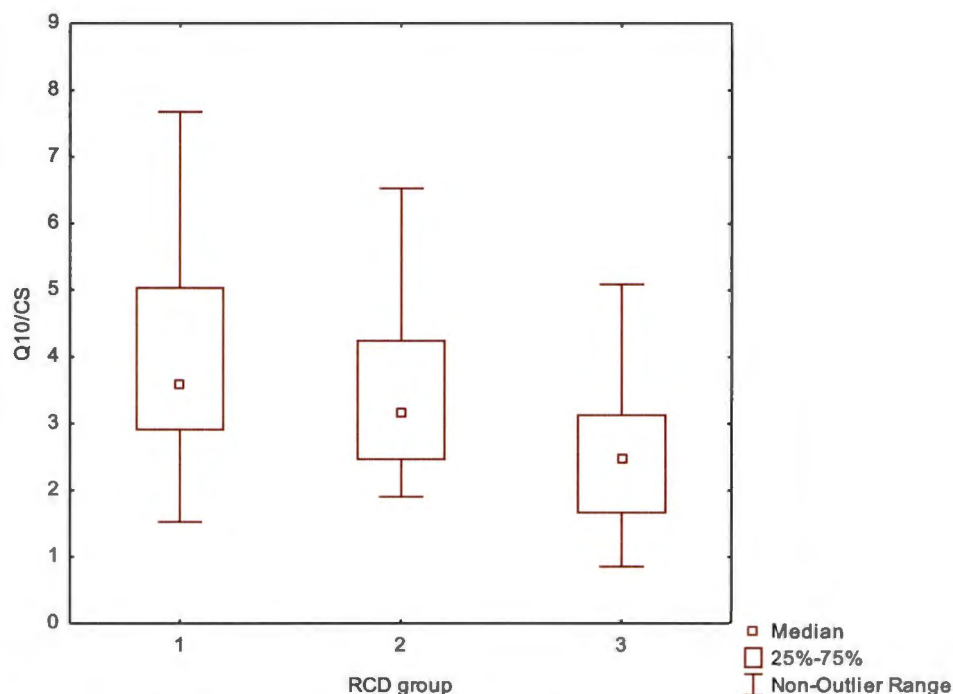


Figure 4.10: Interquartile box plots of median muscle CoQ₁₀ concentrations in a cohort of South African clinically referred controls and RCD patients. A statistically significant difference was indicated between the CRC and CII+III deficiency groups (Kruskal-Wallis, $p < 0.001$). Outliers were excluded from this figure for graphical representation purposes. 1: clinically referred controls (CRC group), 2: patients diagnosed with respiratory chain deficiencies, excluding CII+III deficiencies (Other RCD group), 3: CII + III deficient patients (CII+III defect group).

Table 4.2 summarizes the statistical findings derived from analysis of mean CoQ₁₀/CS ratios and the median CoQ₁₀/CS ratios of the sample groups. The Other RCD group and the CRC group were more alike in their median CoQ₁₀/CS ratios ($p = 0.095$) than in their mean CoQ₁₀/CS ratios ($p = 0.072$), and thus no significant difference between the Other RCD group and the CRC group was inferred. The CII+III defect group radically differed from the CRC group in their median CoQ₁₀/CS ratios ($p = 0.001$) and mean CoQ₁₀/CS ratios ($p < 0.001$). The Other RCD group and the CII+III defect group also differed significantly in their median CoQ₁₀/CS ratios ($p = 0.019$) as well as in their mean CoQ₁₀/CS ratios ($p = 0.036$). This is also illustrated by Figure 4.10. The CII+III defect group consisted of patients with CII+III deficiency and normal levels of their isolated ETC complexes, while other patients in this group had CII+III deficiencies with various other RCDs. The Other RCD group consisted of the patients with multiple RCDs (excluding CII+III deficiencies), and therefore the relative decrease in the mean CoQ₁₀/CS ratio of the Other RCD group compared to the mean control value was expected because of the frequent association of decreased CoQ₁₀ concentrations and mitochondrial encephalomyopathies (Montero *et al.*, 2005; Sacconi *et al.*, 2010).

The Other RCD group showed a 12% decrease in median CoQ₁₀/CS ratio compared to the control median value, while the CII+III defect group measured the lowest median CoQ₁₀/CS ratio which was significantly decreased 31% below the control median value. Literature supported this observation with results for muscle CoQ₁₀ concentrations stratified within sample groups similar to the sample groups in this study (Montero *et al.*, 2005; Miles *et al.*, 2008). Montero *et al.* (2005) measured decreased muscle CoQ₁₀ concentrations in six patients within a group of thirteen with low CI+III and CII+III activities. A second group of nine patients had definite RCDs, but the mean muscle CoQ₁₀ concentration of this group was still higher compared to the group of thirteen with low CI+III and CII+III activities. Miles *et al.* (2008) also found that the lowest CoQ₁₀ concentrations compared to the mean control value were within a group of twelve patients with probable RCDs (all with low CII+III and one patient with low CIII activity). This highlights the usefulness of CI+III and CII+III deficiencies in the detection of a possible CoQ₁₀ deficiency.

CoQ₁₀ deficiency is also associated with various other RCDs, up-regulation of antioxidant defenses, and apoptosis (Quinzii *et al.*, 2008a). The pathogenic mechanisms in patients with decreased CoQ₁₀ concentrations involve impaired electron transport via CI and CII (and other dehydrogenases) to CIII of the respiratory chain which causes an impairment of OXPHOS and therefore decreased ATP production in the affected cells (Quinzii *et al.*, 2008a). In an investigation on the pathogenic mechanisms of CoQ₁₀ deficiency in skin fibroblasts, Quinzii *et al.* (2008b) found that severe CoQ₁₀ deficiency causes variable degrees of impaired ATP synthesis and oxidative stress (lipid and protein oxidation), and that there was a direct correlation between the degree of CoQ₁₀ deficiency and the decrease in ATP synthesis. Further studies on the topic revealed an additional direct correlation between the degree of CoQ₁₀ deficiency and ROS production so that partial CoQ₁₀ deficiencies (between 30 – 50% of residual CoQ₁₀) may also have harmful effects on the affected cell (Quinzii *et al.*, 2010). These findings were supported by Duberley *et al.* (2013b) who found that an induced decrease in CoQ₁₀ (reduced to 46% residual CoQ₁₀ concentrations at cellular level) was accompanied by a decrease in total cellular ATP synthesis in neuronal cells, and a concomitant progressive decrease in ETC activities (CI, CII+III, and CIV).

In light of these pathogenic mechanisms of CoQ₁₀ deficiency, and according to the significant differences between the three sample groups of this study (Table 4.2), it may be that the decreased median CoQ₁₀/CS ratio found within the Other RCD group (3.171 nmol/U) could have caused the RCD deficiencies in the patients, but would not have caused such a great decrease in ETC activities as would the significantly low median CoQ₁₀/CS ratio (2.474 nmol/U) found in the CII+III defect group.

The greater decrease in the muscle CoQ₁₀/CS ratios in the CII+III defect group could have caused a greater decrease in ETC activity, which resulted in combined CII+III RCDs (and other associated RCDs) (Duberley *et al.*, 2013b). Mitochondrial oxidative stress due to abnormal ROS generation has been said to decrease endogenous levels of CoQ₁₀ in humans (Flint Beal *et al.*, 2002), and CoQ₁₀ is an electron transport molecule itself, and therefore its decrease would also contribute to the pathogenesis of a present RCD and consequential oxidative stress (Quinzii *et al.*, 2010). The determination of the combined CI+III and CII+III ETC deficiencies in patients with CoQ₁₀ deficiency is supportive in diagnosis (Montero *et al.*, 2005), but patients with proven CoQ₁₀ deficiencies may present with normal activities of all their ETC enzymes (Hirano *et al.*, 2012). Therefore, establishment of a reference interval can identify a CoQ₁₀ deficiency despite the variability in the clinical phenotypes and different RCDs found in the patients with CoQ₁₀ deficiency (Buján *et al.*, 2014). Hence, a reference interval was established for the observed range of CoQ₁₀/CS ratios within the CRC group (n=79).

4.6 Establishing a CoQ₁₀ reference interval for 600 x g muscle homogenate supernatants

The reference interval (RI) was established to aid in the identification of patients with a CoQ₁₀ deficiency. Partitioning of the CoQ₁₀/CS ratios according to ethnicity, gender, and age was considered in Sections 4.4.1 – 4.4.3, and because these variables had no significant influence on total muscle CoQ₁₀/CS ratios there was no need for separate sub-divisional partitioning of the RI range. The RI was calculated according to the International Federation of Clinical Chemistry (IFCC) guidelines (CLSI C28-A3c) as a 95% central reference interval. With skewed normality, non-parametric ascending rank order statistics were used to determine the reference limits (the 2.5th and 97.5th percentiles) of the CRC group data set. Having less than 120 individuals in the CRC group (n= 79), the bootstrap method was also applied. This would ensure a proper reference interval estimate, but an exact lower 2.5th limit of 1.71 nmol/U was again calculated.

4.6.1 Results and discussion

The reference interval for CoQ₁₀/CS concentrations in quadriceps (vastus lateralis) muscle homogenate 600 x g supernatants was derived as 1.710 - 8.460 nmol/U, with a median CoQ₁₀/CS ratio of 3.606 nmol/U. This observed reference interval for muscle CoQ₁₀ was similar to the reference interval (2.680 - 8.470 nmol/U, mean value 5.380 nmol/U) of skeletal muscle supernatants of pediatric controls (n=37) quantified with an LC-ECD method, but the preparation and the muscle type of the homogenates were not specified (Montero *et al.*, 2008, 2009; Buján *et al.*, 2014). Because total CoQ₁₀ content may differ significantly between muscle type (Benoist *et al.*, 2003), there were limited comparable RIs in literature.

Table 4.3: CoQ₁₀/CS ratios and RCDs in patients identified with CoQ₁₀ deficiencies.

