The Role of Mevalonate Derived Metabolites (Isoprenoids) in Statin – related Myopathies

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This thesis is submitted to Newcastle University for the degree of Doctor of Philosophy

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September 2017

Abstract

The statins, inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase, are the mainstay of the treatment of hypercholesterolaemia, atherosclerosis and coronary artery disease. However, some patients develop myopathy which may be related to pre-disposing factors such as high cellular statin uptake, or effects on other, non-sterol isoprenoids, such as CoQ10, prenylation and geranylgeranylation of proteins, dolichols, or due to alterations in the distribution of cholesterol precursors. Mitochondrial dysfunction has been associated with statin-related myopathy and it is possible that this arises secondary to inhibition of CoQ10 biosynthesis.

This project was designed to develop methods for the measurement of CoQ10, and to enable differentiation between cholesterol precursors specific to the Bloch or Kandutsch-Russell pathways. Tandem mass spectrometry was used to develop the CoQ10 assay and gas chromatography mass spectrometry was used for the quantitation of cholesterol and the sterol intermediates. These methods were then used to compare results from a large cohort of patients to test the hypothesis that modulated isoprenoid metabolism due to treatment with statins is involved in the aetiology of myopathy.

To complement this the possible influence of statins *in vitro* was examined and showed decreased CoQ10 synthesis, together with evidence of increased mitochondrial superoxide production and an enhanced cellular mitochondrial mass. Despite this, using the parameters investigated in this study no definitive association to statin-related myopathy could be found, suggesting this process is a complex one and further investigations are needed.

Declaration

This thesis is submitted for the degree of Doctor of Philosophy to Newcastle University. The research was performed within the Institute for Ageing and Health, Newcastle University, and the Blood Sciences Department, Newcastle-upon Tyne NHS Foundation Trust. Funding was awarded from the NIHR CSO Healthcare Scientist Research Fellowship Programme.

I certify that none of the material offered in this thesis has been previously submitted by me for a degree or any other qualification at this or any other university. Any views expressed in this dissertation are those of the author. All figures taken from other sources have been checked to confirm they are available from open access sources for reproduction within this thesis with appropriate citation.

SIGNED…….. DATE……

Acknowledgements

A huge debt of gratitude is owed to Dr Kim Bartlett, my long time mentor who recommended me for the challenge of undertaking a PhD and put his faith in me that I could see it through, even when I had my own doubts. My one regret is that I never finished it before the laid back life of retirement beckoned, but I hope I lived up to your high standards and just remember Kim…this was all your fault! Thanks a million.

This project would not have been possible without the support and guidance of Dr Dermot Neely. His enthusiasm for lipids is immeasurable, he inspired this project and his clinical expertise has been overwhelming but invaluable.

To Professor Sir Doug Turnbull, I bow to your willingness to take on the challenging role as my academic supervisor, and to Dr Robert McFarland for following suit. If not for you both, this work would have been for nothing and I am grateful for all the guidance you offered over years.

I am also indebted to Dr Ann Bowron. I know you came late to the party but your expertise and guidance in preparing this manuscript, endless proof-reading of chapters, positive encouragement throughout and your advice in pulling it all together has been truly inspiring. Really appreciate all the work you put in to it, I don't think I would have made it this far without it.

Professors Robert Taylor and Rita Horvath provided external supervision for this project. Their guidance in keeping me on the right track with the research reports and advice in all matters student related has been gratefully received.

Financial funding for this project was supplied by the NIHR. The CSO Healthcare Scientist Research Fellowship Programme gave me the chance to pursue this independent research project whilst maintaining the job I love. I wouldn't have done it any other way.

A particular shout out needs to go to my friends and colleagues in the Blood Sciences department. To Clare, for keeping me sane and giving me encouragement just when I needed it. To the 'NSS girls', you provided the laughs and made the tough times fun…there is no

number I can put on the value of that. And to 'my team' in the paediatric metabolic section, I owe you a huge apology for having to put up with my occasional stress induced mood swings but I am eternally grateful for all the extra hard work you had to put so that I could complete this project. Many, many thanks guys. To all the staff in Blood Sciences who helped with the routine analysis, thank you all.

To Nina, Colin, Matthew and Anya, without our weekly catch-ups I would not have kept my sanity. You guys are the best!

Last but not least, a big thank you to all my family. I can't thank you enough for all the support you have given me over the years. Without your constant encouragement, and many hours of emergency 'babysitting', I doubt I would ever have reached the end. This one is for you. Cheers.

Contents

List of Figures

List of Tables

Abbreviations

7DHC	7-dehydrocholesterol
CAD	Coronary artery disease
CK	Creatine kinase
CO_2	Carbon dioxide
CoQ	Ubiquinone
CoQ10	Ubiquinone (10 isoprene units)
CoQ9	Ubiquinone (9 isoprene units)
$CoQ10H_2$	Ubiquinol
CVD	Cardio vascular disease
DHCR24	24-dehydrocholesterol reductase
DMSO	Dimethyl sulphoxide
EDTA	Ethylenediaminetetraacetic acid
EPIC	Epicoprostanol
ESI	Electrospray ionisation
FBS	Foetal bovine serum
GC	Gas chromatography
HDL	High density lipoprotein
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
HPLC	High performance liquid chromatography
IS#	Internal standard
LDL	Low density lipoprotein
LOD	Limits of detection
LOQ	Limits of quantitation
MEM	Minimal essential media with Earles salt
MRM	multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NAD	Nicotinamide adenine dinuleotide
NADH	Reduced nicotinamide adenine dinuleotide
PBS	Phosphate buffered saline
ROS	Reactive oxygen species

RSD	Relative standard deviation
RT	Retention time
SAMS	Statin associated muscle symptoms
SIM	Selective reaction monitoring
SLOS	Smith Lemli Optiz syndrome
SREBP	Sterol regulatory element-binding proteins
SRM	Selected reaction monitoring
TMRM	Tetramethylrhodamine
VLDL	Very low density lipoprotein

Introduction

Chapter 1. Introduction

1.1 MEVALONATE PATHWAY

The mevalonate pathway is the first stage in a complex series of reactions culminating in the production of a diverse class of organic compounds known as isoprenoids. The pathway starts with the condensation of acetyl-CoA and ends with the production of five-carbon isoprene units, isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). Further condensation reactions and modifications of these molecules serves as the basis for the synthesis of bioactive molecules essential to multiple cellular processes. Cholesterol, for instance, is the principle end product of sterol metabolism and the starting substrate for steroid hormones, bile acids and signalling compounds such as oxysterols. Other end products include: ubiquinone, an antioxidant and important electron carrier in the mitochondrial respiratory chain; dolichol, essential for co-translational modification of proteins; and heme A, a constituent of cytochrome *c* oxidase. Protein prenylation, which facilitates attachment to cell membranes and protein-protein interactions, is also dependant on the synthesis of farnesyl and geranyl-geranyl moieties (Nowicka and Kruk, 2010). The mevalonate pathway is best known as a target for HMG-CoA reductase inhibitors, commonly known as statins, which block the reduction of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to mevalonic acid (Figure 1.1).

Biosynthesis of polyisoprenoid lipids occurs in several organelles, including the endoplasmic reticulum (ER), peroxisomes, and Golgi and mitochondrial membranes. Enzymes prior to the branch point are primarily located in the cytosol although HMG-CoA reductase, the rate limiting enzyme in the pathway, depends on its membrane association for regulation (Brown and Goldstein, 1980; Grünler *et al.*, 1994). Recently it was established that peroxisomes contained all the enzymes needed for conversion of acetyl-CoA to farnesyl pyrophosphate (FPP) (Kovacs *et al.*, 2002). However, work by Appelkvist and colleagues (Appelkvist *et al.*, 1999) using fibroblasts obtained from Zellweger patients in which peroxisomes are known to be absent or reduced in numbers, demonstrated the rates of cholesterol and dolichol synthesis were not significantly different from control cells, suggesting these organelles play a limited role in these processes.

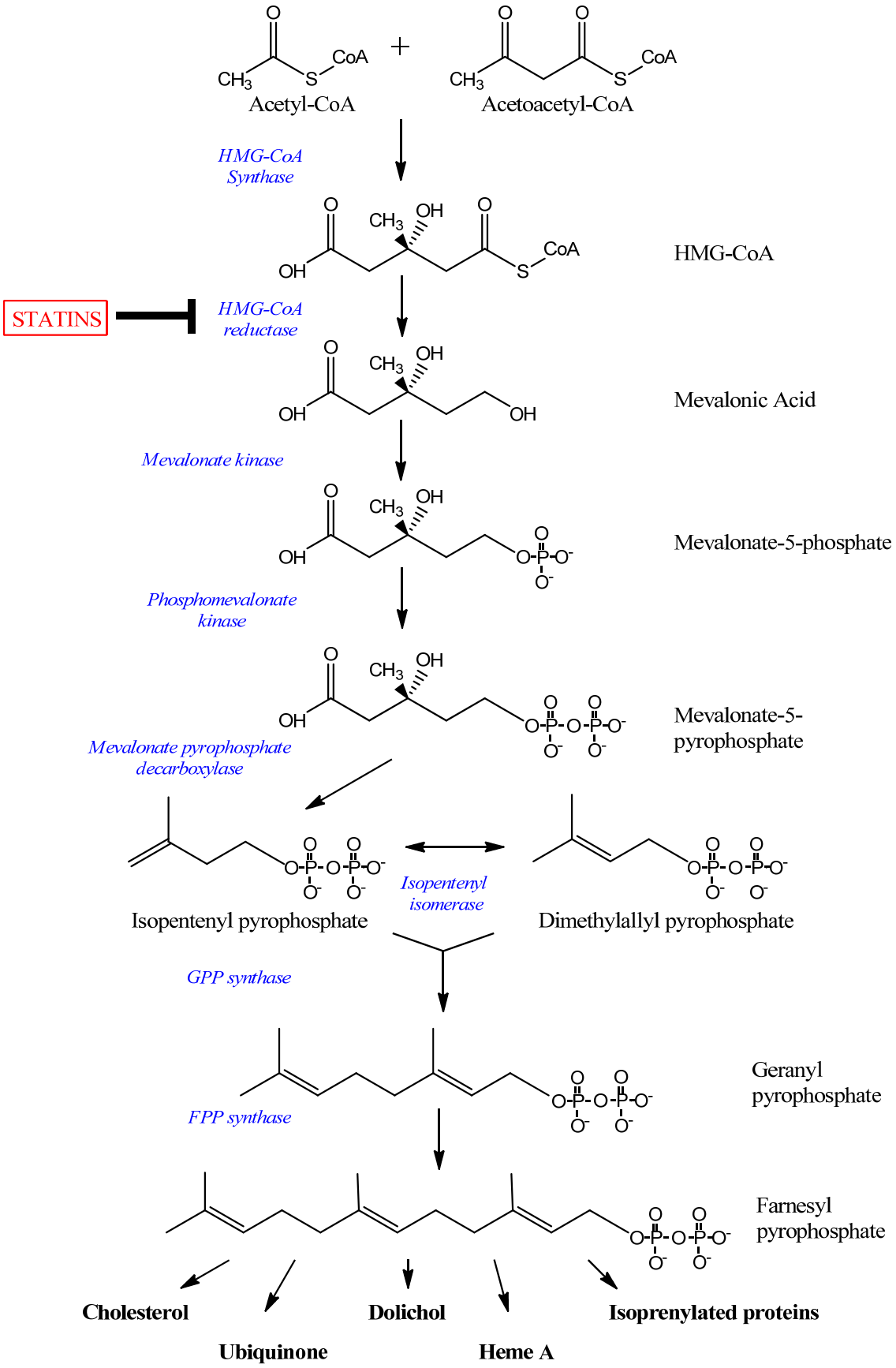


Figure 1.1 The mevalonate pathway.
Sequential condensation reactions convert IPP/DMAPP to polyisoprenoids. FPP is the common substrate for the branch point enzymes in isoprenoid synthesis.

Sterol regulatory element binding protein (SREBP) controls the expression of enzymes in this pathway, in particular HMG-CoA reductase gene transcription, by binding to specific sterol regulatory element (SRE) DNA sequences. SREBP is escorted from the ER to the Golgi for processing to its active form with the aid of a SREBP cleavage-activating protein, SCAP. In the presence of high sterol concentrations, SCAP is anchored in the ER via attachment to the resident protein Insig and is prevented from transporting SREBP therefore synthesis of further sterols is reduced (Figure 1.2) (Griffiths and Wang, 2009).

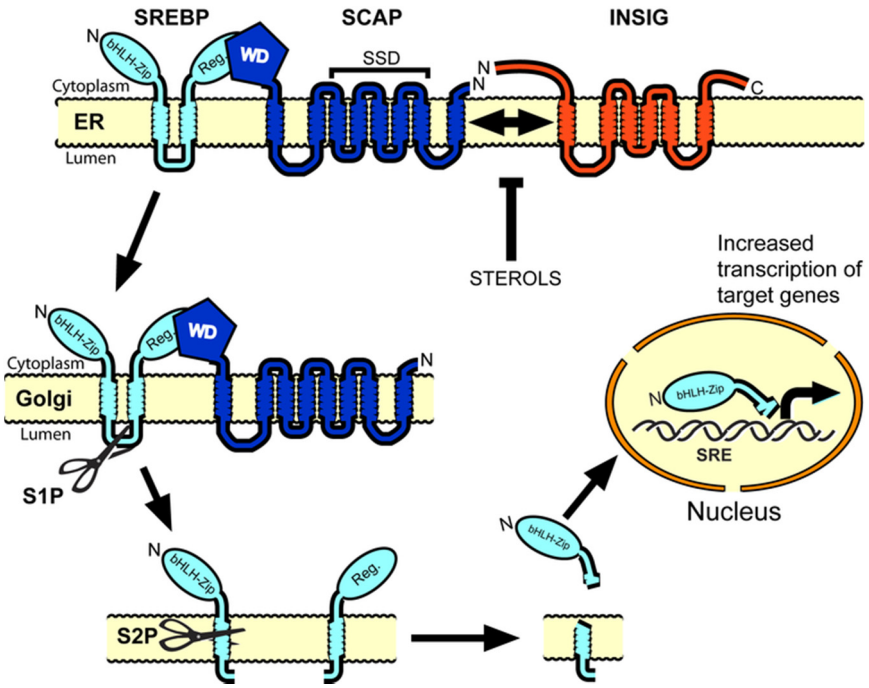


Figure 1.2 The SREBP regulatory pathway.
At low sterol concentrations SCAP is cleaved from the protein Insig and transports SREBP to the Golgi for activation. This in turn increases transcription of target genes such as HMG-Co reductase with the associated increase in sterol production.(Rrawson, 2009)

Branch point reactions occur in multiple cellular locations and it is unclear if a common pool of farnesyl pyrophosphate acts as substrate for mitochondrial biosynthesis of ubiquinone, ER biosynthesis of cholesterol and dolichol as well as cytosolic protein prenylation (Grünler *et al.*, 1994). The enzymes involved in the first committed steps of these compounds provide possible sites for regulation distal to HMG-CoA reductase as they have different affinities for the substrate FPP. Squalene synthase has a low affinity and as such cholesterol biosynthesis

is more attenuated than non-sterol isoprenoids by changes in the pool of isoprenoid substrates (Hargreaves *et al.*, 2005; Littarru and Langsjoen, 2007).

Manipulation of the mevalonate pathway may provide a means for therapeutic interventions for a variety of diseases. The effect of statins, competitive inhibitors of HMG-CoA reductase, has been extensively studied in the battle against cardiovascular disease and lead to statins being among the most widely prescribed pharmaceutical agents (Havel *et al.*, 1987; Hoeg and Brewer, 1987; Black, 2002; Schachter, 2005; Sewright *et al.*, 2007). Nitrogen containing bisphosphonates, used to treat metabolic bone disease, have possible anti-tumour properties by inhibiting FPP synthase and consequently decreasing the formation of FPP and geranyl-geranyl pyrophosphate (GGPP). In turn, prenylation of the small GTPases (Rho, Ras, Rac and Rab) which play a role in malignant transformations is prevented (Buhaescu and Izzedine, 2007).

1.2 COENZYME Q10

One of the most significant end products of the mevalonate pathway is coenzyme Q (CoQ). Isolated and characterised in 1957 by Dr Frederick Crane, CoQ is a lipophilic quinone essential to the mitochondrial respiratory chain (Crane *et al.*, 1957). Shortly thereafter the ubiquitous presence of this molecule in virtually all tissues and cells led to the introduction of the name ubiquinone (Langsjoen, 1995; Crane, 2001). Folkers and colleagues determined the precise chemical structure in 1958 to indicate a quinone ring attached to an isoprenoid side chain which exhibits species specific chain length variation (Crane, 2007). In humans and most mammals the primary form, designated coenzyme Q10 (CoQ10), comprises 10 isoprene units with approximately 2-5 % containing 9 units (Aberg *et al.*, 1992). In rats and mice CoQ9 predominates whilst shorter chain polyprenoids are common in bacteria and yeast (Overvad *et al.*, 1999).