Pateint number	Ethnicity	CoQ ₁₀ /CS concentration* (nmol/U)	ETC deficiencies	Sample group
P24	A	1.204	CI, CII, CIII, CIV, CII+III	CII+III defect
P78	C	0.861	CI, CIII, CIV, CII+III	CII+III defect
P98	A	1.229	CII+III	CII+III defect
P99	C	1.391	CIII, CII+III	CII+III defect
P2	A	1.559	CII, CII+III	CII+III defect
P9	A	1.607	CII+III	CII+III defect
P14	A	1.638	CII+III	CII+III defect
P95	A	1.654	CIV, CII+III	CII+III defect
95% central reference interval		1.710 - 8.460 (3.606)		

*CoQ₁₀/CS concentrations expressed as nmolCoQ₁₀/UCS

The study by Montero *et al.* (2008) of the quantification of CoQ₁₀ concentration in six patients with RCDs, and the case report of CoQ₁₀ deficiency associated with mtDNA depletion by Montero *et al.* (2009) are the only other known studies that employed a similar sample (muscle homogenate supernatant) and the same normalization method as this study, though no reported studies for CoQ₁₀ quantification used supernatants of 600 x g vastus lateralis muscle homogenates.

The reference interval was subsequently used to identify a suspected CoQ₁₀ deficiency in the patients with mitochondrial disorders. Decreased CoQ₁₀ levels were identified as those which were below the lowest limit (2.5th %) of the reference interval observed in this study's reference cohort. CoQ₁₀ was deficient (< 1.710 nmol/U) in six African patients, and two Caucasian patients.

As seen in the results summarized in Table 4.1 and Table 4.2, it is evident that the CII+III defect group differed significantly from both the CRC and the Other RCD group. Buján *et al.* (2014) reported a patient with multiple RCDs who measured a muscle CoQ₁₀/CS ratio (1.2 nmol/U) only slightly decreased below a patient with normal ETC activities (muscle CoQ₁₀/CS ratio of 1.3 nmol/U), and surprisingly, a patient with a combined CII+III deficiency with a higher CoQ₁₀/CS ratio (1.6 nmol/U) than the patient with normal ETC activities. However, all these muscle CoQ₁₀ measurements were below the lowest limit of the established reference interval (2.7 nmol/U). In other words, there are exceptions where patients with CII+III deficiencies do not have low levels of CoQ₁₀ and may even have normal levels of CoQ₁₀ (Hirano *et al.*, 2012), but the results obtained by Buján *et al.* (2014) show that by establishing a reference interval, CoQ₁₀ deficiency can be detected no matter the ETC deficiency.

Also, more often than not, the combined CI+III and CII+III enzyme deficiencies are impaired in the absence of CoQ₁₀, and therefore the presence of these combined RCDs in skeletal muscle are especially predictive of a CoQ₁₀ deficiency (Rahman *et al.*, 2012). The results in this study support this notion because all eight patients identified with decreased CoQ₁₀ concentrations in this study had CII+III RCDs (< 1.710 nmol/U, *i.e.*, below the 2.5th % of the observed reference interval). Table 4.3 gives a summary of the CoQ₁₀/CS ratios and RCDs in patients identified with decreased CoQ₁₀ concentrations, as well as the 95% central reference interval. Three of the eight patients with decreased CoQ₁₀/CS ratios had CII+III deficiencies with normal activities of their isolated ETC complexes, while two patients had associated multiple RCDs, and three patients had CII+III deficiencies with a concomitant CII, CIII and CIV deficiency, respectively. A decrease in muscle CoQ₁₀ concentrations may have caused their CII+III deficiencies and a concomitant decrease in the activity of CI, CII and CIII, and most likely CIV (Duberley *et al.*, 2013b) due to the involvement of cytochrome *c* in the Q-cycle as electron transport molecule from CIII to CIV. Coupled to a defect in the Q-cycle is the consequential impaired extrusion of protons from the mitochondrial matrix by CoQ₁₀ for the reduction of CoQ₁₀ (Mitchell, 1975b).

Some of the patients within the CII+III defect group had fairly abundant CoQ₁₀/CS ratios. For example, two patients diagnosed with the same RCDs, namely a combined CII+III deficiency along with isolated CII, and CIII deficiencies. They also had very similar, normal CoQ₁₀/CS ratio of 3.967 nmol/U and 3.983 nmol/U. Both these CoQ₁₀/CS ratios fell within the 95% central RI. This may indicate a genetic defect in the isolated ETC enzyme complexes, or a structural distortion in any one of the binding sites for CoQ₁₀ in the ETC complexes CI, CII or CIII within these patients, though the precise binding site in CII is unclear (Lenaz *et al.*, 2007). The binding sites are mentioned in Section 2.1.4, and Section 2.2.3 of Chapter 2. Except for cytochrome *b*, all the subunits of CIII are nDNA encoded. Therefore, a mutation in nDNA may also be a possible explanation for the CII+III deficiency apparently not caused by decreased CoQ₁₀ concentrations (Leonard & Schapira, 2000; reviewed by Cannino *et al.*, 2007; El-Hattab & Scaglia, 2013). Also, nDNA mutations in genes functioning in mitochondrial nucleotide synthesis or mtDNA replication may be a cause of the multiple ETC chain deficiencies (El-Hattab & Scaglia, 2013). Therefore, a diagnosis based on the ETC analysis may give a false positive result, which is another reason why direct measurement of muscle CoQ₁₀ concentrations is the most reliable diagnostic test for a CoQ₁₀ deficiency (Rahman *et al.*, 2012), because the degree of CoQ₁₀ in patients with (CII+III) RCDs are very broad (Quinzii *et al.*, 2008a). Decreased levels of CoQ₁₀ in muscle or fibroblasts remain the biochemical hallmark of a CoQ₁₀ deficiency (Montero *et al.*, 2008), though muscle is the tissue of choice (Buján *et al.*, 2014), since determination in fibroblast can be more time consuming (Montero *et al.*, 2008).

4.7 Summary

After quantification of the CoQ₁₀ concentrations with the standardized LC-MS/MS method, and determining a skewed data distribution for the clinically referred controls, statistical investigation of the acquired patient data was performed using non-parametric methods. The LC-MS/MS method used for the acquisition of the patient data had excellent precision described by the intra- and interday CVs of 1.95% and 2.37%, respectively. The convenience of the ETC enzyme activity data (CI, CII, CIII, CIV, CII+III and CS activity) enabled categorization of the samples within the three different groups (CRC group, Other RCD group and the CII+III defect group), and also enabled the investigation of the total CoQ₁₀/CS ratios within these different RCD groups. Statistical inference of the CRC group indicated no differences concerning CoQ₁₀ concentrations between age, gender, or ethnic groups within the context of this study.

Significant differences in the CoQ₁₀/CS ratios were found between the CII+III defect group compared to both the CRC group and the Other RCD group, with the lowest median CoQ₁₀/CS ratio found in the CII+III defect group. The CRC group and the Other RCD group did not differ significantly in their CoQ₁₀/CS ratios. Patients with CII+III deficiencies with normal levels of CoQ₁₀ may have a problem with the stoichiometry at the binding sites of the respective enzyme complexes. Other possible reasons may be mutations in the assembly factors or respiratory subunit genes involved in the assembly and encoding of the ETC enzyme complexes, respectively (Rahman & Hanna, 2009). This raises the question of whether these patients have an isolated case of CoQ₁₀ deficiency and if there are other diseases at play causing the mitochondrial myopathies in these patients (Hargreaves, 2014). The established central 95% central RI (calculated from the CoQ₁₀/CS ratios of the CRC group) enabled the identification of eight patients (two Caucasian, and six African patients) with decreased CoQ₁₀/CS concentrations (below the 2.5th % of the RI) and all eight had CII+III deficiencies (Montero *et al.*, 2009). This supported the findings in a study by Smuts *et al.* (2010) who stated that African patients in South-Africa show predominance for combined CII+III deficiencies. It also showed that the lowest levels of CoQ₁₀/CS ratios are found within the patients with varying degrees of CII+III deficiencies. Five of the eight patients identified with decreased CoQ₁₀/CS concentrations had CII+III deficiencies and various other RCDs. Likewise, in literature patients with decreased CoQ₁₀ concentrations in skeletal muscle presented with variable degrees of different RCDs (Quinzii *et al.*, 2008a; Bujan *et al.*, 2014).

CHAPTER 5: CONCLUSIONS

5.1 Introduction

CoQ₁₀ is a remarkable lipid soluble molecule involved in many functions of the cell (Turunen *et al.*, 2004). Besides being an electron transport molecule during ATP production between CI and CII to CIII, it also accepts electrons into the ETC from various dehydrogenases, such as dehydro-oxaloacetate dehydrogenase involved in pyrimidine biosynthesis, and the ETFDH:QO complex involved in β -oxidation (Lenaz *et al.*, 2007; reviewed by Watmough & Frerman, 2010). In its reduced form it is one of the most potent endogenously synthesized lipid soluble antioxidants within all cellular membranes and membranous organelles (Turunen *et al.*, 2004), and CoQ₁₀ is also involved in the modulation of apoptosis and uncoupling proteins (Quinzii *et al.*, 2008a). These functions of CoQ₁₀ explain its crucial role in the ETC and its association with ETC deficiencies (Quinzii *et al.*, 2008b; Quinzii *et al.*, 2010). CoQ₁₀ deficiencies may arise secondary to pathological conditions such as mutations in genes not directly involved in CoQ₁₀ biosynthesis, mtDNA mutations or deletions, increased susceptibility due to genetic factors (Bujàn *et al.*, 2014), or mitochondrial encephalomyopathies (Montero *et al.*, 2005). Primary CoQ₁₀ deficiencies result from mutations in the genes involved in the biosynthesis of CoQ₁₀ and is known to respond well to CoQ₁₀ supplementation. These mitochondrial disorders present with heterogeneous clinical phenotypes that have decreased activities of the combined CI+III and CII+III deficiencies, and low muscle or fibroblast CoQ₁₀ concentrations (Ogasahara *et al.*, 1989; Rahman *et al.*, 2001; Lalani *et al.*, 2005; Salviati *et al.*, 2005; Rötig *et al.*, 2007; Bujàn *et al.*, 2014).