It is estimated only 25 % of CoQ10 in plasma is of dietary origin (Hargreaves *et al.*, 2005) with less than 5 mg/day CoQ10 coming from dietary intake (Ely and Krone, 2000). Dietary CoQ10 is slowly absorbed from the gut and processed by the liver. Low-density lipoproteins (LDL) are estimated to transport approximately 58 % of the plasma CoQ10 and it appears normal tissue reaches a saturation point at which exogenous supplementation no longer influences tissue levels (Mas and Mori, 2010). The adult body pool contains about 2 g with a turnover rate of

approximately 0.5 g/day (Ely and Krone, 2000). Consequently, a significant contribution is obtained from endogenous biosynthesis. It is evident CoQ10 is involved in numerous cellular activities. Its association with disease pathologies and as a potential treatment modality has warranted much investigation, beginning with the first use of CoQ10 to treat congestive heart failure in the mid-1960s. Analytical techniques able to directly measure CoQ10 in biological samples promoted an increase in the number of clinical trials from the 1980's (Langsjoen, 1995).

1.2.1 Structure and Biosynthesis

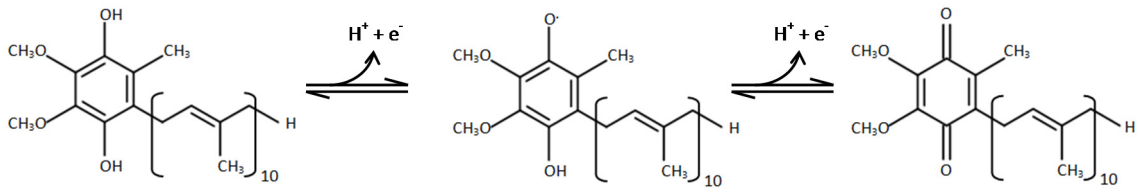
Coenzyme Q10 is 2,3-dimethoxy-5-methyl-6-decaprenyl-1,4 benzoquinone and exists in 3 oxidation states (Figure 1.3):-

Fully oxidised form – ubiquinone (CoQ10)

Free radical – semiquinone (CoQ10·)

Fully reduced form – ubiquinol ($CoQ10H_2$)

With the exception of brain and lung, a high percentage of CoQ10 measured in human plasma and tissue exists in the reduced form (Table 1.1) (Aberg *et al.*, 1992; Kaikkonen, 1999; Turunen *et al.*, 2004b).



Ubiquinol **Semiubiquinone (free radical)** **Ubiquinone**

Figure 1.3 Structure of coenzyme Q10.
Coenzyme Q can exist in three redox states: fully reduced ubiquinol ($CoQH_2$), fully oxidised ubiquinone (CoQ) and the intermediate semiubiquinone radical ($CoQH\cdot$)

Biosynthesis of CoQ10 remains incompletely characterised but requires 3 major steps: synthesis of the benzoquinone ring, synthesis of the isoprenoid chain, and condensation of the two molecules together (Overvad *et al.*, 1999). The ring structure, 4-hydroxybenzoate, originates via vitamin B6 dependent enzymatic transformation from the amino acids tyrosine or phenylalanine (Willis *et al.*, 1999). The polyisoprenoid side chain is generated via intermediates of the mevalonate pathway, a series of reactions common to the production of cholesterol, dolichol, isoprenylated proteins and ubiquinone. 3-Hydroxy-3-methylglutartyl coenzyme A (HMG-CoA) reductase is the rate limiting enzyme and the site for therapeutic interventions in hypercholesterolaemia employing statins as competitive inhibitors. Downstream of the branch point *trans*-prenyl transferase combines FPP with several molecules of IPP all in the *trans* configuration to synthesise the side chain (Teclebrhan *et al.*, 1993; Okada *et al.*, 1996).

Organ	CoQ10 µg/g tissue	% reduced
Lung	7.9	24
Intestine	11.5	93
Brain	13.4	23
Spleen	24.6	87
Muscle	40	60
Liver	55	95
Kidney	66.5	73
Heart	114	47

Table 1.1 Average Coenzyme Q10 Concentrations in Human Tissue.
Results are the mean values obtained for autopsy samples following a rapid extraction procedure and direct injection onto HPLC. (data taken from Aberg *et al.*, 1992)

Parahydroxybenzoate polyprenyltransferase, encoded by *COQ2*, catalyses the condensation reaction to produce polyprenyl-4-hydroxybenzoate (Turunen *et al.*, 2004a). This enzyme is not thought to be substrate specific for chain length (Okada *et al.*, 1996). The ring structure subsequently undergoes a series of 7 modifications including hydroxylations, O-methylations and a decarboxylation step to produce the functional CoQ (Kawamukai, 2002; Turunen *et al.*, 2004b; Quinzii *et al.*, 2007) (Figure 1.4).

Enzymes involved are primarily located in the inner mitochondrial membrane which suggests CoQ transport to other membranes (Nowicka and Kruk, 2010) although this process is still poorly understood. Biosynthesis in the ER-Golgi system is also active and biosynthetic enzymes have been detected in other organelles such as peroxisomes (Turunen *et al.*, 2004b). Genes encoding the terminal part of CoQ biosynthesis have been extensively studied in bacteria and yeast and 16 human homologs identified (Duncan *et al.*, 2009a). To date, mutations in only a few of these genes have been documented as a molecular cause of primary CoQ10 deficiency and these are discussed briefly in section 1.2.3.1.

A consensus model for the arrangement of polyisoprenoids in biological membranes is shown in Figure 1.5. The tail regions of dolichol, dolichyl-P and CoQ anchors the molecules within the hydrophobic region of the lipid bilayer saturating the available space. The functionally active polar head of CoQ angles towards the membrane surface, probably around the third isoprene unit. In its reduced state ubiquinol is more polar than the oxidised ubiquinone, accounting for motility within the membrane and interaction with redox centres in the mitochondrial complexes. Contrary to this linear model, molecular modelling experiments suggest a folded conformation may be preferential. Such a structure has important implications and can explain several experimental observations including the high activation energy requirements during protein binding (Lenaz *et al.*, 2007).

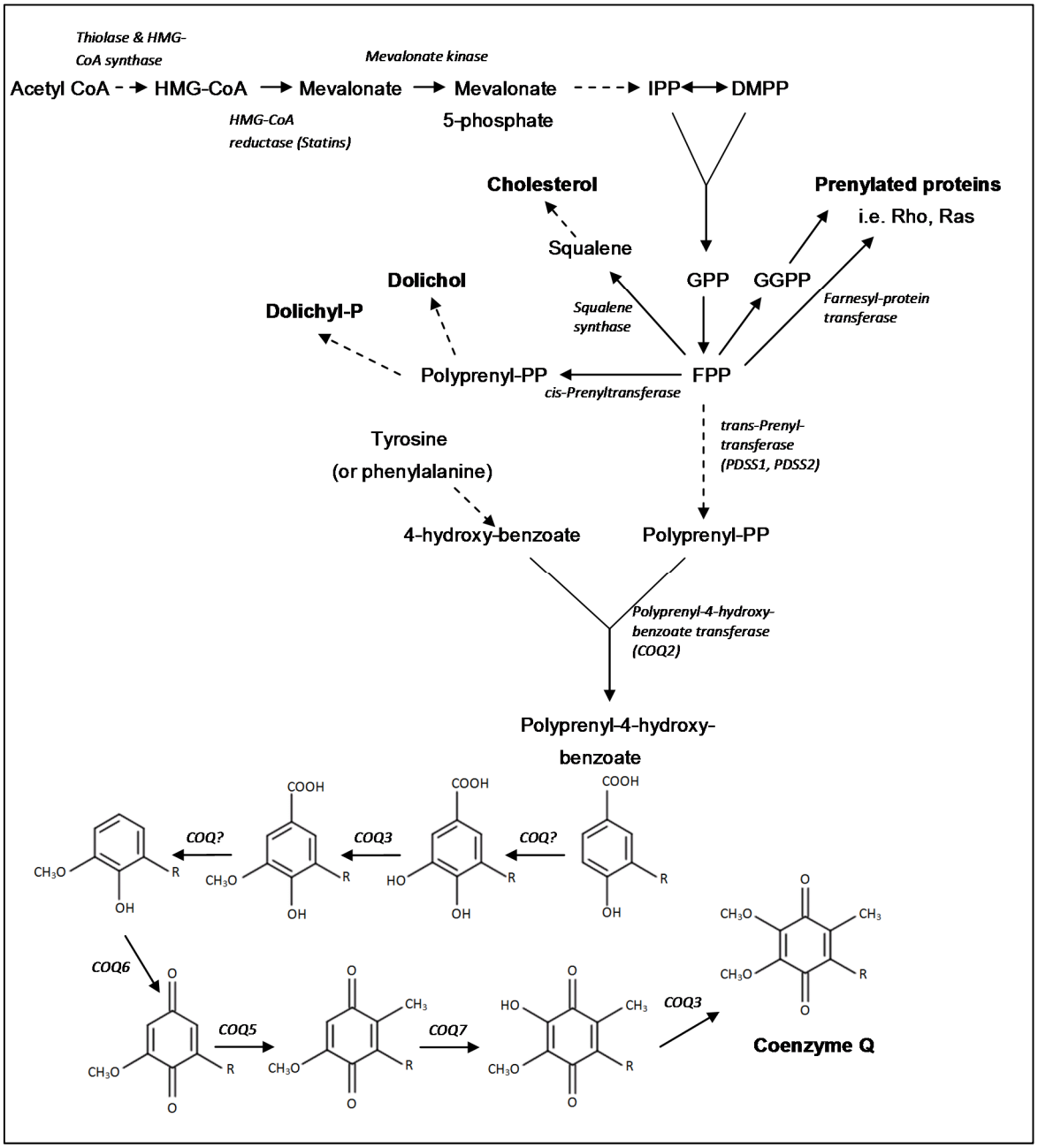


Figure 1.4 The proposed pathway of coenzyme Q biosynthesis.
The polyisoprenoid 'tail' region is synthesized from intermediates of the mevalonic acid pathway and the hydroxybenzoate ring is produced from tyrosine or phenylalanine. After condensation of the two molecules the ring structure undergoes a series of modifications. Significant enzymes and genes identified are shown in italics. Abbreviations: CoA, coenzyme A; HMG, 3-hydroxy-3methylglutaryl; IPP, isopentenyl pyrophosphate; DMPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; FPP, farnesyl pyrophosphate; R depicts the polyisoprenoid chain

In contrast to cholesterol, which is localised between the fatty acids, the polyisoprenoids increase permeability and fluidity and act to destabilise the membrane, hence a disturbed balance between these lipids can affect membrane properties. It would be expected, in the case of decreased endogenous levels for exogenous CoQ10 to be taken up by the cell until the saturation point was reached (Bentinger *et al.*, 2007).

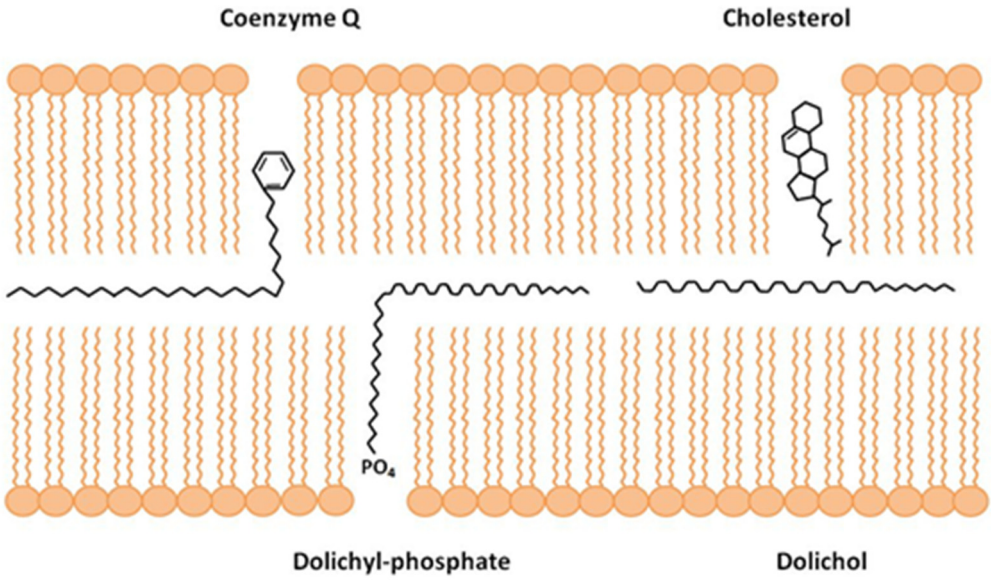


Figure 1.5 Distribution of isoprenoids in the membrane lipid bilayer.
Polyprenoid tails anchor dolichol, dolichyl-P and CoQ within the lipid bilayer whilst the more polar head region angles towards the membrane surface, allowing for redox reactions to occur.

1.2.2 Functions of Coenzyme Q10

Following early focus on the pivotal role of coenzyme Q10 as an electron carrier in the mitochondrial respiratory chain extensive research has focused on additional functions for this ubiquitous lipid. Much has addressed the antioxidant properties and ability to recycle other antioxidants such as tocopherol (Mitchell, 1976). A role in cell signalling and gene expression, uncoupling proteins, apoptosis and anti-inflammatory effects have also been described (Crane, 2001; Bentinger *et al.*, 2010).

1.2.2.1 Energy Coupling

An in-depth review of the fundamental role of CoQ10 in bioenergetics and mitochondrial electron transport is provided by Lenaz *et al* (Lenaz *et al.*, 2007). This vitamin-like nutrient is essential to the cellular energy making process that culminates in the production of adenosine tri-phosphate (ATP). The redox capacity of CoQ10 confers its function as an efficient electron and proton carrier. CoQ10 accepts reducing equivalents, via flavoproteins and iron-sulphur proteins (FeS), from complex I (NADH:CoQ oxidoreductase) and complex II (succinate:CoQ oxidoreductase) and transfers them to complex III (cytochrome *bc1*) with the concomitant translocation of protons across the inner mitochondrial membrane and reduction of 2 cytochrome *c* molecules. In the final reaction cytochrome *c* is reoxidised and oxygen reduced to water through complex IV (cytochrome *c* oxidase) with a further 4 proton transfer into the intermitochondrial space. The resultant proton gradient constitutes the proton motive force that drives the formation of ATP through ATP synthase (oxidative phosphorylation) (Figure 1.6). Complex II does not release sufficient energy and does not contribute to the proton gradient (Kroger and Klingenberg, 1973; Nowicka and Kruk, 2010).

Complex III comprises a Rieske iron-sulphur protein, two *b* cytochromes and one cytochrome *c*. Electrons pass from the reduced CoQ10 (ubiquinol) to cytochrome *c* through the efficiency of the Q cycle formulated by Peter Mitchell (Figure 1.6 inset) (Mitchell, 1975a; Mitchell, 1975b; Mitchell, 1976). The demonstration of unique binding sites within the protein complex for ubiquinol oxidation at the Q_0 site and ubiquinone reduction at the Q_i site, located on the outer and inner side of the inner mitochondrial membrane respectively further cement this mechanism and ensures release of protons in the right direction (Halestrap, 1982; Ding *et al.*, 1995).