Treatment of mitochondrial disorders is limited, but supplementation with CoQ₁₀ (and its analogues) has shown therapeutic benefit in patients with mitochondrial disorders (Hargreaves, 2014; Quinzii & Hirano, 2010). Partial CoQ₁₀ deficiency with normal respiratory chain CI+III and/or CII+III enzyme activities benefit from CoQ₁₀ supplementation. Patients with a primary CoQ₁₀ deficiency respond better to supplementation than patients with a secondary CoQ₁₀ deficiency (Desbats *et al.*, 2014), since sustained treatment may show very good recovery in patients with primary CoQ₁₀ deficiency, and may ameliorate symptoms in patients with secondary CoQ₁₀ deficiency (Montero *et al.*, 2008; Bujàn *et al.*, 2014). Unfortunately, CoQ₁₀ supplementation therapy does not always lead to clinical improvement (Rötig *et al.*, 2000; Rahman *et al.*, 2001; Musumeci *et al.*, 2001). However, all the phenotypes presented in CoQ₁₀ deficiency benefit from high dosage oral CoQ₁₀ supplementation (Quinzii & Hirano, 2010), and diseases not associated with CoQ₁₀ deficiency. Conversely, there are disorders not associated with CoQ₁₀ deficiency, such as Kearns-Sayre Syndrome and hypoparathyroidism, which also show clinical improvement with CoQ₁₀ supplementation (Hargreaves, 2014).

Due to the therapeutic potential of oral CoQ₁₀ supplementation, it was recommended that muscle CoQ₁₀ concentrations be quantified in a cohort of South African patients with suspected mitochondrial disorders to identify candidates with possible CoQ₁₀ deficiency, *i.e.* patients with decreased CoQ₁₀ concentrations who would benefit from CoQ₁₀ replacement therapy (Rahman *et al.*, 2012).

5.2 Problem statement, aim & objectives

In the experimental group of this study 38% of the patients with RCDs had CII+III deficiencies. Given that CoQ₁₀ participates as an electron transport molecule between these complexes, and because CoQ₁₀ deficiency has been frequently found among patients with mitochondrial encephalomyopathies (Montero *et al.*, 2005; Sacconi *et al.*, 2010; Rahman *et al.*, 2012), the question arose whether the patients with RCDs had lower levels of CoQ₁₀, particularly the patients with combined CII+III deficiency. The **aim** of this study was to quantify the total CoQ₁₀ concentrations in a cohort of South African patients with ETC deficiencies. Therefore, an analytical approach with LC-MS/MS was designed to determine the CoQ₁₀ concentrations in skeletal muscle of a cohort of South African (mostly pediatric) patients, to subsequently address the above raised question, and possibly identify patients with CoQ₁₀ deficiency who would benefit from CoQ₁₀ supplementation for the restoration of efficient electron flow within their ETC (Montero *et al.*, 2008). The LC-MS/MS method would also be used in the accomplishment of the study objectives. Two **objectives** were set, namely i) to optimize the quantification of total CoQ₁₀ using LC-MS/MS, and ii) to investigate the levels of CoQ₁₀ in ETC deficiencies by quantifying total CoQ₁₀ concentrations in South African patient muscle samples with a standardized LC-MS/MS method.

5.3 Conclusions

In this section, the approach and method used (Section 5.3.1), and the results obtained during this study (Section 5.3.2) are thoroughly evaluated and interpreted in order to formulate reliable conclusions (Section 5.4) and propose feasible future prospects in Section 5.5.

5.3.1 The experimental approach with an LC-MS/MS method

CoQ₁₀ values have been quantified in various tissue types using different well established methods (Barshop & Gangoiti, 2007). In this subsection, a general understanding of the different methods used in the quantification of CoQ₁₀ and the rationale for the compilation of the HPLC-MS/MS method used in this study are briefly discussed. Finally, the features of the standardized and validated HPLC-MS/MS method utilized to accomplish the aim of this study is discussed to point out the suitability of the method for quantification of total CoQ₁₀ in biological samples.

For this study, an LC-MS/MS method with ESI in positive mode was chosen for the quantification of CoQ₁₀ concentrations in 600 x g (vastus lateralis) skeletal muscle homogenate supernatants.

The electrochemical detection (ECD) technique is commonly used in the determination of CoQ₁₀ in human plasma (Hansen *et al.*, 2004), because an LC-ECD method is useful in the sensitive determination of the redox status of electroactive compounds like CoQ₁₀ (Kishikawa & Kuroda, 2014), simultaneously measuring ubiquinone and ubiquinol or the total CoQ₁₀ content. However, the electrochemical cells are vulnerable to high concentrations of the lipophilic ubiquinone (CoQ₁₀) because the lipids may aggregate onto the electrochemical cells increasingly inactivating the ECD over time (Hansen *et al.*, 2004). Unlike the ECD technique, LC-MS/MS provides quantitative (mass/concentration) and qualitative (structural) information about the analyte after ionization (reviewed by Ho *et al.*, 2003; Kishikawa & Kuroda, 2014). Though the instrumentation is expensive and not widespread, the use of LC-MS/MS has become more popular (El-Najjar *et al.*, 2011; Kishikawa & Kuroda, 2014), especially in the quantification of CoQ₁₀ in skeletal muscle (Duncan *et al.*, 2005; Teshima & Kondo, 2005; Duberley *et al.*, 2013a; Itkonen *et al.*, 2013), providing higher selectivity and comparative sensitivity to the HPLC-ECD method. The diverse choices of ionization modes for CoQ₁₀ measurement makes it hard to reach consensus on the best ionization mode for CoQ₁₀ measurement (Kishikawa & Kuroda, 2014). Therefore, improvement of poor ionization of the lipophilic CoQ₁₀ molecule in this study rested on standardization of the ionization agent (ammonium formate) more than on the ionization mode to increase sensitivity of the LC-MS/MS method (Teshima & Kondo, 2005). During the compilation of the LC-MS/MS, the economical option of using a UPLC separation technique (Palaikov *et al.*, 2009) was not available for detection of CoQ₁₀. Along with the use of the stable isotope (deuterium labeled CoQ₁₀, CoQ₁₀[²H₆]), the ionization agent (ammonium formate) improved ionization to such an extent that it practically overcame the suppression of analyte ionization (Barshop & Gangoi, 2007).

A common limitation of the methods in CoQ₁₀ quantification was the use of physiological internal standards already present in humans (ubidecarenone, CoQ₆, CoQ₇, CoQ₉). Other analogues of CoQ₁₀ such as CoQ₁₁, diethoxy-CoQ₁₀, and dipropoxy-CoQ₁₀ are chemically synthesized and are also inadequate for the same reason as CoQ₆₋₉ (Duncan *et al.*, 2005; Duberley *et al.*, 2013a), and the synthesis of radio-labeled CoQ₁₀ is difficult (Hamamura *et al.*, 2002a,b). Only two reports used a non-physiological stable isotope (Duberley *et al.*, 2013a; Itkonen *et al.*, 2013) with LC-MS/MS for CoQ₁₀ quantification in skeletal muscle. Stable isotopes are preferred in mass spectrometry because they fragment in a similar way that produces the same unique product ion of the analyte during CID.

For this study, CoQ₁₀[²H₆] was used as internal standard for the quantification of CoQ₁₀ concentrations in vastus lateralis 600 x g skeletal muscle homogenate supernatants. The stable isotope closely co-eluted with CoQ₁₀ and thus minimized matrix effects and increased sample recovery which was very beneficial, because quantification of CoQ₁₀ has the drawback of ionization suppression (Itkonen *et al.*, 2013). The calibration curve constructed using CoQ₁₀[²H₆] gave excellent linearity with an R² value > 0.999 for the standardized LC-MS/MS quantification method. Therefore, standardizing the LC-MS/MS method enabled the achievement of the first objective of this study, that is, to optimize the quantification of total CoQ₁₀ using LC-MS/MS. The standardized LC-MS/MS method was also validated for the analysis of biological samples and the successive quantification of CoQ₁₀ concentrations in patients with respiratory chain deficiencies (n= 76) and the clinically referred controls (n= 79). Calculated from 5 QC samples analyzed on the same day in a single sample batch, the inter-day CV was 1.96% and the intra-day CV was calculated as 2.37% from 18 QC samples analyzed in 18 different batches over a period of 4 months. These values described the decent precision of the standardized LC-MS/MS method which was therefore fit to analyze biological samples with repeatable and reliable results. Hence, the validation of the LC-MS/MS method enabled the quantification of CoQ₁₀ concentrations in the experimental group of the study so as to address the second objective, namely to investigate the levels of CoQ₁₀ in ETC deficiencies by quantifying total CoQ₁₀ concentrations in South African patient muscle samples with a standardized LC-MS/MS method.

5.3.2 CoQ₁₀ concentrations in patients with RCDs and in clinically referred controls

The investigated muscle samples were grouped into three groups according to their respiratory chain activities. The Clinically Referred Control (CRC) group were patients expected of having a respiratory chain deficiency but no RCD was detected on enzymatic level (n=79). The 'Other RCD' group compiled all patients diagnosed with a respiratory chain deficiency, excluding a CII+III defect (n=47), and the CII+III defect group consisted of patients with combined CII+III deficiencies, including defects in other RC complexes in some patients (n=29). The measured CoQ₁₀/CS ratios of the clinically referred controls were not significantly influenced by ethnicity ($p= 0.074$), gender ($p= 0.631$), or age ($p= 0.601$), as affirmed by previous reports (Benoist *et al.*, 2003; Miles *et al.*, 2008; Itkonen *et al.*, 2013). Overall statistical analysis between the CRC, the Other RCD and CII+III defect group revealed that the CoQ₁₀/CS ratios differed significantly between these sample groups ($p < 0.001$). Further statistical analysis between the sample groups revealed that the median CoQ₁₀/CS ratio for the CII+III defect group was significantly different compared to both the CRC group ($p < 0.001$) and the Other RCD group ($p= 0.019$). The mean CoQ₁₀/CS ratio of the CII+III defect group was also significantly different compared to both the CRC group ($p < 0.001$) and the Other RCD group ($p= 0.036$).

The CII+III defect group had the lowest calculated median (and mean) muscle CoQ₁₀/CS ratio of the three sample groups, confirming the statements in previous reports (Montero *et al.*, 2005; Miles *et al.*, 2008; Duberley *et al.*, 2013b). The median and mean CoQ₁₀/CS ratios of the sample groups are summarized in Table 4.1 of Chapter 4, and the inferred statistical differences between the groups are given in Table 4.2 of Chapter 4.

After establishing a 95% central reference interval (RI) for the 600 x g muscle homogenate supernatants of the clinically referred controls, the measured muscle CoQ₁₀/CS ratios in the other two patient groups could be compared thereto. In the CII+III defect group, patients also presented with normal levels of CoQ₁₀/CS ratios. This suggested that their CII+III deficiencies were not related to the pathogenic mechanisms caused by decreased CoQ₁₀ concentrations or to any defects in the ubiquinone biosynthesis protein (COQ) genes involved in CoQ₁₀ biosynthesis, but possibly related to other molecular defects (Montero *et al.*, 2005; Quinzii *et al.*, 2008b, 2010; Duberley *et al.*, 2013b). However, the eight patients identified with decreased muscle CoQ₁₀/CS ratios (below the 2.5th % of the established 95% central RI, *i.e.* < 1.710 nmol/U) were all in the CII+III defect group, supporting the notion that CII+III deficiencies are a good indication of a CoQ₁₀ deficiency (Montero *et al.*, 2005; Miles *et al.*, 2008; Trevisson *et al.*, 2011). This also emphasizes the usefulness of the central RI as a diagnostic tool, because other reports also confirm that patients with combined CII+III RCDs in muscle often have CoQ₁₀ deficiency (Ogasahara *et al.*, 1989; Sobreira *et al.*, 1997; Boitier *et al.*, 1998; Musumeci *et al.*, 2001; Rahman *et al.*, 2001; Lalani *et al.*, 2005; Montero *et al.*, 2005; Salviati *et al.*, 2005; Artuch *et al.*, 2006; López *et al.*, 2006; Gempel *et al.*, 2007; Rötig *et al.*, 2007; Miles *et al.*, 2008; Montero *et al.*, 2009; Montero *et al.*, 2013; Bujàn *et al.*, 2014).

The significant differences in the CoQ₁₀ concentrations between the three sample groups indicate that the CoQ₁₀ concentrations were most likely an important factor in the ETC deficiencies of the sample groups. This is supported by the results reported in other studies stating that total CoQ₁₀ concentrations correlated with deficit (CII+III) ETC activity (Montero *et al.*, 2005; Miles *et al.*, 2008; Duberley *et al.*, 2013b), and that the quantification of total CoQ₁₀ concentration is the best indicator of a possible ETC deficiency (Miles *et al.*, 2008), especially total CoQ₁₀/CS concentration (Itkonen *et al.*, 2013). Total CoQ₁₀ measurement was also found to be the most reliable predictor of an ETC dysfunction as opposed to the redox status of the oxidized and reduced form of CoQ₁₀ or their individual values (Miles *et al.*, 2008), and the determination of ETC enzyme activities prior to CoQ₁₀ determination (Montero *et al.*, 2005). Statistical analyses of the sample groups enabled the accomplishment of the second objective, namely to investigate the levels of CoQ₁₀ in ETC deficiencies by quantifying total CoQ₁₀ concentrations in South African patient muscle samples with a standardized LC-MS/MS method.

5.4 Concluding remarks

In conclusion, the standardized analytical approach with LC-MS/MS was utilized to accomplish the aim of this study, namely to quantify total CoQ₁₀ levels in a cohort of South African pediatric patients with ETC deficiencies. The quantification of total CoQ₁₀ enabled the statistical investigation of the of skeletal muscle CoQ₁₀ levels in the South African patients so as to address the question of whether the patients with RCDs had lower levels of CoQ₁₀, particularly the patients with combined CII+III deficiency. Patients with CoQ₁₀ deficiency classically present with the CII+III ETC deficiencies (Sacconi *et al.*, 2010; Bujàn *et al.*, 2014). The CII+III deficiencies in patients of this study may have resulted from a decrease in CoQ₁₀, owing to the pathogenic mechanisms associated with CoQ₁₀ deficiency (Quinzii *et al.*, 2008b; Duberley *et al.*, 2013b). Accordingly, the CoQ₁₀ concentration differences between the sample groups may have had an effect on the respiratory chain enzyme activities within these groups (Quinzii *et al.*, 2008b, 2010; Duberley *et al.*, 2013b). Establishing a central 95% RI enabled the identification of eight patients with decreased CoQ₁₀/CS ratios, and because direct quantification of CoQ₁₀ in muscle is a very reliable diagnostic test for CoQ₁₀ deficiency (Rahman *et al.*, 2012), these patients are very likely to benefit from CoQ₁₀ replacement therapy (Quinzii & Hirano, 2010). Patients with mitochondrial disorders with normal levels of CoQ₁₀ benefit from supplementation which suggests the involvement of CoQ₁₀ outside of its function in the ETC (Desbats *et al.*, 2014). Therefore, the LC-MS/MS and the newly established RI for CoQ₁₀ in 600 x g skeletal muscle supernatants contributed to this significant application of detecting a CoQ₁₀ deficiency (Itkonen *et al.*, 2013), and can be used for the early detection of a CoQ₁₀ deficiency with successive replacement therapy, possibly reversing the irreparable tissue damage and/or poor clinical manifestation in patients with early onset muscle CoQ₁₀ deficiency (Montero *et al.*, 2008). Because patients respond differently to supplementation, it is however uncertain whether supplementation will ensure amelioration of their symptoms.

To summarize, the aim and set objectives for this study had been accomplished.

5.5 Future prospects

Concerning the method for CoQ₁₀ analysis, the use of an ultra high pressure liquid chromatography (UPLC) separation technique (Palaikov *et al.*, 2009) would serve as a more economical option. Determination of the limit of detection and limit of quantification of the standardized LC-MS/MS method would aid in reliable diagnosis of patients with severe (< 20% of normal CoQ₁₀ concentration) CoQ₁₀ deficiency (Quinzii *et al.*, 2008b; Itkonen *et al.*, 2013).

Though measurement of CoQ₁₀ in muscle biopsy is the most reliable diagnostic test for a CoQ₁₀ deficiency, it is still an invasive procedure, especially for infants. Miles *et al.* (2008) suggested a needle muscle biopsy for a very small specimen (20 – 40 mg). Other less invasive options ought to be explored such as the use of blood mononuclear cells in plasma that reflect alterations in the cellular CoQ₁₀ concentrations, also after oral supplementation (Duncan *et al.*, 2005; Rahman *et al.*, 2012). Biosynthetic assays in fibroblasts are a non-invasive option to confirm a primary CoQ₁₀ deficiency (Bujàn *et al.*, 2014). This would support timely administration of CoQ₁₀ supplementation prior to the disease threshold being reached, and to possibly reverse the induced tissue damage of the severe, infantile onset primary CoQ₁₀ deficiency (Trevisson *et al.*, 2011).

REFERENCE LIST

- Acín-Pérez, R., Fernández-Silva, P., Peleato, M.L., Pérez-Martos, A. & Enriquez, J.A. 2008. Respiratory active mitochondrial supercomplexes. *Molecular cell*, 32(4):529-539.
- Adams, P. & Turnbull, D. 1996. Disorders of the electron transport chain. *Journal of inherited metabolic disease*, 19(4):463-469.
- Aeby, A., Sznajder, Y., Cavé, H., Rebuffat, E., Van Coster, R., Rigal, O. & Van Bogaert, P. 2007. Cardiofaciocutaneous (CFC) syndrome associated with muscular coenzyme Q₁₀ deficiency. *Journal of inherited metabolic disease*, 30(5):827-827.
- Agilent Technologies. 2008. Agilent 6460 series triple quad LC/MS system concepts: quick start guide. 6th ed. Santa Clara, Calif.: Agilent Technologies.
- Agilent Technologies. 2010. MassHunter optimizer software for Automated MRM Method Development using the agilent 6400 series Triple Quadrupole mass spectrometers: technical overview. Santa Clara, Calif.: Agilent technologies. (5990-5011EN.).
- Anderson, S., Bankier, A.T. & Barrell, B.G. 1981. Sequence and organization of the human mitochondrial genome. *Nature*, 290:457-465. (Abstract.)
- Andersson, S.G.E., Karlberg, O., Canbäck, B. & Kurland, C.G. 2002. On the origin of mitochondria: a genomics perspective. *Philosophical Transactions of the Royal Society of London, Series B: biological sciences*, 358(1429):165-179.
- Artuch, R., Brea-Calvo, G., Briones, P., Aracil, A., Galván, M., Espinós, C., Corral, J., Volpini, V., Ribes, A. & Andreu, A.L. 2006. Cerebellar ataxia with coenzyme Q₁₀ deficiency: diagnosis and follow-up after coenzyme Q₁₀ supplementation. *Journal of the neurological sciences*, 246(1):153-158.
- Ashraf, S., Gee, H.Y., Woerner, S., Xie, L.X., Vega-Warner, V., Lovric, S., Fang, H., Song, X., Cattran, D.C. & Avila-Casado, C. 2013. ADCK4 mutations promote steroid-resistant nephrotic syndrome through CoQ₁₀ biosynthesis disruption. *Journal of clinical investigation*, 123(12):5179-5189.

Barshop, B.A. & Gangoiti, J.A. 2007. Analysis of coenzyme Q in human blood and tissues. *Mitochondrion*, 7:S89-S93.

Benard, G., Faustin, B., Galinier, A., Rocher, C., Bellance, N., Smolkova, K., Casteilla, L., Rossignol, R. & Letellier, T. 2008. Functional dynamic compartmentalization of respiratory chain intermediate substrates: implications for the control of energy production and mitochondrial diseases. *International journal of biochemistry & cell biology*, 40(8):1543-1554.

Benoist, J., Lombès, A., Rigal, O., Nivoche, Y., Martin, C. & Biou, D. 2003. Differences in coenzyme Q₁₀ content in deltoid and quadriceps muscles. *Clinica chimica acta*, 329(1):147-148.

Bentinger, M., Dallner, G., Chojnacki, T. & Swiezewska, E. 2003. Distribution and breakdown of labeled coenzyme Q₁₀ in rat. *Free radical biology and medicine*, 34(5):563-575.

Bentinger, M., Tekle, M. & Dallner, G. 2010. Coenzyme Q–biosynthesis and functions. *Biochemical and biophysical research communications*, 396(1):74-79.

Blier, P.U., Dufresne, F. & Burton, R.S. 2001. Natural selection and the evolution of mtDNA-encoded peptides: evidence for intergenomic co-adaptation. *Trends in genetics*, 17(7):400-406.

Boitier, E., Degoul, F., Desguerre, I., Charpentier, C., François, D., Ponsot, G., Diry, M., Rustin, P. & Marsac, C. 1998. A case of mitochondrial encephalomyopathy associated with a muscle coenzyme Q₁₀ deficiency. *Journal of the neurological sciences*, 156(1):41-46.