The transient formation of the partially oxidised semiquinone and recycling of the ubiquinol doubles the efficiency of coenzyme Q10s contribution to the proton gradient. The ultimate process can be summarised as:-

$$CoQH_2 + 2\text{cyt } c_{\text{(oxidised)}} + 2H^+_{\text{(matrix)}} \rightarrow CoQ + 2\text{cyt } c_{\text{(reduced)}} + 4H^+_{\text{intermembrane space}}$$

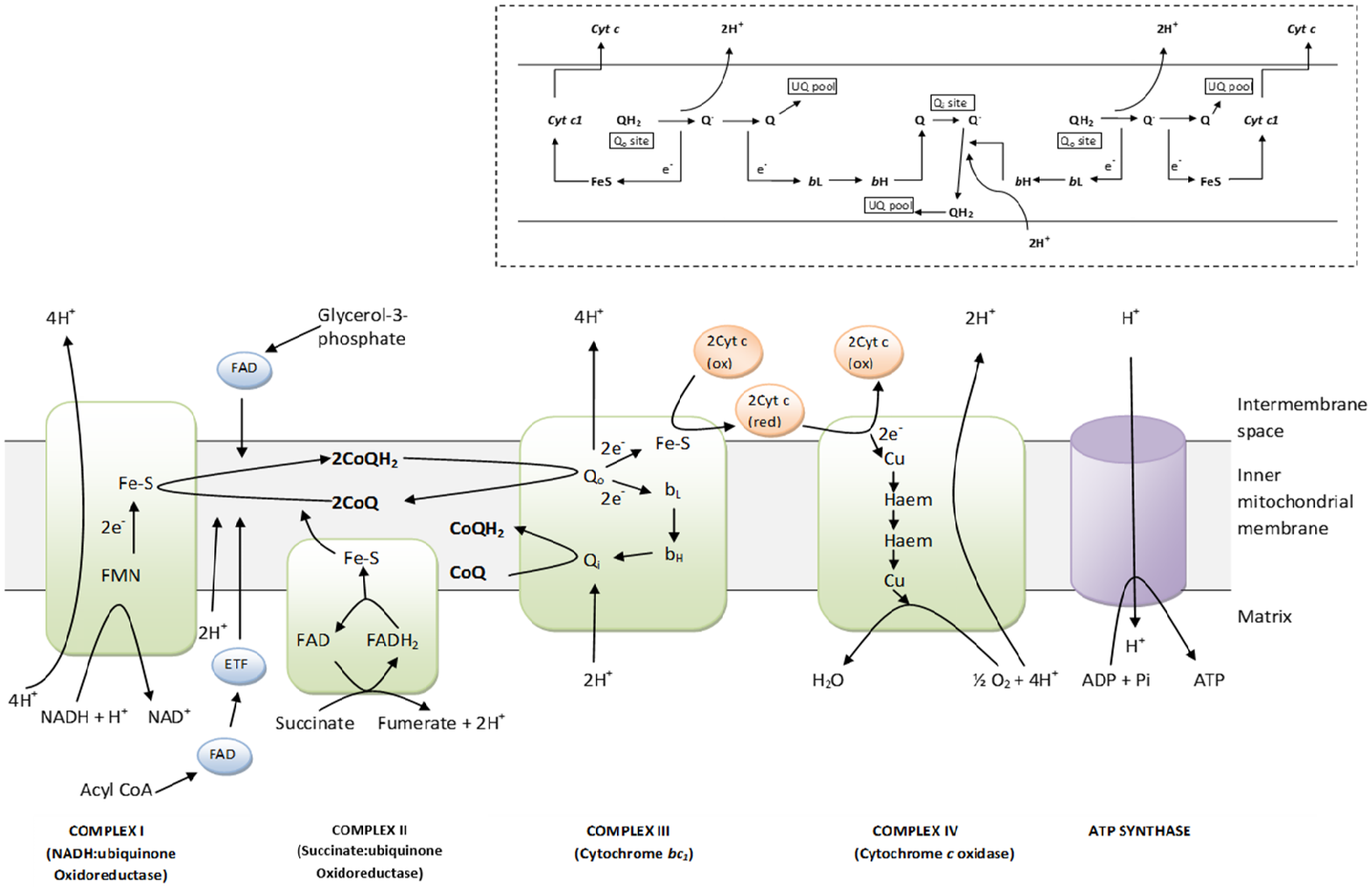


Figure 1.6 **Function of coenzyme Q within the mitochondrial electron transport chain.**

Inset: The Q cycle through complex III. During the oxidation of ubiquinol, 2 H+ are released into the intermembrane space, one electron is donated to cytochrome c and the second electron to ubiquinone forming the semiquinone which remains attached at the Qi site. A similar process occurs with a second ubiquinol but an electron, together with 2 H+ taken up from the matrix, is used to reduce the semiquinone to ubiquinol. Abbreviations: Fe-S, iron sulphur protein; ETF, electron transferring flavoprotein; FAD, flavin adenine dinucleotide; cyt, cytochrome; bL and bH are cytochrome b haem molecules; UQ, coenzyme Q.

Coenzyme Q10 also acts as a direct link into the respiratory chain for the oxidation of fatty acids and amino acids. Electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO) catalyses CoQ10 reduction and the flow of electrons from acyl-CoA via FAD and FeS clusters to complex III (Gempel *et al.*, 2007). This enzyme does not span the lipid bilayer but attaches to the membrane surface. It accepts electrons from various acyl-CoA dehydrogenases and is responsible for the human metabolic condition multiple acyl-CoA dehydrogenase deficiency (MADD, also known as glutaric aciduria type II) (Zhang *et al.*, 2006). Gempel and co-workers described 7 cases of isolated myopathy (age 12 - 32 years at disease onset) with significantly reduced muscle CoQ10 and acylcarnitine profiles indicative of MADD. Subsequent identification of recessive mutations in the ETF-QO gene suggested the CoQ10 deficiency was secondary due to down regulation via a feedback mechanism or enhanced degradation due to faulty binding (Gempel *et al.*, 2007).

Glycerol-3-phosphate, from triglyceride catabolism and glycolysis, also contributes to reduction of the CoQ pool through flavin linked dehydrogenases. In contrast to ETF-QO, glycerol phosphate dehydrogenase is located on the outer surface of the inner membrane (Klingenberg, 1970) and shuttles reducing equivalents from the cytosol to CoQ in a pathway distinct from that of complexes I and II (Lenaz *et al.*, 2007). Another enzyme, dihydroorotate dehydrogenase, is also known to reduce CoQ during pyrimidine biosynthesis (Nowicka and Kruk, 2010).

1.2.2.2 Extra-mitochondrial electron transport

In addition to its location in mitochondria, CoQ10 is found throughout the membrane network. In plasma membranes, an externally attached NADH-oxidase (NOX) regulates cytosolic NAD^+/NADH ratios. This CoQ10 dependent enzyme helps maintain cellular reduced CoQ (Turunen *et al.*, 2004b) and is also involved in reduction of ascorbate and formation of superoxide radicals, regulating cell growth and differentiation (Sun *et al.*, 1992).

Lysosomes have been shown to contain CoQ10 dependant redox chains (Gille and Nohl, 2000). In this instance hydrolytic enzymes, used for debris digestion, are activated by proton translocation creating an acidified environment. The CoQ10 is reduced in the cytoplasm by NADH in two one-electron transfer steps and reoxidised by oxygen (Crane, 2001).

1.2.2.3 Anti- and Pro- oxidant functions of CoQ10

Reactive oxygen species (ROS) and free radicals are derived from internal metabolic processes or as a consequence of external stimuli, such as radiation and smoking. If left unchecked, these oxidants react with cellular macromolecules causing damage to proteins, lipids, carbohydrates and DNA leading to a plethora of pathological conditions. Oxidative stress is a state of imbalance between the rate of ROS formation and counteractive measures of antioxidant molecules or defensive enzymes, such as superoxide dismutases (SOD), catalase and peroxidises. The best known non-enzymatic antioxidants include α-tocopherol (vitamin E), ascorbate (vitamin C), glutathione and ubiquinol of which the latter is the only one endogenously synthesized (Bagchi and Puri, 1998; Turrens, 2003).

Mitochondria use approximately 90 % of the oxygen consumed and are a major source of superoxide ($O_2\cdot^-$) and consequently hydrogen peroxide (H_2O_2) production (Sheu *et al.*, 2006). Electron leaks from complex III (approximately 1 - 2 %) (Bentinger *et al.*, 2007) are widely regarded as the major contributor but a study by McLennon and Esposti (McLennan and Esposti, 2000) suggests complex I and II also have a significant input. As stated previously, CoQ10 plays a vital role in electron transfer through the respiratory chain. Redox reactions proceed in a controlled manner but a disturbed equilibrium in which the influx of electrons exceeds efflux leads to increased semiquinone formation and autoxidation to superoxide (James *et al.*, 2004). Low levels of free radicals are necessary for normal cellular structure and signalling, including cell cycle regulation and hormone production (Turrens, 2003; Bentinger *et al.*, 2007), but are inversely correlated with disease pathologies and ageing (Lass *et al.*, 1997).

Paradoxically to its pro-oxidant role, an antioxidant function for CoQ10 has been extensively documented and primarily relies on the reduced form ubiquinol (James *et al.*, 2004; Bentinger *et al.*, 2007). Studied in detail is the ability to prevent both initiation and propagation of lipid peroxidation (Figure 1.7), a function reflected in the localization of CoQ10 within close proximity to potential radicals in the lipid bilayer (Mellors and Tappel, 1966; Do *et al.*, 1996). Experiments involving pentene extraction of lipids from submitochondrial particles, then reinsertion of CoQ10 prevented peroxidation, even in the absence of other antioxidants such as tocopherol, indicating CoQ10 has independent antioxidant capacity (Bentinger *et al.*, 2007). Reduction of the perferryl radical, with the subsequent formation of semiubiquinone and

H_2O_2, prevents formation of the lipid peroxyl radical (LOO·). Evidence also suggests CoQ10 interferes with the propagation process through direct elimination of LOO· or indirectly by regeneration of α-tocopherol from the tocoperoxyl radical. Conversely, the antioxidant property of α-tocopherol lies in its chain breaking role but does not prevent initiation (Do *et al.*, 1996). CoQ10 has also been shown to prevent proteins from carbonyl formation, and to protect DNA strand breaks as a consequence of hydrogen peroxide binding to metal ions on DNA bases forming hydroxyl radicals (Bentinger *et al.*, 2007). Some evidence supports a function in scavenging reactive nitrogen species (James *et al.*, 2004; Nowicka and Kruk, 2010).

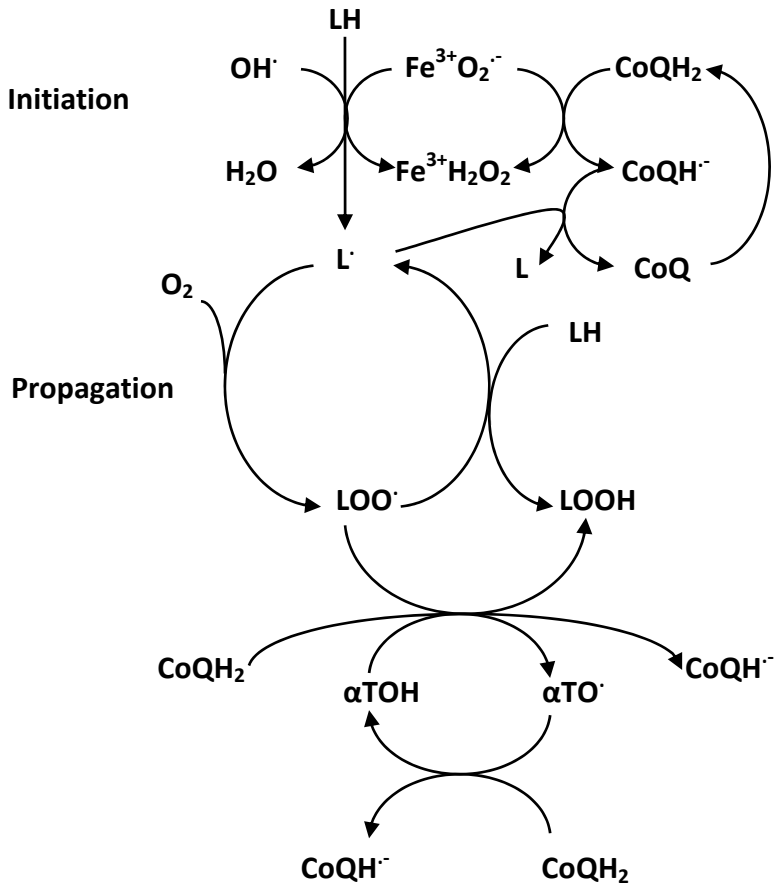


Figure 1.7 Action of CoQ10 on lipid peroxidation.
Reduction of the initiating perferryl radical ($Fe^{3+}O_2^{\cdot-}$) by ubiquinol ($CoQH_2$) prevents abstraction of a hydrogen atom from a polyunsaturated lipid (LH) and subsequent formation of the carbon-centered radical (L·) and lipid peroxyl radical (LOO·). During propagation LOO· reacts with another LH, forming lipid hydroperoxide (LOOH) and regenerating L·. Ubiquinol interferes with propagation by direct quenching of LOO· or regenerating α-tocophorol (αTOH) from a tocopherol radical (αTO·), with the formation of the semiubiquinone (CoQH·-) intermediate

In addition to the direct scavenging of free radicals, ubiquinol plays a role in the regeneration of another lipophilic antioxidant, α-tocopherol (vitamin E) from the tocopheroxyl radical (Stoyanovsky *et al.*, 1995; Gille *et al.*, 2008). Evidence also suggests it can regenerate ascorbate (vitamin C) outside the cell via electron transfer across the plasma membrane

(Gómez-Díaz *et al.*, 1997). Circulating levels of CoQ10 are less than those of α-tocopherol but it has a greater efficiency and is preferentially utilized during oxidative stress suggesting a sparing effect on α-tocopherol (Colquhoun *et al.*, 2005; Hargreaves *et al.*, 2005). In mitochondria, the content of CoQ10 is 10 times that of α-tocopherol (James *et al.*, 2004) and in membranes it may be up to 30 times (Crane, 2001).

Inherent with its lipophilic nature, coenzyme Q10 does not mix well with the aqueous environment of plasma and is therefore bound to lipoproteins, specifically LDL, to aid transport (Figure 1.8). Due to its antioxidant properties, the bound CoQ10 plays a significant protective role against LDL-oxidation. Of clinical note is the oxidation of cholesterol in macrophages which leads to the development of plague lesions resulting in heart disease. The close proximity of CoQ10 serves to protect the cholesterol and thus confers anti-atherosclerotic properties. However not all effects relate solely to an antioxidant function (Nowicka and Kruk, 2010). Experiments have shown CoQ10 reduces β-integrin CD11b levels thereby preventing recruitment of monocytes to the lesion by reducing interaction with endothelial cells (Turunen *et al.*, 2004b; Bentinger *et al.*, 2010).

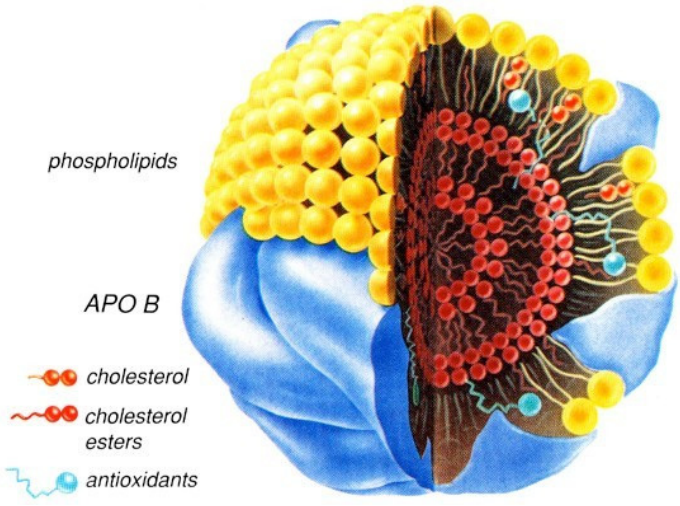


Figure 1.8 Structure of LDL particle.
The lipophilic antioxidant, coenzyme Q10, is encased within the phospholipid layer which enables transport in the plasma. (*Structure of LDL*, 2014)

Concurrent with its universal antioxidant function, a widespread occurrence of enzymes able to maintain CoQ10 in its reduced form exists. These include; glutathione reductase, thioredoxin reductase, NADH cytochrome b5 reductase, NADPH coenzyme Q reductase, lipoamide dehydrogenase and DT-diaphorase (NAD(P)H oxidoreductase) (Xia *et al.*, 2001; Nowicka and Kruk, 2010). The latter enzyme is unique as it can reduce CoQ10 by 2 electrons at a time without formation of the semiquinone (Crane, 2001).

1.2.2.4 Additional functions of CoQ10

Located in the inner mitochondrial membrane, uncoupling proteins (UCP) translocate protons across the membrane and consequently uncouple the proton gradient of the electron transport chain from oxidative phosphorylation. The most widely studied is UCP1 found in brown adipose tissue and used for thermogenesis (Kawamukai, 2002). UCPs have recently been shown to require the oxidized form CoQ10 as an obligatory cofactor with the proposed interaction occurring in the lipid bilayer as short chain homologues of CoQ do not influence UCP action (Turunen *et al.*, 2004b).

Under conditions of calcium ion accumulation, an inner mitochondrial complex, the membrane permeability transition pore, opens and increases membrane permeability to macromolecules. This in turn causes collapse of the membrane potential, disruption of the ionic status and cell apoptosis. CoQ10 appears to prevent opening of this pore through binding to the channel (Walter *et al.*, 2002).

Expression of the transcription factor NFκB1, a primary responder to harmful events is influenced by CoQ10 uptake into lymphocytes and monocytes (Bentinger *et al.*, 2010) and it has been shown that H_2O_2 from autoxidation of semiquinone is an important source for activation in malignant melanoma cells (Brar *et al.*, 2001).

1.2.3 Clinical Importance

Altered levels of CoQ10 have been reported in a variety of conditions including cardiovascular disease, neurodegenerative disorders, and cancer, as well as ageing and during physical exercise. Primary deficiencies involving genes of the CoQ10 biosynthetic pathway are recognised. Insufficient dietary intake, decreased biosynthesis and excess utilization can

contribute to changes in the CoQ10 pool and correction of the CoQ10 levels in some cases may lead to dramatic clinical improvement.

1.2.3.1 Inborn Errors of Metabolism

1.2.3.1.1 Primary Deficiency of Coenzyme Q10 Metabolism

Primary CoQ10 deficiency constitutes a rare group of autosomal recessive mitochondrial respiratory chain disorders. Fewer than 40 patients were identified in 17 years since the first reported case (Rotig *et al.*, 2007). Of the 15 genes required for CoQ10 biosynthesis, pathogenic mutations have been identified in at least 8 of them with substantial variation in both the clinical and biochemical phenotypes (Rahman *et al.*, 2012; Desbats *et al.*, 2015). For instance, in comparison to controls, fibroblast cells harbouring mutations in the *PDSS2* gene, responsible for elongation of the isoprenoid chain, have 12 % CoQ10 content and markedly reduced ATP synthesis but are not associated with increased ROS production. In contrast, mildly affected ATP synthesis and diminished CoQ10 levels of 30 % are observed in *COQ2* mutant cells with significant production of ROS and lipid oxidation (Quinzii and Hirano, 2010).