Boreková, M., Hojerová, J., Koprda, V. & Bauerová, K. 2008. Nourishing and health benefits of coenzyme Q₁₀: a review. *Czech journal of food sciences*, 26(4):229-241.

Braggio, S., Barnaby, R., Grossi, P. & Cugola, M. 1996. A strategy for validation of bioanalytical methods. *Journal of pharmaceutical and biomedical analysis*, 14(4):375-388.

Bressolle, F., Bromet-Petit, M. & Audran, M. 1996. Validation of liquid chromatographic and gas chromatographic methods applications to pharmacokinetics. *Journal of chromatography B: Biomedical sciences and applications*, 686(1):3-10.

Buján, N., Arias, A., Montero, R., García-Villoria, J., Lissens, W., Seneca, S., Espinós, C., Navas, P., De Meirleir, L. & Artuch, R. 2014. Characterization of CoQ₁₀ biosynthesis in

fibroblasts of patients with primary and secondary CoQ₁₀ deficiency. *Journal of inherited metabolic disease*, 37(1):53-62.

Cannino, G., Di Liegro, C.M. & Rinaldi, A.M. 2007. Nuclear-mitochondrial interaction. *Mitochondrion*, 7(6):359-366.

Crane, F.L. 1957. Electron transport and cytochromes of sub-cellular particles from cauliflower buds. *Plant physiology*, 32(6):619-625.

Criddle, R.S., Bock, R.M., Green, D.E. & Tisdale, H. 1962. Physical characteristics of proteins of the electron transfer system and interpretation of the structure of the mitochondrion. *Biochemistry*, 1(5):827-842.

Dallner, G. & Sindelar, P.J. 2000. Regulation of ubiquinone metabolism. *Free radical biology & medicine*, 29(3/4):285-294.

D'Aurelio, M., Gajewski, C.D., Lenaz, G. & Manfredi, G. 2006. Respiratory chain supercomplexes set the threshold for respiration defects in human mtDNA mutant cybrids. *Human molecular genetics*, 15(13):2157-2169.

Dehahn, T., Barr, R. & Morré, D.J. 1997. NADH oxidase activity present on both the external and internal surfaces of soybean plasma membranes. *Biochimica et biophysica Acta (BBA)-Biomembranes*, 1328(2):99-108.

Deichmann, R., Lavie, C. & Andrews, S. 2010. Coenzyme Q₁₀ and statin-induced mitochondrial dysfunction. *Ochsner journal*, 10:16-21.

Desbats, M.A., Lunardi, G., Doimo, M., Trevisson, E. & Salviati, L. 2014. Genetic bases and clinical manifestations of coenzyme Q₁₀ (CoQ₁₀) deficiency. *Journal of inherited metabolic disease*: 1-12. (Presented at the workshop "Diagnostic Approach, and Classification of IEM Affecting the Synthesis and Catabolism of Complex Lipids" in Paris, France, June 14-15, 2013.)

Di Mauro, S. 2010. Pathogenesis and treatment of mitochondrial myopathies: recent advances. *Acta Myologica*, 29(2):333-338.

Di Mauro, S., Bonilla, E., Mancuso, M., Filosto, M., Sacconi, S., Salviati, L. & Hirano, M. 2003. Mitochondrial myopathies. *Basic and applied myology*, 13(3):145-155.

Di Mauro, S., Quinzii, C.M. & Hirano, M. 2007. Mutations in coenzyme Q10 biosynthetic genes. *Journal of clinical investigation*, 117(3):587-589.

Di Mauro, S. & Rustin, P. 2009. A critical approach to the therapy of mitochondrial respiratory chain and oxidative phosphorylation diseases. *Biochimica et Biophysica Acta (BBA)-molecular basis of disease*, 1792(12):1159-1167.

Di Mauro, S. & Schon, E.A. 2003. Mitochondrial respiratory-chain diseases. *New England journal of medicine*, 348(26):2656-2668.

Dolan, J.W. 2003. Why do peaks tail. *LCGC North America*, 21(7):612-616.

Duberley, K.E., Hargreaves, I.P., Chaiwatanasirikul, K., Heales, S.J., Land, J.M., Rahman, S., Mills, K. & Eaton, S. 2013. Coenzyme Q₁₀ quantification in muscle, fibroblasts and cerebrospinal fluid by liquid chromatography/tandem mass spectrometry using a novel deuterated internal standard. *Rapid communications in mass spectrometry*, 27(9):924-930.

Duberley, K.E., Abramov, A.Y., Chalasani, A., Heales, S.J., Rahman, S. & Hargreaves, I.P. 2013. Human neuronal coenzyme Q₁₀ deficiency results in global loss of mitochondrial respiratory chain activity, increased mitochondrial oxidative stress and reversal of ATP synthase activity: omplications for pathogenesis and treatment. *Journal of inherited metabolic disease*, 36(1):63-73.

Dunbar, D.R., Moonie, P.A., Zeviani, M. & Holt, I.J. 1996. Complex I deficiency is associated with 3243G: C mitochondrial DNA in osteosarcoma cell cybrids. *Human molecular genetics*, 5(1):123-129.

Duncan, A.J., Bitner-Glindzicz, M., Meunier, B., Costello, H., Hargreaves, I.P., López, L.C., Hirano, M., Quinzii, C.M., Sadowski, M.I. & Hardy, J. 2009. A nonsense mutation in COQ9 causes autosomal-recessive neonatal-onset primary coenzyme Q₁₀ deficiency: a potentially treatable form of mitochondrial disease. *American journal of human genetics*, 84(5):558-566.

Duncan, A.J., Heales, S.J.R., Mills, K., Eaton, S., Land, J.M. & Hargreaves, I.P. 2005. Determination of coenzyme Q₁₀ status in blood mononuclear cells, skeletal muscle, and plasma by HPLC with di-propoxy-coenzyme Q₁₀ as an internal standard. *Clinical chemistry*, 51(12):2380-2382.

Echtay, K.S., Winkler, E., Frischmuth, K. & Klingenberg, M. 2001. Uncoupling proteins 2 and 3 are highly active H transporters and highly nucleotide sensitive when activated by coenzyme Q (ubiquinone). *Proceedings of the National Academy of Sciences*, 98(4):1416-1421.

Edlund, P.O. 1988. Determination of coenzyme Q₁₀, α -tocopherol and cholesterol in biological samples by coupled-column liquid chromatography with coulometric and ultraviolet detection. *Journal of chromatography B: biomedical sciences and applications*, 425:87-97.

El-Hattab, A.W. & Scaglia, F. 2013. Mitochondrial DNA depletion syndromes: review and updates of genetic basis, manifestations, and therapeutic options. *Neurotherapeutics*, 10(2):186-198.

El-Najjar, N., Gali-Muhtasib, H., Ketola, R.A., Vuorela, P., Urtti, A. & Vuorela, H. 2011. The chemical and biological activities of quinones: overview and implications in analytical detection. *Phytochemistry reviews*, 10:353-370.

Emmanuele, V., López, L.C., Berardo, A., Naini, A., Tadesse, S., Wen, B., D'Agostino, E., Solomon, M., Dimauro, S. & Quinzii, C. 2012. Heterogeneity of coenzyme Q10 deficiency: patient study and literature review. *Archives of neurology*, 69(8):978-983.

Ernster, L. & Dallner, G. 1995. Biochemical, physiological and medical aspects of ubiquinone function. *Biochimica et biophysica acta*, 1271:195-204.

Ernster, L., Lee, I., Norling, B. & Persson, B. 1969. Studies with Ubiquinone-Depleted submitochondrial particles. *European journal of biochemistry*, 9(3):299-310.

Fernández-Vizarra, E., Tiranti, V. & Zeviani, M. 2009. Assembly of the oxidative phosphorylation system in humans: what we have learned by studying its defects. *Biochimica et biophysica acta*, 1793(1):200-211.

Festenstien, G.N., Heaton, F.W., Lowe, J.S. & Morton, A.R. 1955. A constituent of the unsaponifiable portion of animal tissue lipids (λ_{max} 272 m μ). *Biochemical journal*, 59(4):558-566.

Flint Beal, M. 2002. Coenzyme Q₁₀ as a possible treatment for neurodegenerative diseases. *Free radical research*, 36(4):455-460.

Fontaine, E., Ichas, F. & Bernardi, P. 1998. A ubiquinone-binding site regulates the mitochondrial permeability transition pore. *Journal of biological chemistry*, 273(40):25734-25740.

Gempel, K., Topaloglu, H., Talim, B., Schneiderat, P., Schoser, B.G., Hans, V.H., Palmaf, B., Kale, G., Tokatli, A., Quinzii, C., Hirano, M., Naini, A., Dimauro, S., Prokisch, H., Lochmuller, H. & Horvath, R. 2007. The myopathic form of coenzyme Q10 deficiency is caused by mutations in the electron-transferring-flavoprotein dehydrogenase (ETF DH) gene. *Brain: a journal of neurology*, 130(8):2037-2044.

Geromel, V., Darin, N., Chrétien, D., Bénit, P., Delonlay, P., Rötig, A., Munnich, A. & Rustin, P. 2002. Coenzyme Q₁₀ and idebenone in the therapy of respiratory chain diseases: rationale and comparative benefits. *Molecular genetics and metabolism*, 77(1-2):21-30.

Gómez-Díaz, C., Rodríguez-Aguilera, J.C., Barroso, M.P., Villalba, J.M., Navarro, F., Crane, F.L. & Navas, P. 1997. Antioxidant ascorbate is stabilized by NADH-coenzyme Q10 reductase in the plasma membrane. *Journal of bioenergetics and biomembranes*, 29(3):251-257.

Greaves, L.C., Reeve, A.K., Taylor, R.W. & Turnbull, D.M. 2012. Mitochondrial DNA and disease. *Journal of pathology*, 226(2):274-286.

Gritti, F. & Guiochon, G. 2011. Kinetic investigation of the relationship between the efficiency of columns and their diameter. *Journal of chromatography A*, 1218(12):1592-1602.

Hägerhäll, C. 1997. Succinate: quinone oxidoreductases: variations on a conserved theme. *Biochimica et biophysica acta*, 1320(2):107-141.