In almost all cases individual mitochondrial complex activities are normal but combined complexes I+III and/or II+III, which are quinone dependent exhibit reduced activity (Duncan *et al.*, 2009a). Confirmation of a primary deficiency is made by direct CoQ10 measurement in muscle although recent evidence suggests that the concentration in mononuclear cells correlates well with muscle concentrations (Duncan *et al.*, 2005). Furthermore, confirmation of primary deficiency has been shown by low rates of CoQ10 biosynthesis in fibroblast cells incubated with ^{14}C-4-hydroxybenzoate (Duncan *et al.*, 2009a) and with ^{3}H-mevalonate, which is incorporated into cholesterol and dolichol in patient fibroblasts but not evident in CoQ10 (Rötig *et al.*, 2000).

Five major clinical phenotypes have been described: (1) encephalomyopathic, (2) multisystemic infantile form, (3) predominantly cerebellar form, (4) isolated myopathic form, and (5) nephritic syndrome (Table 1.2). The cerebellar ataxia form represents the most common phenotype (Quinzii and Hirano, 2010) and primary defects associated with the *ADCK3/CAB1* gene (Mollet *et al.*, 2008), or secondary deficiency due to mutations in the *APTX* gene encoding the nuclear DNA repair protein aprataxin have been described (Quinzii and Hirano, 2010). Adult onset presentations have been associated with the myopathic form

(Horvath *et al.*, 2006). In the encephalomyopathic form, CoQ10 depletion was only observed in muscle homogenate with normal levels detected in cultured lymphoblasts and fibroblast (Boitier *et al.*, 1998). In a neonatal multisystemic form, exogenous CoQ restored mitochondrial complex activity *in vitro* but therapy did not improve the clinical outcome and the patient died at age 2 years, possibly due to the severe neurological damage sustained before diagnosis (Rahman *et al.*, 2001). In four cases of pure myopathic CoQ10 deficiency, citrate synthase levels were significantly elevated indicating increased mitochondrial proliferation and CoQ10 was only reduced in muscle (Lalani *et al.*, 2005; Horvath *et al.*, 2006). Interestingly, 7 patients with this phenotype from 5 independent families had mutations identified in the *ETF-QO* gene suggesting the myopathic form of CoQ10 deficiency and late-onset glutaric aciduria type II (MADD) are allelic diseases (Gempel *et al.*, 2007). With few exceptions most patients responded favourably to oral CoQ10 treatment (Horvath *et al.*, 2006; Quinzii *et al.*, 2007; Mollet *et al.*, 2008) which emphasises the need for early diagnosis and treatment to maximise efficacy.

Phenotype	Year identified	Onset	Q levels (% of controls)	Symptoms	Genes	reference
Encephalomyopathy	1989[a]	6 months – 3 years[b]	Muscle 3.7- 40%[b]	Mitochondrial myopathy, recurrent myoglobinuria, encephalopathy, ↑CK and lactate		[a](Quinzii and Hirano, 2010) [b](Teshima and Kondo, 2005)
Multisystemic infantile	2000[c]	Neonatal period	Fibroblasts 0-18%[b]	Neurological, seizures, nystagmus, optic atrophy, hearing loss, weakness, ataxia, nephropathy	*COQ2*[a] *COQ9*[d] *PDSS1 and PDSS2*[a]	[c](Li *et al.*, 2007) [d](Duncan *et al.*, 2009a)
Cerebellar ataxia	2001[a]	Variable childhood	Muscle 10-53%[b] Fibroblasts 38-63%[b]	Cerebellar ataxia, atrophy, seizures, neuropathy, mental retardation	*ADCK3/CABC1*[e] *APTX* (secondary deficiency)[a]	[e](Ruiz-Jimenez *et al.*, 2007)
Isolated myopathy	2005[f]	6-32 years[g]	Muscle 22-63%[g]	Exercise intolerance, fatigue, proximal myopathy, ↑CK and lactate	*ETF-QO*	[f](Niklowitz *et al.*, 2007) [g](Paliakov *et al.*, 2009)
Nephrotic [a]	2007	5 days – 18 months		Oliguria, renal disease	*COQ2*[a]	

Table 1.2 Primary Deficiencies of Coenzyme Q10

Evaluation of CoQ10 and electron transport chain (ETC) enzymes in muscle biopsies from 82 children with suspected but unproven mitochondrial myopathy suggests total CoQ10 may be a good predictor of mitochondrial disease in children which often does not conform to the "classical" presentation seen in adult cases (Miles *et al.*, 2008). CoQ10 values below 185 nmol/g protein suggested a greater likelihood of a complex I+III or II+III defect. Direct measurement of CoQ10 has the advantage of requiring much less tissue, approximately 1/5th of that needed for ETC analysis. The latter measurements are also highly susceptible to variations in collection and storage conditions (Tang *et al.*, 2004). Very low CoQ10 content in muscle i.e. <107 nmol/g protein should warrant further investigation of a primary or secondary deficiency (Miles *et al.*, 2008).

1.2.3.1.2 Mevalonate Kinase Deficiency

Mevalonic aciduria (MVA) and hyper-IgD with periodic fever syndrome (HIDS) represent two ends of the spectrum of an autosomal recessive condition caused by a defect in the cholesterol and isoprenoid biosynthetic pathway at the level of mevalonate kinase (MK). MVA is often fatal and presents with psychomotor retardation, failure to thrive and recurrent fever, vomiting and diarrhoea. Patients have very low or undetectable residual activity of MK in contrast to the more benign HIDS which have activity of MK up to 7 % of the control value (Houten *et al.*, 2003; Haas *et al.*, 2009).

As they share a common pathway it is not inconceivable for the biosynthesis of both cholesterol and non-sterol isoprenoids to be affected in MVA. However, cholesterol levels remain normal or only slightly lowered, suggesting it is not pathological for the condition and determination of flux through the pathway in MVA fibroblast cells indicates an up-regulation of HMG-CoA reductase activity with elevated mevalonate compensating for the reduced MK activity to maintain the supply of downstream intermediates (Houten *et al.*, 2003). Although slightly decreased concentrations of CoQ10 have been reported in plasma of MVA patients little is known of intracellular levels. One report suggests levels in fibroblasts are indistinguishable from controls (Haas *et al.*, 2009) but this contrasts an earlier report suggesting combined CoQ10 and dolichol synthesis in MVA cell lines is less than 10 % of normal (Hargreaves, 2007).

In contrast to MVA, methylmalonic aciduria (MMA) appears to result in suppression of CoQ10 via indirect inhibition of the respiratory chain and tricarboxylic acid cycle by propionyl-CoA (Haas *et al.*, 2009). Of note, trials with lovastatin aimed at reducing the mevalonate levels induced severe crisis in MVA patients possibly indicating a shortage of downstream isoprenoids is the major pathological factor and not mevalonate level *per se* (Houten *et al.*, 2003).

1.2.3.2 The Role of Coenzyme Q10 in Health

The diverse functions of CoQ10 and its ubiquitous presence constitute the basis for its clinical applications. Oxidative stress has been implicated in many fields of study, including cardiovascular disease, neurodegeneration disorders and diabetes, and experiments suggest CoQ10 may play a protective role in the prevention of cellular damage as deficiency is associated with increased disease severity.

Numerous clinical trials have also investigated the potential of CoQ10 as an adjuvant therapy in both the prevention and treatment of a diverse range of disease states from cancer to ageing. Table 1.3 identifies the possible roles for CoQ10 summarised from a Google Scholar literature review using the search terms “coenzyme Q10” and “human health”. Although benefits of oral CoQ10 have been ascribed, with maximum benefit coinciding with highest dose, correction of the biochemical deficits does not always relate to improved clinical outcome. For instance, exogenous CoQ10 can ameliorate the high lactate and decreased complex I activity in patients with Huntington’s disease but fails to show significant improvement in functional capacity, although long term treatment does appear to attenuate progression (Boitier *et al.*, 1998). Current data on the benefit of CoQ10 is inconclusive and often conflicting but the relative safety of oral CoQ10 appears to be mutually agreed. Further clinical trials are warranted to confirm the effectiveness of CoQ10 in clinical applications.

Condition	Significance	CoQ10 therapy	Reference
Cardiovascular Disease		Scavenges free radicals and provides protective role during surgery and cardiac reperfusion. Pre-operative doses associated with fewer post-operative complications. Improves survival rates and neurological outcomes.	(Folkers *et al.*, 1985; Langsjoen *et al.*, 1994; Lerman-Sagie *et al.*, 2001; Senes *et al.*, 2008; Ho *et al.*, 2009)
cardiomyopathy arteriosclerosis chronic heart failure ischemic heart disease hypertension ischemic reperfusion injury	Oxidative stress, mitochondrial dysfunction and energy depletion. CoQ deficiency correlates with increased disease severity. Beta-blockers may cause secondary CoQ deficiency.		
Neurodegeneration Disorder	Oxidative damage to DNA and proteins, mitochondrial impairment. CoQ levels positively correlate with neuronal protection.		(Ely *et al.*, 1998; Young *et al.*, 2007a)
Huntington's disease	Decreased complex I activity and increased lactate levels in brain.	Improves biochemistry but no improvement in functional capacity. May attenuate progression.	(Matthews *et al.*, 1998; The Huntington Study Group, 2001)
Parkinson's disease	Decreased complex I activity and CoQ levels leading to ROS generation and neuronal susceptibility to mitochondrial damage.	Improves visual symptoms and decreases rate of decline	(Shults *et al.*, 1997; Beal *et al.*, 1998; Shults *et al.*, 2002; Müller *et al.*, 2003)
Friedrich's ataxia	Deficiency of frataxin protein and depletion of iron-sulphur proteins results in free radical generation.	Stabilization of neurological functions and improves cardiac and skeletal muscle bioenergetics.	(Geromel *et al.*, 2002; Cooper and Schapira, 2007)
Alzheimer's disease	Increased CoQ and dolichyl phosphate and decreased dolichol levels in brain. Up-regulation of FPP and GGPP, essential for prenylation of GTPases, linked to generation of amyloid-beta plaque.	Inhibits formation of amyloid-beta peptide therefore enhanced CoQ levels have been interpreted as an attempt to protect the brain.	(Dhanasekaran and Ren, 2005; Hooff *et al.*, 2010)
Cancer	Low blood CoQ levels reported in several types of malignancy. Immunoglobulin G levels affected by exogenous administration of CoQ.	Suppression of lesion development and tumour regression. Counteracts adverse effects of chemotherapy.	(Lockwood *et al.*, 1994; Folkers *et al.*, 1997; Hodges *et al.*, 1999; Sachdanandam, 2008; Hertz and Lister, 2009; Kim and Park, 2010)
Diabetes	Pancreatic β-cells are particularly susceptible to oxidative damage thus insulin secretion disrupted.	Inconsistent results but may improve long-term glycaemic control.	(Henriksen *et al.*, 1999; Hodgson *et al.*, 2002; Green *et al.*, 2004)
Ageing	CoQ levels decline with age. The 'mitochondrial theory of ageing' advocates ROS from oxidative phosphorylation cause oxidant insults resulting in mitochondrial DNA mutation accumulation and compromised bioenergetics.	Antioxidant property has led to anti-ageing therapies but evidence is conflicting.	(Lönnrot *et al.*, 1998; Quiles *et al.*, 2004; Aguilaniu *et al.*, 2005; Sohal *et al.*, 2006)
Myoclonic dystrophy type 1 (Steinert's disease)	Autosomal dominate multisystemic disease - progressive muscle wasting and weakness with cardiac and neurological involvement.	Number of CTG triplicates is inversely associated with CoQ levels and correlated with disease severity.	(Siciliano *et al.*, 2001)
Reproduction Health			
Pre-eclampsia	Associated with hypertension and oxidative stress. Low CoQ levels detected in plasma	Supplementation may reduce risk in susceptible women.	(Palan *et al.*, 2004; Teran *et al.*, 2009)
Asthenospermia	Low CoQ levels detected	Improves motility and sperm count. Improves fertility rate.	(Lewin and Lavon, 1997)
Peridontal disease	CoQ levels decrease in gums with age and disease	Topically applied as prophylactic agent or during treatment may reduce microorganisms and inflammation.	(Nakamura *et al.*, 1974; Hanioka *et al.*, 1994)
Migraine	74.6% paediatric and adolescent patients reported to have low CoQ levels in blood.	Reduces frequency, duration and severity of attacks.	(Sandor *et al.*, 2005; Hershey *et al.*, 2007)

Table 1.3 Possible Roles for Coenzyme Q10 in Health and Disease.

1.3 NON-STEROL ISOPRENOIDS

In addition to the main mevalonate derived end products, over 23,000 isoprenoids have been identified that play key roles in a variety of processes (Edwards and Ericsson, 1999). Also referred to as terpenoids, they consist of elongated chains of the basic building unit isoprene, a 5 carbon atom hydrocarbon. 'Head-to-head' or 'head-to-tail' additions of sequential units and deviations in the functional groups give rise to the thousands of complex isoprenoids. In most organisms, isoprenoids are synthesized via the mevalonic pathway as described above. Plants and certain eubacteria can also produce the terpenoid precursors via an alternative, non-mevalonate pathway known as the deoxy-xylulose phosphate (DOXP) pathway (Eisenreich *et al.*, 2004).

The biphosphate derivatives of isopentenyl, geranyl, farnesyl and geranylgeranyl as well as farnesol and geranylgeraniol, have proved to be essential bioactive molecules for transcriptional and post-transcriptional regulation of genes, including controlling the degradation of HMG-CoA reductase (Holstein and Hohl, 2004). Induction of apoptosis by activation of caspase-3 has been attributed to the isoprenoid alcohols, and the biphosphates have been suggested to play a role in the immune system via T cell recognition (Edwards and Ericsson, 1999).

The post-translational modification of proteins by addition of farnesyl or geranylgeranyl moieties plays a key role in membrane attachment and protein-protein interactions. Over 300 such prenylated proteins are known and have key roles in signal transduction for cell growth and differentiation. Isoprenylation of small GTPases, such as Ras, Rab and Rho, anchor the protein and prevent solubilisation and transfer to cell membranes, and farnesylated Ras proteins have implications for tumour progression and maintenance (McTaggart, 2006). Inhibition of isoprenylation, as a consequence of depleted mevalonic acid via HMG-CoA reductase inhibitors, or reduced FPP via nitrogen-containing bisphosphonate inhibition of FPP synthase (van Beek *et al.*, 1999), also upregulates production of Ras-related proteins. The loss of activity of prenylated GTPases has been linked to reduced bone resorption in a variety of bone diseases following bisphosphonate therapy (Holstein and Hohl, 2004).

1.4 STEROLS

1.4.1 Biosynthesis

Cholesterol, and its biosynthetic precursors, represent a major group of compounds that have their origins embedded in the mevalonate pathway. The chemical structure of cholesterol consisting of four cycloalkane rings with a hydroxyl group at position-3, was elucidated in the early 1930s and followed by a remarkable series of investigations using labelled isotopes which enabled the prediction that the sole building blocks for cholesterol were a two-carbon metabolite of acetate (Bloch, 1965). The standard nomenclature used to identify sterol related compounds is shown in Figure 1.9.

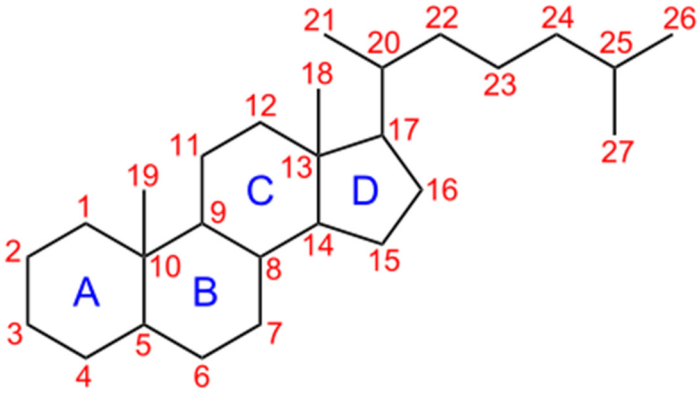


Figure 1.9 Standard nomenclature for a steroid skeleton.
Structure shown is cholestane, a saturated 27-carbon steroid precursor. Adapted from (Moss, 1989)

As mentioned previously, the early steps in the biosynthesis of cholesterol encompass the mevalonate pathway. The enzyme squalene synthase provides the first committed step in cholesterol production resulting in the formation of squalene, a 6-isoprene unit compound containing 30 carbon atoms. Cyclisation of this molecule produces lanosterol, which then undergoes a series of reactions to give the final 27 carbon cholesterol molecule (Russell, 1992). The ultimate precursor for cholesterol can be either desmosterol or 7-dehydrocholesterol and two major routes, known as the Bloch and Kandutsch-Russell pathways, have been proposed depending on when the reduction of the double bond at position C24 occurs (Figure 1.10).

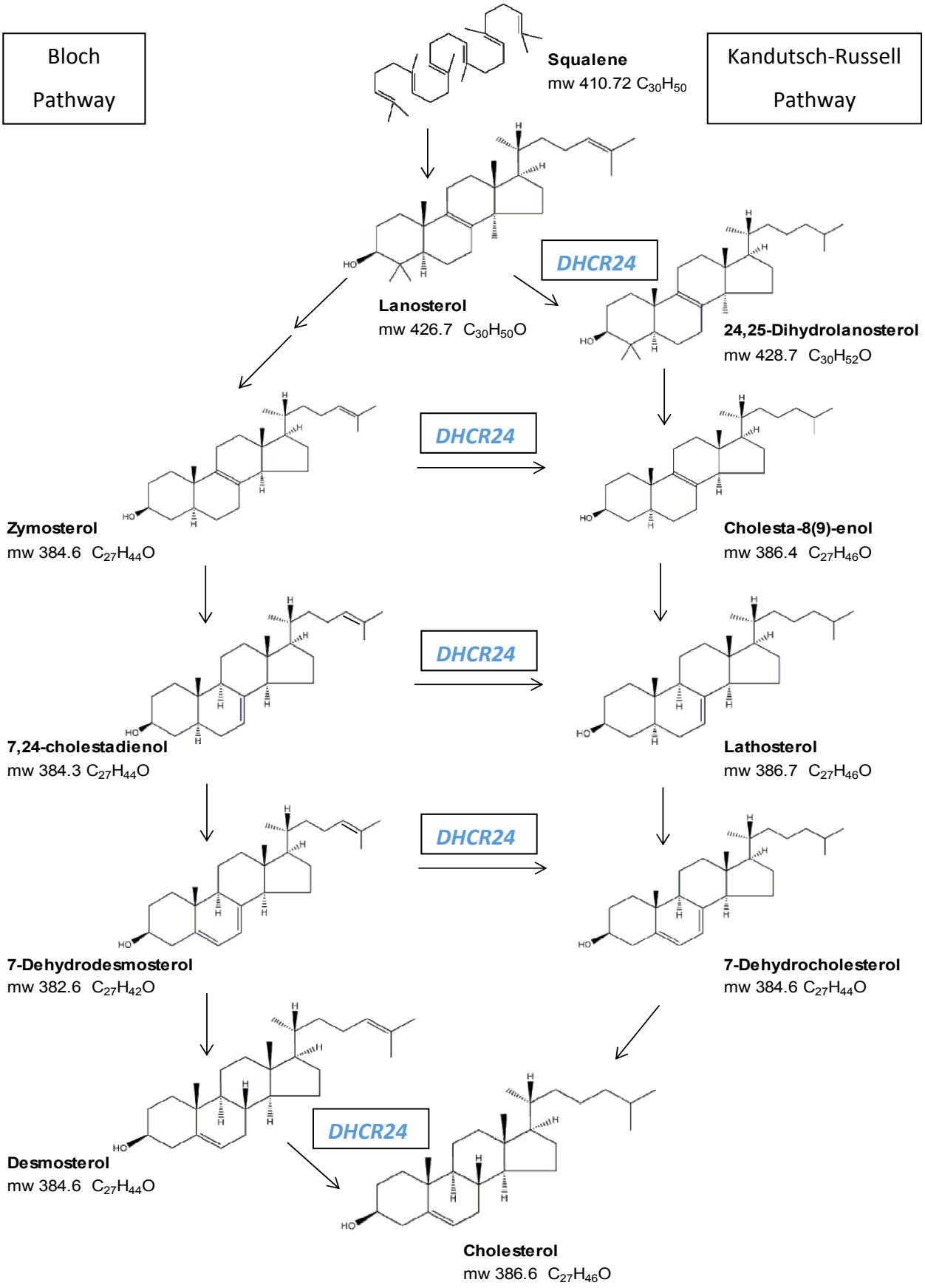


Figure 1.10 Cholesterol biosynthesis.
The two major routes are shown depending on when reduction of the C24 double bond occurs. DHCR24, sterol-Δ^{24}-reductase. All chemical structures are taken from ChemSpider (available at www.chemspider.com)

1.4.2 Homeostasis

Cholesterol is a critical lipid in supporting mammalian life. As well as absorbing from the diet, humans synthesise approximately 700 - 900 mg a day, primarily in the liver, gastrointestinal tract and tissue with high rates of turnover such as skin and bone marrow (Russell, 1992). In addition to being the precursor molecule for bile acids, oxysterols and steroid hormones, it is also required to maintain membrane fluidity and integrity, supports receptor-mediated signalling activities and movement of molecules through membrane compartments, and is involved in myelination (Calandra *et al.*, 2011). Embryonic development is also dependant on cholesterol via interaction with the Hedgehog protein (Stevenson and Brown, 2009).

However, excess levels of cholesterol are harmful so biosynthesis of this vital molecule is under tight regulation via multivalent feedback mechanisms. HMG-CoA reductase is controlled at the level of transcription, translation and degradation. It is suppressed by negative feedback from cholesterol derived from low density lipoproteins (LDL) and very low density lipoproteins (VLDL) but other steroid compounds, in particular 7-ketocholesterol, have also been found to be potent inhibitors of this enzyme (Brown and Goldstein, 1974; Brown and Goldstein, 1980).

Liver X receptors (LXR), activated by ligation with various oxysterols, control transcription of SREBP and genes involved in the export of cholesterol from the cell. High affinity binding appears to depend on the position of the hydroxyl group in the cholesterol side chain and 22-, 24- and 27-hydroxycholesterol are considered endogenous LXR ligands (Yang *et al.*, 2006; Bensinger *et al.*, 2008; Bensinger and Tontonoz, 2008). 24(S),25-epoxycholesterol, derived from a parallel shunt pathway during early cholesterol biosynthesis rather than from cholesterol itself has been shown to influence cholesterol homeostasis at multiple levels. This compound binds to both Insig and LXRs influencing the transcription of cholesterol-related genes, and also accelerates HMG-CoA degradation via the proteasome system (Brown and Jessup, 2009). Recently, a novel mode for cholesterol control by this oxysterols suggested it interfered with DHCR24, resulting in accumulation of desmosterol at the expense of cholesterol (Zerenturk *et al.*, 2012).

1.4.3 Clinical Importance

Cholesterol, specifically LDL cholesterol, plays a well-recognised role in cardiovascular disease via promotion of atherosclerotic plaques leading to myocardial infarction (García-Otín *et al.*, 2007). Since the elucidation of the pathway, several inherited disorders leading to increased intermediates of cholesterol biosynthesis have been recognised (Table 1.4) (Hoffmann *et al.*, 1986; Kelley, 1995; Kelley and Herman, 2001; Waterham *et al.*, 2001; Pappu *et al.*, 2006; Waterham, 2006). These disorders cause a variety of morphogenic and congenital abnormalities affecting the skeleton, skin and internal organs and underline the importance of cholesterol and the sterol intermediates in a variety of cellular processes. It has also been suggested that elevated levels of lathosterol and lanosterol, indicative of high rates of cholesterol biosynthesis, have a causal relationship with cognitive performance in an aging population (Teunissen *et al.*, 2003). It has also been suggested that elevated levels of lathosterol and lanosterol, indicative of high rates of cholesterol biosynthesis, have a causal relationship with cognitive performance in an aging population (Teunissen *et al.*, 2003).

Disorders involving non-cholesterol sterols, such as phytosterols, and bile acid biosynthesis also result in excess accumulation of steroid molecules but without a noticeable effect on cholesterol levels. Sitosterolaemia, a rare autosomal recessive disease, has impaired functioning of pathways involved in absorption and retention of plant sterols, namely sitosterol and campesterol, which are closely related in structure to cholesterol. Clinical features resemble hypercholesterolaemia with the presence of tendon xanthomas and premature atherosclerosis (Hidaka *et al.*, 1990; Kidambi and Patel, 2008). Cholestanol, the saturated analogue to cholesterol, is produced by the conversion of a bile acid intermediate under control of a cytochrome P-450 species, sterol 27-hydroxylase (CYP27A1) (Rystedt *et al.*, 2002). Deficient activity of this enzyme causes accumulation and excretion of 25-hydroxylated bile acids as well as cholestanol in the urine and plasma. The result is the production of tendinous xanthomas, progressive cerebellar ataxia and mental retardation which are the hallmark of cerebrotendinous xanthomatosis (CTX).

Disorder	Inheritance	Enzyme deficient	Gene	Metabolite	Cholesterol	Phenotype
Mevalonic kinase deficiency -Mevalonic aciduria (MIM 251170) -HIDS[a] (MIM 260920)	Autosomal recessive	Mevalonate kinase	*MVK*	Mevalonic acid	low normal	Recurrent episodes of fever, abdominal pain, hepatosplenomegaly, ataxia, mental retardation.
Smith-Lemli-Opitz syndrome (SLOS) (MIM 270400)	Autosomal recessive	3β-hydroxysterol Δ^7 reductase	*DHCR7*	7-dehydrocholesterol and 8-dehydrocholesterol	low	distinctive SLOS face (microcephaly, ptosis, short nasal root), 2/3 toe syndactyly, hypogenitalism, internal organ malformations
Desmosterolosis (MIM 602398)	Autosomal recessive	3β-hydroxysterol Δ^{24} reductase	*DHCR24*	Desmosterol	low	similar to SLOS
Lathosterolosis (MIM 607330)	Autosomal recessive	3β-hydroxysterol Δ^5 desaturase	*SC5D*	Lathosterol	low	similar to SLOS
Greenberg skeletal dysplasia (MIM 215140)	Autosomal recessive	3β-hydroxysterol Δ^{14} reductase	*LBR*	cholesta-8,14-dienol		Fetal hydrops, short limbs, 'moth-eaten' appearance of bones. Early in utero lethality
Conradi-Hunermann-Happle syndrome (CDPX2) (MIM 302960)	X-linked	sterol D8-D7 isomerase	*EBP*	cholesta-8(9)-enol	normal	Bilateral and asymmetric skeletal and skin abnormalities
CHILD syndrome (MIM 308050)	X-linked	sterol C4 demethylase	*NSDHL*	4-methyl sterols		Unilateral (right side) skeletal and skin abnormalities

[a]**HIDS, Hyperimmunoglobulinemia D and periodic fever syndrome**

Table 1.4 Inherited disorders of cholesterol biosynthesis.
The table identifies the diagnostic analyte(s) and cholesterol levels associated with cholesterol biosynthesis disorders. Data taken from (Waterham, 2006)

1.5 HYPERLIPIDAEMIA

Concern and preoccupation with obesity play a major influence on dietary habits but nonetheless, dyslipidaemia, in particular hyperlipidaemia, are still highly prevalent in modern society. Abnormally high levels of lipids in the blood are directly associated with an increased incidence of atherosclerosis and cardiovascular disease (CVD) and levels of cholesterol and triglycerides can be used as the major predictors of cardiovascular events (Gylling *et al.*, 2014). To enable lipids to be transported in the aqueous medium of the blood, they are encased in protein capsules known as lipoproteins. Four main classes of lipoproteins exist based on the protein to lipid content which determines size and density. Chylomicrons and VLDL contain the greater proportion of triglycerides whereas LDL, which are generally regarded as the 'bad' lipoproteins, are rich in cholesterol. High density lipoproteins (HDL) are responsible for the transfer of cholesterol from peripheral tissue back to the liver for elimination in bile (Jairam *et al.*, 2012).

Hyperlipidaemias may be caused by genetic abnormalities or acquired secondary to an underlying cause such as diabetes or a high fat diet. As atherosclerosis is a progressive disease, any interventions aimed at lowering LDL-cholesterol levels have a positive impact on reducing the risk of CVD (Besseling *et al.*, 2015). Similarly low levels of HDL cholesterol have been inversely related to disease risk (Rosenson *et al.*, 2016). Thus monitoring levels of cholesterol in both LDL and HDL is particularly beneficial in assessment and treatment to reduce mortality and morbidity of CVD.

The benefit of the diet should not be over looked in the management of hyperlipidaemia (Gylling *et al.*, 2014). However, this is not always sufficient to control cholesterol concentrations, particularly in the primary lipidaemias, so pharmacological intervention is often required.

Lipid lowering treatment may involve the use of several classes of drugs. Although rarely used as a monotherapy, fibrates act by increasing lipid metabolism and are predominately used to decrease triglyceride levels (Hodel, 2002). Ezetimibe reduces the absorption of cholesterol in the small intestine and can reduce LDL-C by 15 – 20 %. A similar reduction is also seen with the use of bile acid sequestrants (JBS3, 2014). A novel therapy under investigation involves injections of antibodies to inhibit the action of proprotein convertase subtilisin/kexin type 9

(PCSK9). This protein normally binds to the LDL receptor and targets it for degradation, thus its inhibition leads to increased LDL uptake from the circulation. Clinical trials have seen significant reductions in LDL-C of 50 - 60 % without noteworthy injection site reactions or adverse effects (Stroes *et al.*, 2015; Fitzgerald *et al.*, 2017). By far the most commonly prescribed lipid lowering medications are statins. Natural statins include simvastatin, pravastatin and lovastatin, while atorvastatin, fluvastatin, rosuvstatin and cerivastatin are synthetic compounds (Schachter, 2005). The benefit of statins in lowering both LDL cholesterol and cardiovascular risk is well established, but adverse effects are noted in a minority of patients which may lead to some to stop taking the medication.

1.6 STATIN-ASSOCIATED MUSCLE SYMPTOMS

1.6.1 Statins

HMG-CoA reductase inhibitors, also known as statins, are a class of lipid-altering agents which act by reducing de novo cholesterol synthesis. As a consequent, LDL receptor expression is induced with increased extraction of LDL-cholesterol from the blood (Schachter, 2005). Lovastatin was the first to be introduced in 1987 as a treatment for hypercholesterolaemia (Havel *et al.*, 1987; Hoeg and Brewer, 1987). Primary concerns with the use of the drugs focused on liver toxicity and cataract development, but adverse effects of muscle pathology soon emerged and statin-associated muscle symptoms (SAMS) remain the primary reason for non-adherence or discontinuation of statin therapy. A review of other effects of statins, besides their cholesterol lowering ability, suggests these most likely relate to inhibited isoprenoid synthesis and include inflammation, increased nitric oxide biosynthesis and immunosuppression (Massy and Guijarro, 2001).

Several statins are in clinical use with excellent safety profiles and are generally well tolerated (Black, 2002). Cerivastatin was withdrawn from the market in August 2001 owing to a high rate (>3 per 1 million) of fatal rhabdomyolysis (Seehusen *et al.*, 2006). Bioavailability varies between statins (5 - 60 %) (Schachter, 2005) and they effectively inhibit HMG-CoA reductase by 45 - 95 % (Mas and Mori, 2010). Although adverse effects have been noted, beneficial outcome, estimated at 25 - 34 % reduction of cardiovascular disease (CVD) outweigh the associated risk (Sewright *et al.*, 2007), (Joy and Hegele, 2009).

As it shares part of the same biosynthetic pathway as cholesterol, an acquired CoQ10 deficiency with statin use would not be unexpected. Patients prescribed statins often describe a feeling of lack of energy which may be ameliorated with CoQ10 supplements. As ATP production necessitates the use of CoQ10 it is conceivable they literally do lack energy (Reidenberg, 2005).

1.6.2 Statin Related Myopathies

The varied criteria used to define myopathy (Table 1.5) often make interpretation of clinical trials complex (Pasternak *et al.*, 2002; McKenney *et al.*, 2006). Myopathy can be generalized to infer all muscle disease but the National Lipid Association (NLA) and U.S Food and Drug Administration (FDA) confines the term to established creatine kinase (CK) levels above 10 times the upper limit of normal (ULN) (Joy and Hegele, 2009). Statin-associated myopathy can range from mild muscle pain to frank rhabdomyolysis. Muscle biopsies often show characteristic ragged red fibres, suggesting increased mitochondrial biogenesis, although muscle pathology is not restricted to overt myopathy (Harper and Jacobson, 2010). Biopsy analysis from 83 patients demonstrated evidence of muscle damage, evaluated by the degree of structural abnormailities in the muscle fibres including vacuolization and subsarcolemmal detatchment of myofibrils, in 25/44 patients with statin-associated myopathy and 1/19 patients prescribed statin but without symptoms. No damage was observed in the control group and CK was only elevated (x10 ULN) in 1 case. Consequently normal or slightly raised CK does not rule out muscle injury (Mohaupt *et al.*, 2009).

	ACC/AHA/NHLBI(Pasternak *et al.*, 2002)	FDA(Sewright *et al.*, 2007)	NLA(McKenney *et al.*, 2006)
Myopathy	Any disease of the muscle, which may be acquired or inherited	Creatine kinase ≥10 times the upper limit of normal	Muscle pain, weakness, soreness or cramps with creatine kinase >10 times the upper limit of normal
Myalgia	Muscle ache or weakness without increases in creatine kinase levels		
Myositis	Muscle symptoms with creatine kinase elevations		
Rhabdomyolysis	Muscle symptoms with significant elevations of creatine kinase (>10 times the upper limit of normal). As a result of muscle destruction, myoglobinuria is usually present	Creatine kinase >50 times the upper limit of normal with evidence of organ damage	Creatine kinase >10,000 IU/L or >10 times the upper limit of normal, plus elevated serum creatinine

ACC/AHA/NHLBI = American College of Cardiology/American Heart Association/National Heart, Lung, and Blood Institute; FDA = U.S. Food and Drug Administration; NLA = National Lipid Association.