Hamamura, K., Yamagishi, Y., Kijima, S., Suzuki, T., Mori, Y. & Nakamura, T. 2002. Synthesis of deuterium-labelled coenzyme Q₁₀. *Journal of labelled compounds and radiopharmaceuticals*, 45(10):831-839.

Hamamura, K., Yamatsu, I., Minami, N., Yamagishi, Y., Inai, Y., Kijima, S. & Nakamura, T. 2002. Synthesis of [3'-¹⁴C] coenzyme Q₁₀. *Journal of labelled compounds and radiopharmaceuticals*, 45(10):823-829.

Hansen, G., Christensen, P., Tüchsen, E. & Lund, T. 2004. Sensitive and selective analysis of coenzyme Q10 in human serum by negative APCI LC-MS. *Analyst*, 129(1):45-50.

Hargreaves, I. 2014. Coenzyme Q₁₀ as a therapy for mitochondrial disease. *International journal of biochemistry & cell biology*, 49:105-111.

Hatefi, Y., Haavik, A.G., Fowler, L.R. & Griffiths, D.E. 1962. Studies on the electron transfer system: XLII. Reconstitution of the electron transfer system. *Journal of biological chemistry*, 237:2661-2669.

Heron, C., Ragan, C. & Trumpower, B. 1978. The interaction between mitochondrial NADH-ubiquinone oxidoreductase and ubiquinol-cytochrome c oxidoreductase: restoration of ubiquinone-pool behaviour. *Biochemical journal*, 174:791-800.

Hirano, M., Garone, C. & Quinzii, C.M. 2012. CoQ₁₀ deficiencies and MNGIE: two treatable mitochondrial disorders. *Biochimica et biophysica acta - general subjects*, 1820(5):625-631.

Ho, C.S., Lam, C.W., Chan, M.H., Cheung, R.C., Law, L.K., Lit, L.C., Ng, K.F., Suen, M.W. & Tal, H.L. 2003. Electrospray ionisation mass spectrometry: principles and clinical applications. *Clinical biochemist*, 24(1):3-12.

Itkonen, O., Suomalainen, A. & Turpeinen, U. 2013. Mitochondrial coenzyme Q10 determination by isotope-dilution liquid chromatography-tandem mass spectrometry. *Clinical chemistry*, 59(8):1260-1267.

Ježek, P. & Hlavatá, L. 2005. Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism. *International journal of biochemistry & cell biology*, 37:2478-2503.

Kaikkonen, J., Nyssönen, K. & Salonen, J. 1999. Measurement and stability of plasma reduced, oxidized and total coenzyme Q10 in humans. *Scandinavian journal of clinical & laboratory investigation*, 59(6):457-466.

Kaikkonen, J., Tuomainen, T., Nyssönen, K. & Salonen, J.T. 2002. Coenzyme Q10: absorption, antioxidative properties, determinants, and plasma levels. *Free radical research*, 36(4):389-397.

Kamzalov, S., Sumien, N., Forster, M.J. & Sohal, R.S. 2003. Coenzyme Q intake elevates the mitochondrial and tissue levels of coenzyme Q and alpha-tocopherol in young mice. *Journal of nutrition*, 133(10):3175-3180.

Keilin, D. & Hartree, E.F. 1939. Cytochrome and cytochrome oxidase. *Proceedings of the Royal Society of London*, 847(127):167-191.

Kishikawa, N. & Kuroda, N. 2014. Analytical techniques for the determination of biologically active quinones in biological and environmental samples. *Journal of pharmaceutical and biomedical analysis*, 87:261-270.

Koene, S., Smeitink, J. & Hirano, M. 2011. Mitochondrial medicine: a clinical guideline. Nijmegen: Khondrion.

Koopman, W.J., Willems, P.H. & Smeitink, J.A. 2012. Monogenic mitochondrial disorders. *New England journal of medicine*, 366(12):1132-1141.

Korzeniewski, B. & Zoladz, J.A. 2001. A model of oxidative phosphorylation in mammalian skeletal muscle. *Biophysical chemistry*, 92(1):17-34.

Kröger, A., Klingenberg, M. & Schweidler, S. 1973. Further evidence for the pool function of ubiquinone as derived from the inhibition of the electron transport by antimycin. *European journal of biochemistry*, 39(2):313-323.

Lalani, S.R., Vladutiu, G.D., Plunkett, K., Lotze, T.E., Adesina, A.M. & Scaglia, F. 2005. Isolated mitochondrial myopathy associated with muscle coenzyme Q₁₀ deficiency. *Archives of neurology*, 62(2):317-320.

Lancaster, C.R.D. 2002. Succinate: quinone oxidoreductases: an overview. *Biochimica et biophysica acta*, 1553(1):1-6.

Lang, J.K., Gohil, K. & Packer, L. 1986. Simultaneous determination of tocopherols, ubiquinol, and ubiquinones in blood, plasma, tissue homogenates, and subcellular fractions. *Analytical biochemistry*, 157(1):106-116.

Lass, A. & Sohal, R.S. 1999. Comparisons of coenzyme Q bound to mitochondrial membrane proteins among different mammalian species. *Free radical biology and medicine*, 27(1):220-226.

Le Cam, L. 1986. The central limit theorem around 1935. *Statistical science*, 1(1):78-91.

- Lekli, I., Das, S., Das, S., Mukherjee, S., Bak, I., Juhasz, B., Bagchi, D., Trimurtulu, G., Krishnaraju, A. & Sengupta, K. 2008. Coenzyme Q9 provides cardioprotection after converting into coenzyme Q10. *Journal of agricultural and food chemistry*, 56(13):5331-5337.
- Lenaz, G., Fato, R., Formiggini, G. & Genova, M.L. 2007. The role of coenzyme Q in mitochondrial electron transport. *Mitochondrion*, 7:S8-S33.
- Lenaz, G. & Genova, M.L. 2009. Mobility and function of coenzyme Q (ubiquinone) in the mitochondrial respiratory chain. *Biochimica et biophysica acta-bioenergetics*, 1787(6):563-573.
- Leonard, J. & Schapira, A.H. 2000. Mitochondrial respiratory chain disorders I: mitochondrial DNA defects. *The Lancet*, 355(9200):299-304.
- Letellier, T., Heinrich, R., Malgat, M. & Mazat, J. 1994. The kinetic basis of threshold effects observed in mitochondrial diseases: a systemic approach. *Biochemistry journal*, 302:171-174.
- Li, K., Shi, Y., Chen, S., Li, W., Shang, X. & Huang, Y. 2006. Determination of coenzyme Q10 in human seminal plasma by high-performance liquid chromatography and its clinical application. *Biomedical chromatography*, 20(10):1082-1086.
- Li, L., Pabbisetty, D., Zhu, S., Avery, M.A. & Avery, B.A. 2008. Urinary excretion study of coenzyme Q10 in rats by ultra-performance liquid chromatography-mass spectrometry. *Journal of chromatographic science*, 46(3):215-219.
- Link, T.A. 1997. The role of the 'Rieske' iron sulfur protein in the hydroquinone oxidation (Q_p) site of the cytochrome bc_1 complex: the 'proton-gated affinity change' mechanism. *FEBS letters*, 412(2):257-264.
- Linnane, A.W., Kios, M. & Vitetta, L. 2007. Coenzyme Q₁₀: its role as a prooxidant in the formation of superoxide anion/hydrogen peroxide and the regulation of the metabolome. *Mitochondrion*, 7:S51-S61.
- Linnane, A.W., Kopsidas, G., Zhang, C., Yarovaya, N., Kovalenko, S., Papakostopoulos, P., Eastwood, H., Graves, S. & Richardson, M. 2002. Cellular redox activity of coenzyme Q₁₀: effect of CoQ₁₀ supplementation on human skeletal muscle. *Free radical research*, 36(4):445-453.

Loeffen, J., Smeitink, J., Trijbels, J., Janssen, A., Triepels, R., Sengers, R. & Van Den Heuvel, L. 2000. Isolated complex I deficiency in children: clinical, biochemical and genetic aspects. *Human mutation*, 15(2):123-134.

Loeffen, J., Triepels, R., Van den Heuvel, L., Schuelke, M., Buskens, C., Smeets, R., Trijbels, J. & Smeitink, J. 1998. cDNA of eight nuclear encoded subunits of NADH: Ubiquinone oxidoreductase: human complex I cDNA characterization completed. *Biochemical and biophysical research communications*, 253(2):415-422.

López, L.C., Schuelke, M., Quinzii, C.M., Kanki, T., Rodenburg, R.J., Naini, A., Dimauro, S. & Hirano, M. 2006. Leigh syndrome with nephropathy and CoQ₁₀ deficiency due to *decaprenyl* diphosphate synthase subunit 2 (PDSS2) mutations. *American journal of human genetics*, 79(6):1125-1129.

López-Martín, J.M., Salviati, L., Trevisson, E., Montini, G., Dimauro, S., Quinzii, C., Hirano, M., Rodriguez-Hernandez, A., Cordero, M.D., Sánchez-Alcázar, J.A., Santos-Ocaña, C. & Navas, P. 2007. Missense mutation of the COQ2 gene causes defects of bioenergetics and *de novo* pyrimidine synthesis. *Human molecular genetics*, 16(9):1091-1097.

Macinga, D.R., Cook, G.M., Poole, R.K. & Rather, P.N. 1998. Identification and characterization of *aarF*, a locus required for production of ubiquinone in *Providencia stuartii* and *Escherichia coli* and for expression of 2'-N-acetyltransferase in *P. stuartii*. *Journal of bacteriology*, 180(1):128-135.

Manfredi, G. & Beal, M.F. 2000. The role of mitochondria in the pathogenesis of neurodegenerative diseases. *Brain pathology*, 10(3):462-472.

Manfredi, G., Schon, E., Moraes, C., Bonilla, E., Berry, G., Sladky, J. & Dimauro, S. 1995. A new mutation associated with MELAS is located in a mitochondrial DNA polypeptide-coding gene. *Neuromuscular disorders*, 5(5):391-398.

McCarty, M. 1999. Coenzyme Q versus hypertension: does CoQ decrease endothelial superoxide generation? *Medical hypotheses*, 53(4):300-304.

McMillan, H.J., Schwartzenruber, J., Smith, A., Lee, S., Chakraborty, P., Bulman, D.E., Beaulieu, C.L., Majewski, J., Boycott, K.M. & Geraghty, M.T. 2014. Compound heterozygous mutations in glycyl-tRNA synthetase are a proposed cause of systemic mitochondrial disease. *BMC medical genetics*, 15(1):36-41.

Menke, T., Niklowitz, P., Adam, S., Weber, M., Schlüter, B. & Andler, W. 2000. Simultaneous detection of ubiquinol-10, ubiquinone-10, and tocopherols in human plasma microsamples and macrosamples as a marker of oxidative damage in neonates and infants. *Analytical biochemistry*, 282(2):209-217.

Milaeva, E.R., Filimonova, S.I., Meleshonkova, N.N., Dubova, L.G., Shevtsova, E.F., Bachurin, S.O. & Zefirov, N.S. 2010. Antioxidative activity of ferrocenes bearing 2,6-di-tert-butylphenol moieties. *Bioinorganic chemistry and applications*, 2010(165482):1-6.

Miles, M.V., Horn, P.S., Morrison, J.A., Tang, P.H., Degrauw, T. & Pesce, A.J. 2003. Plasma coenzyme Q₁₀ reference intervals, but not redox status, are affected by gender and race in self-reported healthy adults. *Clinica chimica acta*, 332(1):123-132.

Miles, M.V., Miles, L., Tang, P.H., Horn, P.S., Steele, P.E., Degrauw, A.J., Wong, B.L. & Bove, K.E. 2008. Systematic evaluation of muscle coenzyme Q10 content in children with mitochondrial respiratory chain enzyme deficiencies. *Mitochondrion*, 8(2):170-180.

Mitchell, P. 1975a. Protonmotive redox mechanism of the cytochrome bc_1 complex in the respiratory chain: protonmotive ubiquinone cycle. *FEBS letters*, 56(1):1-6.

Mitchell, P. 1975b. The protonmotive Q cycle: a general formulation. *FEBS letters*, 59(2):137-139.

Mitchell, P. 1979. Keilin's respiratory chain concept and its chemiosmotic consequences. *Science*, 206(4423):1488-1159.

Mollet, J., Delahodde, A., Serre, V., Chretien, D., Schlemmer, D., Lombes, A., Boddaert, N., Desguerre, I., De Lonlay, P. & Ogier De Baulny, H. 2008. CABC1 gene mutations cause ubiquinone deficiency with cerebellar ataxia and seizures. *American journal of human genetics*, 82(3):623-630.

Mollet, J., Giurgea, I., Schlemmer, D., Dallner, G., Chretien, D., Delahodde, A., Bacq, D., De Lonlay, P., Munnich, A. & Rotig, A. 2007. Prenyldiphosphate synthase, subunit 1 (PDSS1) and OH-benzoate polyprenyltransferase (COQ2) mutations in ubiquinone deficiency and oxidative phosphorylation disorders. *Journal of clinical investigation*, 117(3):765-772.

Molyneux, S.L., Young, J.M., Florkowski, C.M., Lever, M. & George, P.M. 2008. Coenzyme Q₁₀: is there a clinical role and a case for measurement? *Clinical biochemist*, 29(2):71-82.

Montero, R., Artuch, R., Briones, P., Nascimento, A., García-Cazorla, A., Vilaseca, M., Sánchez-Alcázar, J., Navas, P., Montoya, J. & Pineda, M. 2005. Muscle coenzyme Q10 concentrations in patients with probable and definite diagnosis of respiratory chain disorders. *Biofactors*, 25(1-4):109-115.

Montero, R., Pineda, M., Aracil, A., Vilaseca, M., Briones, P., Sánchez-Alcázar, J., Navas, P. & Artuch, R. 2007. Clinical, biochemical and molecular aspects of cerebellar ataxia and coenzyme Q10 deficiency. *Cerebellum*, 6(2):118-122.

Montero, R., Sánchez-Alcázar, J.A., Briones, P., Hernández, Á.R., Cordero, M.D., Trevisson, E., Salvati, L., Pineda, M., García-Cazorla, A. & Navas, P. 2008. Analysis of coenzyme Q₁₀ in muscle and fibroblasts for the diagnosis of CoQ₁₀ deficiency syndromes. *Clinical biochemistry*, 41(9):697-700.

Montero, R., Sánchez-Alcázar, J.A., Briones, P., Navarro-Sastre, A., Gallardo, E., Bornstein, B., Herrero-Martín, D., Rivera, H., Martín, M.A. & Martí, R. 2009. Coenzyme Q₁₀ deficiency associated with a mitochondrial DNA depletion syndrome: a case report. *Clinical biochemistry*, 42(7):742-745.

Moraes, C.T., Ricci, E., Bonilla, E., Dimauro, S. & Schon, E.A. 1992. The mitochondrial tRNA^{Leu(UUR)} mutation in mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes (MELAS): genetic, biochemical, and morphological correlations in skeletal muscle. *American journal of human genetics*, 50(5):934-949.

Mordehai, A. & Fjeldsted, J. 2009. Agilent jet stream thermal gradient focusing technology. Agilent technologies. [Technical note, publication number (5990-3494EN).]

Morré, D.J. & Morré, D.M. 2011. Non-mitochondrial coenzyme Q. *Biofactors*, 37(5):355-360.

Musumeci, O., Naini, A., Slonim, A.E., Skavin, N., Hadjigeorgiou, G.L., Krawiecki, N., Weissman, B.M., Tsao, C.Y., Mendell, J.R., Shanske, S., De Vivo, D.C., Hirano, M. & Dimauro, S. 2001. Familial cerebellar ataxia with muscle coenzyme Q10 deficiency. *Neurology*, 56(7):849-855.

Ogasahara, S., Engel, A.G., Frens, D. & Mack, D. 1989. Muscle coenzyme Q deficiency in familial mitochondrial encephalomyopathy. *Proceedings of the National Academy of Sciences of the United States of America*, 86(7):2379-2382.

Ordovas, J.M. 2007. Gender, a significant factor in the cross talk between genes, environment, and health. *Gender medicine*, 4:S111-S122.

Paliakov, E.M., Crow, B.S., Bishop, M.J., Norton, D., George, J. & Bralley, J.A. 2009. Rapid quantitative determination of fat-soluble vitamins and coenzyme Q-10 in human serum by reversed phase ultra-high pressure liquid chromatography with UV detection. *Journal of chromatography B*, 877(1):89-94.

Papucci, L., Schiavone, N., Witort, E., Donnini, M., Lapucci, A., Tempestini, A., Formigli, L., Zecchi-Orlandini, S., Orlandini, G. & Carella, G. 2003. Coenzyme Q₁₀ prevents apoptosis by inhibiting mitochondrial depolarization independently of its free radical scavenging property. *Journal of biological chemistry*, 278(30):28220-28228.

Potgieter, M., Pretorius, E. & Pepper, M.S. 2013. Primary and secondary coenzyme Q10 deficiency: the role of therapeutic supplementation. *Nutrition reviews*, 71(3):180-188.

Quinzii, C.M. & Hirano, M. 2010. Coenzyme Q and mitochondrial disease. *Developmental disabilities research reviews*, 16(2):183-188.

Quinzii, C.M. & Hirano, M. 2011. Primary and secondary CoQ10 deficiencies in humans. *Biofactors*, 37(5):361-365.

Quinzii, C.M., Kattah, A.G., Naini, A., Akman, H.O., Mootha, V.K., Dimauro, S. & Hirano, M. 2005. Coenzyme Q deficiency and cerebellar ataxia associated with an aprataxin mutation. *Neurology*, 64(3):539-541.

Quinzii, C.M., López, L., Naini, A., Dimauro, S. & Hirano, M. 2008. Human CoQ10 deficiencies. *Biofactors*, 32(1):113-118.

Quinzii, C.M., Lopez, L.C., Gilkerson, R.W., Dorado, B., Coku, J., Naini, A.B., Lagier-Tourenne, C., Schuelke, M., Salviati, L., Carrozzo, R., Santorelli, F., Rahman, S., Tazir, M., Koenig, M., Dimauro, S. & Hirano, M. 2010. Reactive oxygen species, oxidative stress, and cell death correlate with level of CoQ10 deficiency. *FASEB journal*, 24(10):3733-3743.

Quinzii, C.M., Lopez, L.C., Von-Moltke, J., Naini, A., Krishna, S., Schuelke, M., Salviati, L., Navas, P., Dimauro, S. & Hirano, M. 2008. Respiratory chain dysfunction and oxidative stress correlate with severity of primary CoQ₁₀ deficiency. *FASEB journal*, 22(6):1874-1885.

Quinzii, C.M., Naini, A., Salviati, L., Trevisson, E., Navas, P., Dimauro, S. & Hirano, M. 2006. A mutation in *Para-hydroxybenzoate-polyprenyltransferase (COQ2)* causes primary coenzyme Q₁₀ deficiency. *American journal of human genetics*, 78(2):345-349.

Rahman, S., Clarke, C.F. & Hirano, M. 2012. 176th ENMC international workshop: Diagnosis and treatment of coenzyme Q₁₀ deficiency. *Neuromuscular disorders*, 22(1):76-86.

Rahman, S. & Hanna, M.G. 2009. Diagnosis and therapy in neuromuscular disorders: diagnosis and new treatments in mitochondrial diseases. *Journal of neurology, neurosurgery, and psychiatry*, 80(9):943-953.

Rahman, S., Hargreaves, I., Clayton, P. & Heales, S. 2001. Neonatal presentation of coenzyme Q₁₀ deficiency. *Journal of pediatrics*, 139(3):456-458.

Reinecke, C.J., Koekemoer, G., Van der Westhuizen, F.H., Louw, R., Lindeque, J.Z., Mienie, L.J. & Smuts, I. 2012. Metabolomics of urinary organic acids in respiratory chain deficiencies in children. *Metabolomics*, 8(2):264-283.

Reinecke, F., Smeitink, J.A. & Van der Westhuizen, F.H. 2009. OXPHOS gene expression and control in mitochondrial disorders. *Biochimica et biophysica acta - molecular basis of disease*, 1792(12):1113-1121.

Rhee, S.G. 2006. H₂O₂, a necessary evil for cell signalling. *Science*, 577(312):1882-1883.

Rosenblatt, M. 1956. A central limit theorem and a strong mixing condition. *Proceedings of the National Academy of Sciences of the United States of America*, 42(1):43-47.