Table 1.5 Proposed Definitions for Statin-associated Muscular Adverse Effects

Predisposing factors for risk of statin-associated myopathy are extensively reviewed by Chatzizisis and colleagues and summarised in Table 1.6 (Chatzizisis *et al.*, 2010). Patient demographics, family history, age, sex, low body weight, major surgery and co-morbidities e.g. McArdles disease, carnitine palmitoyltransferase deficiency type II, impaired renal and liver function, all contribute to the risk assessment (Black, 2002; Sewright *et al.*, 2007; Harper and Jacobson, 2010). Excess consumption of grapefruit juice, an inhibitor of the intestinal cytochrome P-450 3A4 system (Kane and Lipsky, 2000) is also contraindicated during statin use. The 2005 prediction of muscular risk in observational conditions (PRIMO) study (Bruckert *et al.*, 2005) highlights dose and pharmacokinetic properties of statins as major contributing factors. Time to onset of symptoms and duration post cessation of therapy varies between reports but a common trigger appears to be physical exertion (Sewright *et al.*, 2007). Drug interactions can interfere with clearance of the statin thereby increasing plasma

concentrations and toxicity. Simvastatin, lovastatin and atorvastin are metabolized by cytochrome CYP3A4 and competing drugs on this metabolic path include blood pressure medication (verapamil), antiarrthyhmic medication (amiodarone) and the immunosuppressant cyclosporine (Joy and Hegele, 2009; Mas and Mori, 2010). Concomitant administration of fibrates, which influence triglyceride levels by reducing the livers production of VLDL, has also shown to increase risk so the choice of statin needs careful consideration and should be started and maintained at the lowest effective dose (Seehusen *et al.*, 2006). Algorithms for defining statin-associated muscle symptoms based on clinical and biochemical phenotypes have been developed to aid clinicians (Alfirevic *et al.*, 2014). A 2015 European Atherosclerosis Society Consensus Panel recommends patients exhibiting continued muscle symptoms when re-challenged with at least three different statins should be considered for alternative therapies to achieve the desired LDL cholesterol targets (Stroes *et al.*, 2015).

Endogenous risk	Exogenous risk
Family history	Alcohol consumption
Age (> 75 years)	Vigorous exercise
Sex (female)	Pharmacokinetics
Low body mass index	Drug interactions
Co-morbidities (CPTII deficiency, diabetes, hypothyroidism, renal or liver dysfunction)	Excess consumption of grapefruit juice (inhibits cyt P450)
Genetic polymorphisms of CYP 450 isoenzymes	Major surgery with severe metabolic demand

Table 1.6 Risk factors for statin myopathy (Chatzizisis *et al.*, 2010)

1.6.3 Pathogenesis

The aetiology of statin-associated myopathy remains poorly understood (Figure 1.11). Possible mechanisms include decreased membrane cholesterol resulting in disturbed membrane fluidity and ionic channel function (Mas and Mori, 2010). Evidence is not supported however, as inherited cholesterol synthesis disorders have low cholesterol levels but no myopathy and non-statin lipid lowering agents (fibrates) can also induce myopathy (Joy and Hegele, 2009). CoQ10 deficiency has gained much interest, particularly in relation to the

influence on the mitochondrial respiratory chain, but muscle level assessment is variable (Littarru and Langsjoen, 2007; Larsen *et al.*, 2013) and limited data exists in patients with myopathy (Rosenson, 2004). Statins can inhibit muscle proliferation independently of cholesterol concentrations, implicating decreases in the isoprenoids FPP and GGPP and attenuated protein prenylation of regulatory GTP-binding proteins (Ras, Rac and Rho), needed to promote cell growth and inhibit apoptosis, in pathogenesis (Miettinen and Björklund, 2016). Supplementation *in vitro* with FPP and GGPP has been shown to prevent statin-induced apoptosis (Johnson *et al.*, 2004). Another cause may be an accumulation of lipids in myocytes as a consequence of a predisposing metabolic abnormality (Rosenson, 2004).

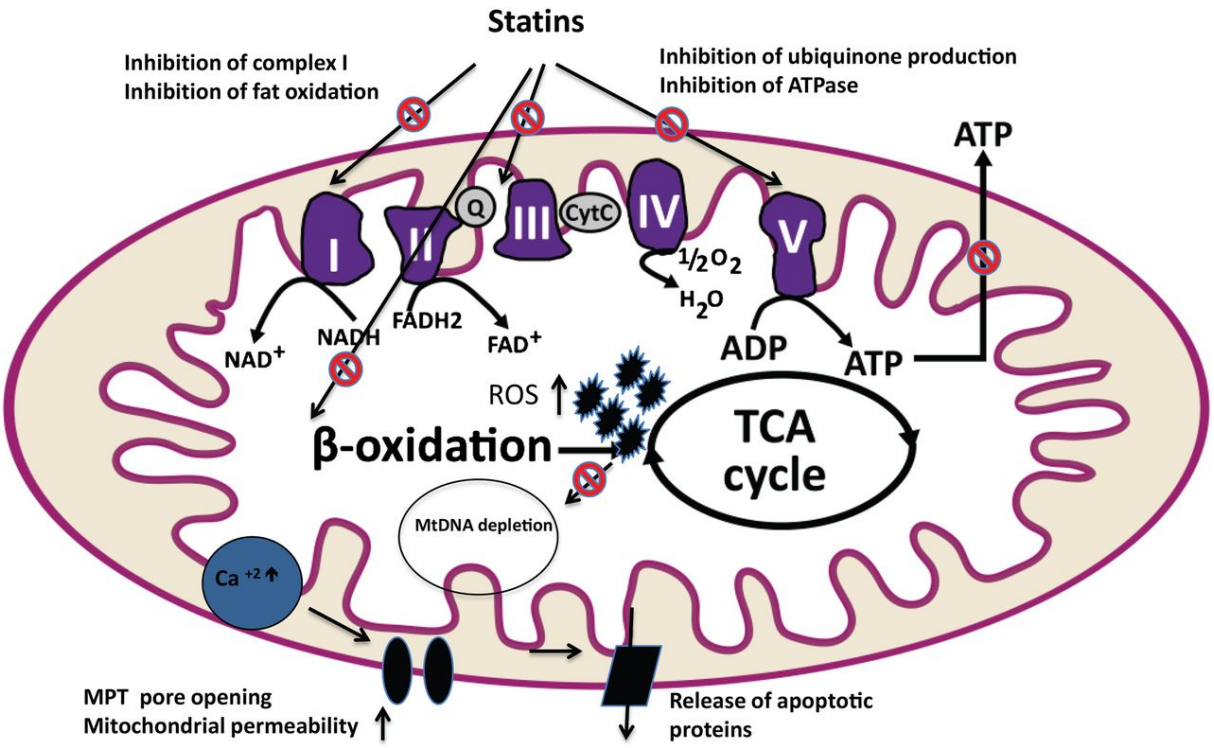


Figure 1.11 Possible targets of statins in the mitochondrion with deleterious effects on muscle function. Statin interaction may involve (i) a reduction in coenzyme Q with participates in the mitochondrial electron transport chain (ETC) (ii) impaired cell growth and autophagy due to subnormal levels of farnesyl pyrophosphate and geranylgeranyl pyrophosphate (iii) low membrane cholesterol content affecting membrane fluidity and ion channels, and (iv) impaired calcium signalling due to attenuated mitochondrial permeability. ADP, adenosine diphosphate; ATP, adenosine triphosphate; Cyt C, cytochrome *c*; FAD, flavin adenine dinucleotide; FADH2, flavin adenine dinucleotide reduced; MPT, mitochondrial permeability transition; MtDNA, mitochondrial DNA; NAD, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide reduced form; ROS, reactive oxidative species; TCA cycle, tricarboxylic acid cycle. Image taken from (Stroes *et al.*, 2015)

Recently, genetic factors have emerged. Polymorphisms in the CoQ10 biosynthetic gene *COQ2*, responsible for severe inherited myopathy, have been related to variation in statin tolerance (Oh *et al.*, 2007). A genome-wide association study by the SEARCH Collaborative group (2008) identified a nucleotide polymorphism in the *SLCO1B1* gene located on chromosome 12, to be strongly associated with increased risk of statin-induced myopathy. This gene encodes a peptide responsible for hepatic uptake and whilst this trial was restricted to those taking simvastatin, it can likely be applied to other statins.

The pathogenesis of statin-associated myopathy is not well understood and much debated in the literature. Complications are dose related with predisposing factors and should be carefully addressed by the clinician. For most patients the risk to benefit ratio favours therapy with statins (Newman and Tobert, 2015).

1.6.4 Clinical assessment

Randomised control trials suggest incidence rates for statin myopathy of up to 5 % (Seehusen *et al.*, 2006) but these values may not reflect the true rate in the clinical setting. Observational studies indicate 10 - 15 % of people prescribed statins exhibit muscle complaints (Mohaupt *et al.*, 2009; Harper and Jacobson, 2010). Myalgia is the most common with serious myopathy with CK elevations affecting 1 per 10,000 patient-years on standard dose (2008). Cases of rhabdomyolysis are rare and the mortality rate is estimated at 1.5 deaths per 10 million prescriptions (Marcoff and Thompson, 2007). Variation in reported incidence may reflect inclusion criteria as low tolerance patients and the older age group are often excluded in pre-trial assessments (Sewright *et al.*, 2007; Chatzizisis *et al.*, 2010), and motivated patients might not report mild cases of myalgia (Joy and Hegele, 2009).

The number of self-reporting cases of myopathy through the FDA Adverse Event Reporting System (AERS) has increased from 2002 and may reflect raised awareness following the withdrawal of cerivastatin in 2001. Fluvastatin elicited fewer symptoms but rosuvastatin, launched after cerivastatin withdrawal, consistently scored the highest rate for myopathy, myositis and rhabdomyolysis and could suggest reporting bias (Bruckert *et al.*, 2005). Other adverse effects pertaining to use of statins including rash, dizziness, headaches and gastrointestinal complaints prompt 1 - 5 % of clinical trial patients to discontinue use (Oh *et al.*, 2007).

A recent patient-targeted post-marketing adverse-effect surveillance study (Cham *et al.*, 2010) underscored the negative association between quality of life and muscle related problems. Concordant with other published evidence the survey also highlighted the correlation with drug dose. 100 % of participants re-challenged with high dose statin experienced adverse effect compared with 72 % taking low dose. This suggests amelioration of symptoms and management of statin-associated muscle symptoms may be achieved using lower potency statins.

1.6.5 Coenzyme Q10 and Statins

As an essential component of the electron transport chain, CoQ10 is fundamental to mitochondrial energy (ATP) generation and accordingly to regulating muscle contractility. It is feasible a decrease in CoQ10 can compromise ATP bioavailability. The branched point enzymes of the mevalonate pathway have different affinities for FPP. Cholesterol biosynthesis is more attenuated than non-sterol isoprenoids by slight reductions in HMG-CoA reductase as squalene synthase has a low affinity (Hargreaves *et al.*, 2005; Littarru and Langsjoen, 2007). Conversely, low CoQ10 levels, often accompanied with increased lactate/pyruvate ratios as an indication of mitochondrial respiratory chain dysfunction have been reported with statin use (Caso *et al.*, 2007; Littarru and Langsjoen, 2007).

The first report of low CoQ10 levels with statin was made by Folkers in 1990 (Folkers *et al.*, 1990) . Ghirlanda and colleagues performed the first study in 1993 and demonstrated 30 % and 54 % decrease in plasma CoQ10 in statin treated normal and hypercholesterolaemic patients respectively (Ghirlanda *et al.*, 1993). Numerous animal studies using rodents, dogs, mini pigs and squirrel monkeys have been performed since 1990 examining CoQ in blood and tissue following statin treatment and uniformly present varying degrees of CoQ depletion that may be species and dose related. Decreased CoQ levels observed in heart muscle in older animals but not in younger animals suggests older populations may be more at risk of statin related adverse effects (Littarru and Langsjoen, 2007).

Human studies evaluating statins consistently report low serum/plasma CoQ10, especially at high doses, with reductions of 15 - 54 % reported (Marcoff and Thompson, 2007). Effects however may be nullified by normalizing to cholesterol (Mortensen *et al.*, 1997; Berthold *et al.*, 2006; Mortensen, 2011). Altekin and colleagues (Altekin *et al.*, 2002) reported 17 patients

prescribed simvastatin in who CoQ10 decreased by 30 % after 8 weeks therapy. Increased CK and myoglobin levels and decreased total antioxidant capacity were also noted, though CoQ10 levels were still higher than in the control group and no change in ratio to total or LDL-cholesterol was observed. Contrary to this, two groups with follow-up times of 4 and 6 months statin treatment, indicated CoQ10 concentration was not fully dependent on lipoprotein changes with significantly increased CoQ10/LDL-C ratio (Colquhoun *et al.*, 2005; Pacanowski *et al.*, 2008). Whilst Colquhoun also reported an analogous pattern for plasma vitamin E measurements, a further study failed to duplicate these findings in plasma but did observe a significant decline of vitamin E in lymphocytes, suggesting possible oxidative stress caused by decreased CoQ10 in blood may be insignificant, as levels of other antioxidants remained unchanged with statin challenge (Passi *et al.*, 2003). A randomized trial of ezetimibe, which prevents cholesterol absorption, and simvastatin, administered as monotherapy or in combination, revealed plasma CoQ10 concentrations were not affected by ezetimibe alone and increased CoQ10/LDL-C ratios were observed in all three groups. The authors concluded "changes of CoQ10 levels are independent of cholesterol synthesis and absorption" (Berthold *et al.*, 2006). Of note, a study led by Bleske, indicated no significant change in plasma CoQ10 levels in 12 healthy volunteers treated for 4 weeks with either pravastatin or atorvastatin despite significant reduction in LDL-C (Bleske *et al.*, 2001). Other studies employing longer term therapy with these statins differ in this account (Hargreaves *et al.*, 2005).

Coenzyme Q10 is a known protective agent against LDL oxidation, but conflicting data pertaining to antioxidant composition (CoQ10 and vitamin E), and susceptibility of LDL to oxidation means the effect of statins on LDL oxidation remains debatable (Colquhoun *et al.*, 2005). Baseline plasma CoQ10 concentrations have been assessed as possible predictive values for lipid lowering response to statin treatment and suggest higher baseline CoQ10/LDL-C ratios correlate to diminished LDL-C lowering effects (Pacanowski *et al.*, 2008).

Data on intramuscular CoQ10 levels is limited and variable, and may be drug and dose dependent. An early study of short term simvastatin therapy in hypercholesterolaemic males observed decreased plasma CoQ10 but a concomitant enhancement (47 %) in muscle tissue levels (Laaksonen *et al.*, 1995), supporting the view that non-sterol isoprenoid synthesis is preserved during HMG-CoA reductase suppression. No conclusion as to the effect on muscle

myopathy can be drawn as all subjects remained asymptomatic. A 2005 follow-up study performed by the same group using high dose statin showed a 30 % decrease in muscle CoQ10 with simvastatin but this was not mirrored by atorvastatin (Littarru and Langsjoen, 2007; Mas and Mori, 2010).

Muscle biopsies from 18 patients with symptomatic statin-associated muscle problems collectively did not significantly differ in CoQ10 content from controls. Nonetheless, 3 patients were more than 2 standard deviations (SD) below normal and 7 were more than 1 SD. Whilst not supporting a pathogenic role for CoQ10 deficiency in myopathy it may be suggestive of an association in some patients (Lamperti *et al.*, 2005). Duncan *et al.* demonstrated low muscle CoQ10 and complex IV activity in 2 patients with statin-associated myopathy. These results were subsequently confirmed by analysis of primary astrocytes without the inference from other drugs and were not correlated to loss of mitochondrial volume. Interestingly, activity of complex II-III was not compromised, suggesting a threshold level for CoQ10 and possible detrimental structural effect on complex IV due to the requirement of a farnesyl molecule to anchor the haem group to the enzyme complex (Duncan *et al.*, 2009b).

Evidence of the causative role of decreased CoQ10 in statin-related myopathy is debated, though it may well be a predisposing factor. As such, patients with pre-existing conditions associated with low CoQ10 levels, such as cancer, increased age, diabetes and mitochondrial dysfunction should be carefully monitored for adverse effects and may benefit from concomitant CoQ10 supplementation (Vaklavas *et al.*, 2009).

1.6.6 Coenzyme Q Therapy and Statins

CoQ10 supplementation up to 600 mg/day is recognized to be safe and free of side effects (Mas and Mori, 2010), however clinical trials have so far proved equivocal with respect to the amelioration of myopathy by the concurrent administration of CoQ10 (Schaars and Stalenhoef, 2008). Furthermore, many trials document a significant increase in circulating levels with oral CoQ10, but the correlation to intramuscular CoQ10 and myopathy remains debatable (Harper and Jacobson, 2010). Bargossi and colleagues confirmed CoQ10 reduction by simvastatin in both plasma and platelets and concomitantly established adjunct therapy with 100 mg/day exogenous CoQ10 counteracted these effects without any effect on the lipid

lowering activities of the statin. No side effects were reported in these hypercholesterolemia patients (Bargossi *et al.*, 1994).