Rötig, A., Appelkvist, E., Geromel, V., Chretien, D., Kadhon, N., Edery, P., Lebedeau, M., Dallner, G., Munnich, A. & Ernster, L. 2000. Quinone-responsive multiple respiratory-chain dysfunction due to widespread coenzyme Q₁₀ deficiency. *The Lancet*, 356(9227):391-395.

Rötig, A., Mollet, J., Rio, M. & Munnich, A. 2007. Infantile and pediatric quinone deficiency diseases. *Mitochondrion*, 7:S112-S121.

- Ruiz-Jiménez, J., Priego-Capote, F., Mata-Granados, J., Quesada, J. & Luque De Castro, M. 2007. Determination of the ubiquinol-10 and ubiquinone-10 (coenzyme Q10) in human serum by liquid chromatography tandem mass spectrometry to evaluate the oxidative stress. *Journal of chromatography A*, 1175(2):242-248.
- Sacconi, S., Trevisson, E., Salviati, L., Aymé, S., Rigal, O., Garcia Redondo, A., Mancuso, M., Siciliano, G., Tonin, P. & Angelini, C. 2010. Coenzyme Q₁₀ is frequently reduced in muscle of patients with mitochondrial myopathy. *Neuromuscular disorders*, 20(1):44-48.
- Salviati, L., Sacconi, S., Murer, L., Zacchello, G., Franceschini, L., Laverda, A.M., Basso, G., Quinzii, C., Angelini, C., Hirano, M., Naini, A.B., Navas, P., Dimauro, S. & Montini, G. 2005. Infantile encephalomyopathy and nephropathy with CoQ10 deficiency: a CoQ10-responsive condition. *Neurology*, 65(4):606-608.
- Saraste, M. 1999. Oxidative phosphorylation at the fin de siècle. *Science*, 283(5407):1488-1493.
- Sbisà, E., Tanzariello, F., Reyes, A., Pesole, G. & Saccone, C. 1997. Mammalian mitochondrial D-loop region structural analysis: identification of new conserved sequences and their functional and evolutionary implications. *Gene*, 205(1):125-140.
- Schaefer, A.M., Taylor, R.W. & Turnbull, D.M. 2001. The mitochondrial genome and mitochondrial muscle disorders. *Current opinion in pharmacology*, 1(3):288-293.
- Schägger, H., De Coo, R., Bauer, M.F., Hofmann, S., Godinot, C. & Brandt, U. 2004. Significance of respirasomes for the assembly/stability of human respiratory chain complex I. *Journal of biological chemistry*, 279(35):36349-36353.
- Schägger, H. & Pfeiffer, K. 2000. Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. *The EMBO journal*, 19(8):1777-1783.
- Shah, V.P., Midha, K.K., Findlay, J.W., Hill, H.M., Hulse, J.D., McGilveray, I.J., McKay, G., Miller, K.J., Patnaik, R.N. & Powell, M.L. 2000. Bioanalytical method validation: a revisit with a decade of progress. *Pharmaceutical research*, 17(12):1551-1557.
- Shi, H., Noguchi, N. & Niki, E. 1999. Dynamics of antioxidant action of ubiquinol: a reappraisal. *Biofactors*, 9(2-4):141-148.

- Shoubridge, E.A. 2000. Mitochondrial DNA segregation in the developing embryo. *Human reproduction*, 15(2):229-234.
- Skladal, D., Halliday, J. & Thorburn, D.R. 2003. Minimum birth prevalence of mitochondrial respiratory chain disorders in children. *Brain: a journal of neurology*, 126(8):1905-1912.
- Smuts, I., Louw, R., Du Toit, H., Klopper, B., Mienie, L.J. & Van der Westhuizen, F.H. 2010. An overview of a cohort of South African patients with mitochondrial disorders. *Journal of inherited metabolic disease*, 33(3):95-104.
- Sobreira, C., Hirano, M., Shanske, S., Keller, R., Haller, R., Davidson, E., Santorelli, F., Miranda, A., Bonilla, E. & Mojon, D. 1997. Mitochondrial encephalomyopathy with coenzyme Q10 deficiency. *Neurology*, 48(5):1238-1243.
- Stucki, J.W. 1980. The optimal efficiency and the economic degrees of coupling of oxidative phosphorylation. *European journal of biochemistry*, 109(1):269-283.
- Taanman, J. 1999. The mitochondrial genome: structure, transcription, translation and replication. *Biochimica et biophysica acta (BBA) - bioenergetics*, 1410(2):103-123.
- Tang, P.H., Miles, M.V., Degrauw, A., Hershey, A. & Pesce, A. 2001. HPLC analysis of reduced and oxidized coenzyme Q₁₀ in human plasma. *Clinical chemistry*, 47(2):256-265.
- Tang, P.H., Miles, M.V., Miles, L., Quinlan, J., Wong, B., Wenisch, A. & Bove, K. 2004. Measurement of reduced and oxidized coenzyme Q₉ and coenzyme Q₁₀ levels in mouse tissues by HPLC with coulometric detection. *Clinica chimica acta*, 341(1):173-184.
- Taylor, R.W. & Turnbull, D.M. 2005. Mitochondrial DNA mutations in human disease. *Nature reviews genetics*, 6(5):389-402.
- Teshima, K. & Kondo, T. 2005. Analytical method for ubiquinone-9 and ubiquinone-10 in rat tissues by liquid chromatography/turbo ion spray tandem mass spectrometry with 1-alkylamine as an additive to the mobile phase. *Analytical biochemistry*, 338:12-19.
- Thelin, A., Schedin, S. & Dallner, G. 1992. Half-life of ubiquinone-9 in rat tissues. *FEBS letters*, 313(2):118-120.

- Thorburn, D. 2004. Mitochondrial diseases: not so rare after all. *Internal medicine journal*, 34(1-2):3-5.
- Tran, U.C. & Clarke, C.F. 2007. Endogenous synthesis of coenzyme Q in eukaryotes. *Mitochondrion*, 7:S62-S71.
- Trevisson, E., Dimauro, S., Navas, P. & Salviati, L. 2011. Coenzyme Q deficiency in mussle. *Current opinion in neurology*, 24(5):449-456.
- Trumpower, B.L. & Edwards, C.A. 1979. Purification of a reconstitutively active iron-sulfur protein (oxidation factor) from succinate: cytochrome *c* reductase complex of bovine heart mitochondria. *Journal of biological chemistry*, 254(17):8697-8706.
- Tuppen, H.A., Blakely, E.L., Turnbull, D.M. & Taylor, R.W. 2010. Mitochondrial DNA mutations and human disease. *Biochimica et biophysica acta*, 1797(2):113-128.
- Turunen, M., Olsson, J. & Dallner, G. 2004. Metabolism and function of coenzyme Q. *Biochimica et biophysica acta - biomembranes*, 1660(1):171-199.
- Van Houten, B., Woshner, V. & Santos, J.H. 2006. Role of mitochondrial DNA in toxic response to oxidative stress. *DNA repair*, 5:145-152.
- Vonck, J. & Schäfer, E. 2009. Supramolecular organization of protein complexes in the mitochondrial inner membrane. *Biochimica et biophysica acta – molecular cell research*, 1793(1):117-124.
- Wallace, D.C. 1999. Mitochondrial diseases in man and mouse. *Science*, 283(5407):1482-1488.
- Watmough, N.J. & Frerman, F.E. 2010. The electron transfer flavoprotein: Ubiquinone oxidoreductases. *Biochimica et biophysica acta - bioenergetics*, 1797(12):1910-1916.
- Watts, G., Playford, D., Croft, K., Ward, N., Mori, T. & Burke, V. 2002. Coenzyme Q10 improves endothelial dysfunction of the brachial artery in type II diabetes mellitus. *Diabetologia*, 45(3):420-426.

Wolf, D.E., Hoffman, C.H., Trenner, N.R., Arison, B.H., Shunk, C.H., Linn, B.O., McPherson, J.F. & Folkers, K. 1958. Coenzyme QI structure studies on the coenzyme Q group. *Journal of the American Chemical Society*, 80(17):4752-4752.

Xia, D., Yu, C.A., Kim, H., Xia, J.Z., Kachurin, A.M., Zhang, L., Yu, L. & Deisenhofer, J. 1997. Crystal structure of the cytochrome bc₁ complex from bovine heart mitochondria. *Science*, 277(5322):60-66.

Yamashita, S. & Yamamoto, Y. 1997. Simultaneous detection of ubiquinol and ubiquinone in human plasma as a marker of oxidative stress. *Analytical bio*

APPENDIX A: LIST OF MATERIALS

Materials	CAS number	Manufacturer
A		
Acetonitrile	75-05-8	Acros Organics
Ammonium formate	540-69-2	Sigma-Aldrich
B		
1,4-Benzoquinone	PHR1028	Sigma-Aldrich
Bicinchoninic Acid Kit for Protein Determination	BCA-1	Sigma-Aldrich
Bicinchoninic Acid (BCA)	B9643	Sigma-Aldrich
C		
Coenzyme Q ₁₀	303-98-0	Sigma-Aldrich
Copper sulfate solution (CuSO ₄)	C2284	Sigma-Aldrich
D		
Deuterium labeled CoQ ₁₀	13c-3713-824	13C Molecular
E		
Ethylenediaminetetraacetic acid (EDTA)	03660	Fluka
Ethylene glycol tetraacetic acid (EGTA)	E4378	Sigma-Aldrich
Ethanol (absolute)	64-17-5	Sigma-Aldrich
H		
HEPES	H3375	Sigma-Aldrich
4- (2-hydroxyethyl)-1-piperazine ethanesulphonic acid		
I		
Isopropanol	22032005	Labchem
J		
Jik solution		
M		
Mannitol	M9546	Sigma-Aldrich
Methanol	230-4	Burdick & Jackson
N		
<i>n</i> -hexane	Cat 212-4	Burdick & Jackson
P		
Potassium hydroxide	P5958	Sigma-Aldrich
Protein standard (bovine serum albumin, BSA)	P0914	Sigma-Aldrich
phosphate free soap		
S		
Sodium dodecyl sulphate	151-21-3	Sigma-Aldrich
Sucrose	S7903	Sigma-Aldrich
Miscellaneous		
deionized Milli-Q water (18Ω)	N/A	from a Millipore Milli-Q water purification system