56 patients prescribed lovastatin in cyclical intervals for possible antitumor activity showed a transient parallel decrease in CoQ10 by up to 49 %. Supplemental CoQ10 decreased the severity but not frequency of myopathic toxicity (Thibault *et al.*, 1996). A further study of gastric adenocarcinoma revealed CoQ10 treatment reversed mild myalgia and elevated CK induced by high dose lovastatin and also concluded prophylactic CoQ10 prevented development of myotoxicity (Young *et al.*, 2007b).

Moreover, 16 out of 18 participants in a double-blind randomized trial reported improvement in statin-induced myopathic pain and quality of life when given CoQ10 supplements in addition to their normal medications. In contrast, only 3 out of 14 patients showed improvement with vitamin E supplements (Caso *et al.*, 2007). Conversely, 44 patients with previous statin-related myalgia randomized to receive either CoQ10 (200 mg/day) or placebo showed no difference in tolerance to the statin or severity of symptoms despite a rise in plasma CoQ10 in the treated group (Young *et al.*, 2007b).

Although a few cases have reported resolution of statin-induced muscle symptoms with CoQ10 supplements, results remain inconclusive and contradictory. Marcoff and Thompson (Marcoff and Thompson, 2007) following a systematic review conclude “there is insufficient evidence to prove the etiologic role of CoQ10 deficiency in statin-associated myopathy…..the routine use of CoQ10 cannot be recommended in statin-treated patients”. This sentiment was supported by the EAS Consensus Panel (Stroes *et al.*, 2015). They do concede the lack of detrimental risk associated with CoQ10 and the conceived benefits may make supplementation an option for some patients.

1.7 HYPOTHESIS

The statins, inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), are the mainstay of the treatment of hypercholesterolaemia, atherosclerosis and coronary artery disease. However, some patients develop myopathy which may be related to pre-disposing factors such as high cellular statin uptake or effects on other, non-sterol, isoprenoids (ubiquinone, prenylation and geranylgeranlyation of proteins, and dolichols). Mitochondrial dysfunction has been associated with statin-related myopathy and it is possible that this arises secondary to inhibition of ubiquinone biosythesis or pre-existing impaired mitochondrial function. My hypothesis is that modulated isoprenoid metabolism, whether due to treatment with statins or to inherited defects, is involved in the aetiology of myopathy.

1.8 AIMS

This project aimed to investigate the role of mevalonate derived CoQ10 and sterol intermediates in statin-related myopathy. Firstly, I needed to develop suitable analytical methods to be able to quantitate the analytes of interest. To achieve this, a method for the measurement of CoQ10 in tissue and body fluids using tandem mass spectrometry was developed and optimized. A Gas chromatography mass spectrometry method was developed to investigate a comprehensive range of cholesterol precursor and absorption markers in plasma samples.

The second part of the project focused on the *in vitro* effects of statins on CoQ10 concentrations. Fibroblast cell assays were designed to measure the concentrations of CoQ10 in the presences and absence of a statin. The effect any changes may have on the function of the mitochondria was then briefly addressed As ROS production is closely associated with the electron transport chain I used a mitochondrial superoxide specific fluorescent compound to examine changes in ROS production which are detectable using a flow cytometry technique. Additionally, confocal microscopy was used to visualise cellular changes that may occur with statin treatment of under conditions of specific CoQ10 depletion.

As very little is known regarding the transport of CoQ10 in the plasma, specifically relating to hyperlipidaemic patients, this project also aimed to investigate the distribution of CoQ10 in lipoprotein particles in a cohort of patients with various dyslipidaemias. Lipoproteins were

separated using an ultra-centrifugation method then quantitation of the lipid profile and CoQ10 was performed.

The final part of this project aimed to measure and compare sterol and non-sterol intermediates in a large cohort of samples from patients with evidence of statin-associated muscle symptons, patients treated with statins but with no evidence of myopathy, and control subjects matched for age and gender to investigate if a causal link could be identified.

General Methods

Chapter 2. General Methods

2.1 SPECIMENS

2.1.1 Plasma and dried blood spots

Blood for method development was collected into tubes containing either EDTA or lithium heparin as anticoagulant and plasma separated following centrifugation at 900*g* for 5 minutes. Aliquots were transferred to labelled screw cap microtubes and stored at -80°C.

Dried blood spot samples were prepared by spotting whole blood onto Whatman 903 filter paper in 20 µl aliquots and allowing to air dry for a minimum of 4 hours. Dried blood spot cards were then air sealed in bags and stored at -40°C. All stored cards were allowed to reach room temperature before the seals were broken for analysis to prevent absorption of moisture.

2.1.2 Muscle tissue

Skeletal muscle biopsy samples were provided by Professor Taylor from the Wellcome Centre for Mitochondrial Research, Newcastle upon Tyne. These were anonymised control tissue which were surplus to diagnostic requirement from patients under the care of Professor Turnbull (ethics number 2002/205). These were stored at -80°C until analysis.

2.1.3 Fibroblast cell lines

Skin fibroblast cell lines, donated from healthy volunteers were provided by Newcastle University Biobank facility, MRC Neuromuscular Centre, Institute of Genetic Medicine. Newcastle upon Tyne. These were accessed through the completion of appropriate requests to the Biobank Confluent monolayer cultures were provided then maintained and cultured as described below. Patient anonymity was maintained at all times.

2.2 FIBROBLAST CULTURE

2.2.1 Reagents and Consumables

Minimum Essential Media (MEM, with Earles salt without L-Glutamine), non-essential amino acids, foetal bovine serum (FBS), L-glutamine, MEM vitamins, phosphate buffered saline (PBS), penicillin/streptomycin mix and 0.5 % trypsin-EDTA (x10 strength) were all purchased from Invitrogen (Paisley, UK). Dimethyl sulphoxide (DMSO) was purchased from VWR Int (Leicestershire, UK).

Canted neck closed cap culture flasks (25 cm^2 and 75 cm^2) and internal thread 2 ml cryovials were from Greiner (Stonehouse, UK). Multiple volume stripettes were available from Fisher Scientific (Loughborough, UK), and filtropur 0.2 µm membrane filters and 1 ml pastettes were purchased from Sarstedt (Leicester, UK) and Alpha Labs (Hampshire, UK) respectively. All consumables used were purchased as sterilised packs.

Supplemented media (10 % FBS in MEM), was prepared by combining 500 ml MEM, 50 ml FBS and 5 ml each of glutamine, penicillin/streptomycin, vitamins and non-essential amino acids, then stored at 4°C and used for a maximum of 2 months before being discarded due to degradation of glutamine. To minimise freeze/thaw effects, once the stock bottles were opened, reagents were aliquoted into sterile universal containers and stored at -20°C. Non-essential amino acids were stored at 4°C.

2.2.2 General procedures

Fibroblast cells were maintained and cultured using aseptic techniques with all manipulations performed within a class II microbiological safety cabinet. Cells were incubated in a closed 37°C incubator after prior gassing of individual flasks with 5 % CO_2 in air using a filtered air supply system and disposable filtered 10 ml stripette. Cell growth was monitored using an inverted phase contrast microscope.

Cultures were maintained in supplemented media in either 25 cm^2 or 75 cm^2 closed cap culture flasks and incubated at 37°C. Media was changed every 4 - 5 days, gently gassed with 5 % CO_2 in air for 30 sec and flasks returned to the incubator until cell confluency had reached

approximately 70 %. At this stage cell lines were either sub-cultured, harvested for analysis or cryogenically frozen for future use.

2.2.3 Sub-culture of fibroblast cell lines

Medium was decanted to waste, the cell monolayers washed twice with PBS then passaged using 0.05 % trypsin diluted with PBS. The trypsin was added to the flask, gently swirled to coat the base then excess trypsin decanted to waste. The flask was re-capped and left, lying flat, at room temperature for 3 - 5 min. The base of the flask was then gentle tapped against the heel of the hand to detach the cells and the trypsin quenched by addition of 5 ml fresh supplemented media per new flask required. As a general rule, cells from one confluent 25 cm^2 flask were seeded to one 75 cm^2 flask or three 25 cm^2, and a 75 cm^2 flask was split to either three 75 cm^2 or ten 25 cm^2 flasks. The volume was then adjusted to 8 ml or 20 ml, for 25 cm^2 or 75 cm^2 flasks respectively, with fresh supplemented media. Each flask was gently gassed with 5 % CO_2 in air for 30 sec prior to incubation at 37°C and the cells allowed to settle. After 24 h the medium was changed to fresh supplemented medium then routine culture performed as described previously.

2.2.4 Harvesting of fibroblast cell lines

Cells for analysis were harvested by trypsination as described above. To ensure maximal recovery of cells, following detachment 6 ml supplemented media was added to the flask and the contents decanted into a 15 ml conical tube. A further 3 ml media was added to the flask to wash off any remaining cells and the contents transferred to the tube. This was repeated a further time and all the contents combined. The cell suspension was centrifuged at 700 g for 5 min to form the cell pellet. The media was decanted and the cells washed twice using PBS. The final cell pellet was resuspended in 0.5 ml PBS for storage at -40°C.

2.2.5 Cryopreservation of fibroblast cell lines

Cells to be frozen were stripped from the flask using trypsin as described previously and suspended in 10 ml of supplemented media. The cell suspension was transferred to a sterile 30 ml universal container, centrifuged at 700 g for 10 min and the media carefully discarded. The pellet was re-suspended in 1 ml 20 % FBS in supplemented media containing 10 % (v/v) DMSO, transferred to a labelled cryovial and immediately placed in a Mr. Frosty™ freezing

unit (Nalgene, Thermo Fisher) containing isopropanol. This was placed at -80°C for 24 hours to achieve the recommended controlled freezing rate for biological samples of -1°C/minute. Frozen cyrovials were then transferred to liquid nitrogen for long term storage.

2.2.6 Rejuvenation of frozen fibroblast cell lines

Samples retrieved from liquid nitrogen were thawed by placing the vial in a water bath at 37°C for 5 mins then carefully transferring the contents into a 25 cm^2 flask containing 5 ml warmed supplemented media. The contents were mixed, gassed with 5 % CO_2 in air for 30 sec then placed in an incubator at 37°C and left overnight to allow the cells to settle. The media was changed the following morning then the regular routine for maintaining a culture was followed.

2.2.7 Cell counts

Manual cell counts were performed using FastRead 102 disposable counting slides (Immune Systems Ltd, Devon, UK). Each chamber on the slide contains ten 4 x 4 counting grids with a total capacity of 1 µl. Samples were suspended in 1 ml sterile PBS and introduced into the counting chamber as a drop. Capillary action draws the liquid into the chamber and excess fluid is expelled into the overflow reservoir. Using an inverted phase contrast microscope, cells that lay within the grids were counted and used to determine the concentration (counts/ml). Following centrifugation, the final cell pellet was then suspended in the required volume of phosphate buffered saline.

2.3 FLOW CYTOMETRY

2.3.1 Principle of the method

Approximately 1 - 2 % of mitochondrial oxygen is incompletely reduced and forms the superoxide anion, the predominant reactive oxygen species (ROS) in mitochondria. MitoSOX™ Red is a fluorogenic dye specifically targeted for the detection of superoxide in the mitochondria of live cells (Mukhopadhyay *et al.*, 2007). The red fluorescence produced by the oxidation of the reagent by superoxide can be measured using flow cytometry, a technique that can analyze multiple physical characteristics of single particles as they flow in a fluid stream.

In the fluidics system, the sample travels up the sample tube and hydrodynamic focusing within the flow cell aligns the suspended cells into single file (Figure 2.1). The cells are intercepted by a laser and disrupt and scatter the light proportionally to their size which enables gating of discrete populations within a mixture of cells. The size of cells and their level of complexity, such as presence of granules, can be determined by measuring both forward scatter (FSC) and side scattered (SSC) light respectively. The presence of a fluoroprobe enables measurement of fluorescent signals as they pass the light source.

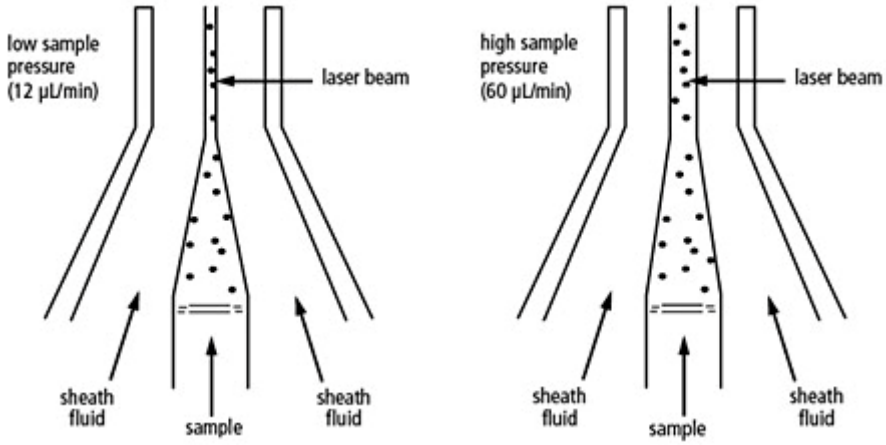


Figure 2.1 Hydrodynamic focusing.
Low flow rates are generally used for optimal resolution and sensitivity. High flow rates acquire data more quickly but is less resolved. (source: FACS Canto user guide, available at www.bdbiosciences.com)

2.3.2 Materials and equipment

MitoSOX™ Red was obtained from Invitrogen (Paisley, UK), prepared in DMSO to a stock concentration of 200 µmol/L and stored in an amber bottle at -20°C. The fluorescent excitation and emission wavelengths for MitoSOX™ Red are λ = 510 nm and 580 nm respectively. All other reagents were as stated in fibroblast culture. Flow cytometry was performed using a FACSCanto II cell analyser with Diva operating software (BD Biosciences, Oxford, UK). Cell counts were performed using FastRead-102 counting chambers purchased from ImmuneSystems Ltd.

2.3.3 Sample preparation

Fibroblast cells were subjected to treatment conditions, and cultured as stated previously. Cells were then harvested by trypsination, washed twice with phosphate buffered saline and re-suspended to a concentration of 1 x 10^6 cells/ml. Aliquots (0.5 ml) were transferred to sterile FACS tubes, spun down and the pellet incubated at 37°C for 15 min in MEM containing 5 µmol/L MitoSOX^{TM} Red. Following a final spin at 700 g for 5 min the cells were re-suspended in 0.5 ml PBS and analysed within one hour by flow cytometry.

2.3.4 Sample acquisition

The flow cytometer is routinely operated by the Immunology section of Blood Sciences based at the Royal Victoria Infirmary, Newcastle Upon Tyne NHS Foundation Trust. Settings are optimised daily using tracking beads and quality control checks are regularly performed.

Specific parameters settings were optimised for fibroblast cells using unstained cells suspended in PBS. Data were acquired on a medium flow rate and voltages adjusted (FSC, SSC, and FL-2 detector) to ensure sufficient data capture based on cell size and fluorescent shift. Cell debris is generally represented by low forward and side scatter so this population was gated out of any analysis.

Approximately 6,000 events (cells) were recorded for each sample and the data presented as histograms of fluorescent intensity.

2.4 PROTEIN MEASUREMENTS

2.4.1 Principle of the method

The assay is based on the turbidimetric method described by Luxton and colleagues (Luxton *et al.*, 1989). The sample is pre-incubated in an alkaline solution which denatures the protein. EDTA is incorporated into the mixture to eliminate interference from magnesium ions. Benzethonium chloride then reacts with the proteins producing turbidity. This reaction is considered more stable and evenly distributed than the turbidity produced using trichloroacetic acid (TCA) or sulphosalicylic acid (SSA).

2.4.2 Reagents and equipment

Protein concentration was determined using a Cobas 8000 Modular Analyser fitted with a c702 unit (Roche Diagnostics, Burgesshill, UK). This module enables spectrophotometric analysis, including colorimetry and turbidimetry, to be performed with a high degree of automation for efficient throughput of high sample volumes. All calculations were performed automatically by the on-board software. All reagents used on the Cobas 8000 were provided as CE marked kits from Roche.

2.4.3 Sample analysis

Protein quantitation was determined using a 2-point calibration standardised against a primary standard traceable to NIST. Quality control is performed at regular intervals and the method is accredited to UKAS ISO 15189 International Standards for Medical Laboratories.

2.4.4 Method performance

The assay is linear between 40 – 2000 mg/L with a detection limit of 40 mg/L as represented by the lowest measureable analyte level that can be distinguished from zero. Precision was determined using control samples and both the within and between batch coefficient of variation (CV) was less than 3 %.

2.5 MEASUREMENT OF LIPID PROFILES

The following methods were all performed using a Cobas 8000 Modular Analyser fitted with a c702 unit. Two-point calibrations were used for all quantitative determinations and each assay uses a CE marked kit and is accredited to UKAS ISO 15189 International Standards for Medical Laboratories.

2.5.1 Cholesterol

2.5.1.1 Principle of the method

Cholesterol was determined using an enzymatic, colorimetric method first described in 1974 (Allain *et al.*, 1974). Cholesterol esters in the sample are hydrolysed by the action of cholesterol esterase. The resultant free cholesterol is then oxidised by cholesterol oxidase to produce cholest-4-en-one and hydrogen peroxide (H_2O_2). In the presence of peroxidase, H_2O_2

oxidatively couples phenol and 4-amino-antipyrine (4AAP) to yield a red chromophore which is measurement using excitation (ex) and emission (em) wavelengths of 505/700 nm. The colour intensity of the dye formed is proportional to the concentration of cholesterol in the sample. The reactions are summarised as follows:

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{RCOOH}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholest-4-en-one} + H_2O_2$$

$$2H_2O_2 + 4\text{AAP} + \text{phenol} \xrightarrow{\text{Peroxidase}} \text{Quinone-imine dye} + 4H_2O$$

2.5.1.2 Method performance

The measuring range of the assay is between 0.1 – 20.7 mmol/L. Samples having higher concentrations are re-analysed following dilution with sodium chloride. Assay precision was calculated with CVs less than 1.6 %.

2.5.2 HDL-Cholesterol

2.5.2.1 Principle of the method

Direct determination of HDL-cholesterol relies on the use of dextran sulphate and polyethylene glycol (PEG) modified enzymes which show selective catalytic activities towards lipoprotein fractions. Reactivity increases in the order LDL < VLDL ≈ chylomicrons < HDL. In the presence of magnesium ions, dextran sulphate selectively forms water-soluble complexes with LDL, VLDL and chylomicrons which are resistant to PEG-modified enzymes. The cholesterol concentration of the HDL fraction is then determined enzymatically using reactions with cholesterol esterase and cholesterol oxidase to produce H_2O_2. In the presence of peroxidase, the H_2O_2 generated reacts with 4AAP and HDSA (Sodium N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline) to form a purple-blue dye. The colour intensity of the dye, measured spectrophotometrically at 600/700 nm ex/em, is proportional to the concentration of HDL-cholesterol in the sample. The reactions are summarised as follows:

$$\text{HDL-Cholesterol esters} + H_2O \xrightarrow{\text{PEG-Cholesterol esterase}} \text{HDL-Cholesterol} + \text{RCOOH}$$

$$\text{HDL-Cholesterol} + O_2 \xrightarrow{\text{PEG-Cholesterol oxidase}} \text{Cholest-4-en-one} + H_2O_2$$

$$2H_2O_2 + 4\text{AAP} + \text{HSDA} + H_2O \xrightarrow{\text{Peroxidase}} \text{purple pigment} + 5H_2O$$

2.5.2.2 Method performance

The analytical sensitivity for the assay, determined by the lowest measureable analyte level that can be distinguished from zero is 0.08 mmol/L and the linear range is up to 3.12 mmol/L. Samples having higher concentrations are re-analysed following dilution with sodium chloride. Assay precision demonstrates CV values less than 1.5 %.

2.5.3 Calculated LDL-cholesterol

Low density lipoprotein cholesterol (LDL-C) was estimated indirectly by using the Friedewald equation (Friedewald *et al.*, 1972). Determined in 1972 based on the analysis of 448 patients, the Friedewald formula estimates LDL-C, in mmol/L, according to the following equation;

(total cholesterol) – (high-density lipoprotein cholesterol) – (triglycerides x 0.45)

The Friedewald formula does have some limitations. It assumes virtually all triglyceride is carried on VLDL and that there is a constant ratio of triglyceride to cholesterol in VLDL measured in mmol/L of 2.19:1 (or 5:1 in mg/L). This assumption becomes invalid when triglyceride levels exceed 4.5 mmol/L or the ratio is disturbed for other reasons (e.g. exogenous oestrogen therapy or Type III hyperlipoproteinaemia). Under these circumstances lipoprotein fractionation methods are useful in determining a more accurate estimate of LDL-cholesterol concentration (refer to section 2.7).

2.5.4 Triglycerides

2.5.4.1 Principle of the method

Quantitative determination of triglyceride was based on an enzymatic, colorimetric method using microbial lipoprotein lipase which rapidly and completely hydrolyses triglycerides to their constituent long-chain fatty acids and glycerol. The glycerol is phosphorylated and subsequently undergoes oxidation to dihydroxyacetone phosphate and H_2O_2. In the presence of peroxidase, H_2O_2 reacts with 4-chlorophenol and 4AAP to yield a red chromophore which is measured spectrophotometrically using wavelengths em/ex 505/700 nm. The colour intensity of the dye formed is proportional to the concentration of triglyceride in the sample. The reactions are summarised as follows:

$$\text{Triglycerides} + 3H_2O \xrightarrow{\text{Lipase}} \text{Glycerol} + 3 \text{ fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerokinase, Mg2+}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{Glycerol phosphate oxidase}} \text{Dihydroxyacetone} + H_2O_2$$

$$H_2O_2 + 4\text{AAP} + \text{4-chlorophenol} \xrightarrow{\text{Peroxidase}} \text{Quinone dye} + 2H_2O + HCl$$

2.5.4.2 Method performance

The measuring range of the assay is between 0.1 – 10 mmol/L. Samples having higher concentrations are diluted with sodium chloride before re-analysis. Assay precision demonstrates less than 2 % variation.

2.6 CREATINE KINASE

2.6.1 Principle of the method

Creatine kinase (CK) exists in four different isoforms; mitochondrial, skeletal, brain and myocardial types. Following cellular injury, CK activity increases due to release from damaged cells. Total CK concentration was determined using a Cobas 8000 Modular Analyser and calculated based on the rate of formation of reduced nicotinamide adenine dinucleotide phosphate (NADPH).

Creatine kinase catalyses the transfer of a phosphate group from creatine phosphate to adenosine diphosphate (ADP) to produce adenosine triphosphate (ATP) which then acts upon glucose to yield glucose-6-phosphate (G6P). This reaction is catalysed by hexokinase in the presence of magnesium ions. The enzyme glucose-6-phosphate dehydrogenase (G6P-DH) then oxidises G6P with the concomitant reduction of NADP to form NADPH. The rate at which NADPH is produced is measured using wavelengths 340/546 nm. The test principles can be summarised as shown below.

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow{\text{Creatine Kinase}} \text{Creatine} + \text{ATP}$$

$$\text{ATP} + \text{Glucose} \xrightarrow{\text{Hexokinase}} \text{ADP} + \text{G6P}$$

$$\text{G6P} + NADP^+ \xrightarrow{\text{G6P-DH}} \text{6-phosphogluconate} + NADPH + H^+$$

2.6.2 Sample analysis

Activity of CK was measured, using a 2-point calibration, as an increase in the rate of formation of NADPH and expressed in U/L. Quality control is performed at regular intervals and the method is accredited to UKAS ISO 15189 International Standards for Medical Laboratories.

2.6.3 Method performance

The lower limit of the test, as represented by the lowest measureable analyte level that can be distinguished from zero is 7 U/L. The maximum level is 2000 U/L. Samples having higher activities were re-analysed following dilution with sodium chloride. Precision was determined using control samples and both the within and between batch coefficient of variation (CV) was less than 1.4 %.

2.7 LIPOPROTEIN FRACTIONATION (BETA-QUANTIFICATION)

2.7.1 Principles of the method

Calculating LDL-cholesterol using the Friedewald formula uses the assumption that virtually all plasma cholesterol is contained in the three major lipoprotein classes; VLDL, LDL and HDL. In fact a small contribution from intermediate-density lipoproteins (IDLs) and lipoprotein(a) is included in this LDL fraction. IDLs, often referred to as remnant lipoproteins, are intermediates between VLDLs and LDLs, but unlike LDLs they retain multiple copies of apolipoprotein E (ApoE) which allow IDL to bind to the LDL receptor with high affinity. Type III hyperlipoproteinaemia (HLP) (also known as familial dys-beta-lipoproteinemia) is a rare disorder caused by defective or absent apoE and is atherogenic in nature. Diagnosing such unusual hyperlipidaemias relies on accurate separation of the lipoprotein subclasses followed by measurement of cholesterol and triglyceride within each fraction.

The beta quantification method combines ultracentrifugation (120,000 rpm or 627,000 x g) with polyanionic precipitation to separate the lipoprotein classes according to their hydrated densities and enables accurate measurement of LDL-cholesterol. The ultracentrifugation step achieves partial physical separation of lipoprotein classes into VLDL (d<1.006 kg/L, top) and non-VLDL (d>1.006 kg/L, bottom) fractions. The polyanionic precipitation step, in which heparin and manganese chloride are added, precipitates apolipoprotein B containing lipoproteins, (the LDL) from the non-VLDL fraction. This enables HDL to remain in the

supernatant fraction which can be recovered following low speed centrifugation. Cholesterol and triglyceride are then measured in each fraction. VLDL lipids are not usually measured directly as it can be difficult to recover the VLDL fraction quantitatively. Instead this fraction is estimated by subtraction. An increase in the ratio of VLDL cholesterol to total triglyceride (> 0.69) implies the presence of IDLs, cholesterol enriched forms of VLDL which can subsequently be confirmed using lipoprotein electrophoresis.

2.7.2 Sample analysis

A schematic representation of the fractionation process is shown in figure 2.2. Samples were prepared by adding 1 ml plasma to a Beckman Quick-Seal™ polyallomer tube (Beckman Coulter, High Wycombe, UK) using a blunt needle to ensure the sample reaches the bottom of the tube. A density gradient was created by carefully added 0.195 mol/L sodium chloride (NaCl, VWR International, Leicestershire, UK) to fill the tube without disturbing the interface layer. Any excess was removed using a tissue. The top of the tube was sealed using a Beckman cordless tube topper, placed in the Ultracentifuge pre-set to 4°C (Optima ™ TLX Ultracentrifuge, Beckman Coulter) and spun for 2 hour 50 minutes at 120,000 rpm.

Using a CentriTube slicer (Beckman Coulter) the tube was clamped and cut through at a depth of 1.3 cm from the base of the tube. The top of the tube was pierced to remove the supernatant, the contents transferred to a 1 ml volumetric flask and the top of the tube washed out with NaCl to ensure maximal recovery of the VLDL fraction. After retracting the blade of the tube slicer, the infranatant, including the viscous protein layer at the bottom of the tube was transferred to a 2 ml volumetric flask and the tube washed several times with NaCl to guarantee full recovery of the combined LDL and HDL fractions. Both fractions were left overnight at 4°C to re-dissolve.

To isolate HDL, 10 µl sodium heparin (5000 units/ml, LEO Laboratories, Berkshire, UK) and 12.5 µl manganese chloride (1 molL, VWR Int.), were combined with 250 µl cold infranatant in a 1.5 ml microtube and vortex mixed for 5 sec before incubating on ice for 30 min. Samples then underwent a second ultracentifugation step at 45,000 rpm for 15 min. Supernatant, representing the HDL fraction, was removed using a fine tip pastette and transferred to a clean microtube.

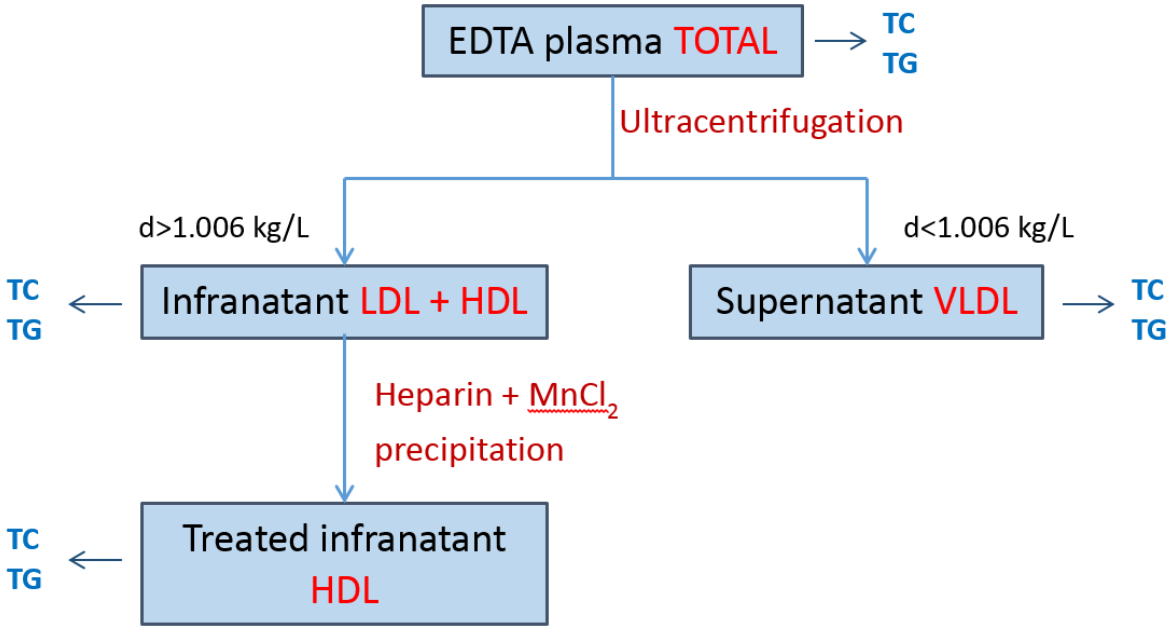


Figure 2.2 Schematic representation of the beta-quantification method.
TC = total cholesterol, TG = Triglycerides

Cholesterol and triglyceride concentrations were measured in each fraction using the methods described in section 2.5. Fractions were identified and correction calculations applied as shown in Table 2.1. Reported VLDL concentrations were calculated as the total value minus the infranatant result (BQ1 – BQ2) but were checked for agreement with the measured VLDL result within 0.5 mmol/L. LDL is calculated as the difference between the infranatant and the measured HDL (BQ2 – BQ4). The percent recovery was calculated as (BQ2 + BQ3)/BQ1 x 100.

Fraction ID	Source/ Contents	Calculation
BQ1	Original plasma sample (Total)	Original result
BQ2	Infranatant (d>1.006) – LDL + HDL	Result x 2/vs
BQ3	Measured VLDL (d<1.006)	Results/vs
BQ4	HDL	1.1(result) x 2/vs

vs – original volume of plasma sample used in mls.

Table 2.1 Identification, contents and calculations applied to measured beta quantification fractions.

2.8 MITOCHONDRIAL IMAGING

2.8.1 Principles of the method

Cellular structure and mitochondrial membrane potential, which controls ATP synthesis and generation of ROS, can be examined using cell permeable molecular probes and dyes. CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) reagent is well suited to monitor cell movement or location. It freely passes through cell membranes then undergoes transformation into a cell-impermeant reaction product. Fluorescence is retained for at least 72 hours, is non-toxic and the excitation and emission spectra are well separated from red fluorescent probe spectra which allows for multiplexing.

TMRM (tetramethylrhodamine) is a cationic molecule which utilises the electrical potential across the mitochondrial membrane to accumulate within the negatively charged matrix of active mitochondria. If membrane potential is reduced, less dye is retained and the fluorescence signal decreases. The red fluorescence of TMRM stained mitochondria is ideally suited to multiplex with green dyes used to stain cell cytoplasm (TMRM user guide – Invitogen). Both dyes can be measured by detection of the corresponding fluorescence signal using confocal microscopy.

2.8.2 Live cell staining

Cells were seeded in a multi-well optical bottom plate (Greiner, Stonehouse, UK) at a density of 2,500 cells per well and allowed to settle for 24 h in standard 10 % supplemented medium. Medium was then changed and the cells incubated for 72 h at 37°C in a humidified atmosphere of 95 % air with 5 % CO_2 in either control or treatment medium. The media was then replaced with mitochondrial dyes, both purchased from Invitrogen (Paisley, UK), diluted to appropriate concentrations as stated below in supplemented media and returned to the incubator for 30 min. The dye containing media was aspirated, the cells washed with PBS then covered with supplemented media without Phenol Red (Invitrogen). Cells were imaged using confocal microscopy using appropriate excitation and emission profiles according to table 2.2.

Dye	Final concentration	Wavelength Ex/Em (nm)
CellTracker Green	5 µmol/L	492/516
TMRM	15 nmol/L	548/574

Table 2.2 Properties of stains used for live cell imaging.

2.8.3 Confocal microscopy

Cells were imaged using an inverted Nikon A1R point scanning confocal microscope equipped with multiple laser lines. Images were captured using a 20x air objective lens and Nikon Elements software. A bio-chamber, supplied with 5 % CO_2 in air and maintained at 37°C, was used during the imaging of live cells. Appropriate laser lines were selected based on the excitation wavelength of the fluorophores and the laser power was adjusted to the lowest acceptable level to prevent bleaching of the fluorophore and photo toxicity. Multiple images were captured and analysed using ImageJ Volocity (Perkin Elmer) software and the intensities of each dye quantitatively determined.

2.9 STUDY SUBJECTS

Ethical approval for the study was provided by NRES Committee North East – County Durham & Tees Valley (number 10/H0908/53). Participants were recruited to the study after written informed consent between May 2011 and June 2013 from patients attending the Lipid and Cardiology Clinics at the Royal Victoria Infirmary, Newcastle upon Tyne, in whom statin therapy for primary or secondary prevention of cardiovascular disease was required. The statin control group were defined as patients who had received a daily dose of statin for at least 6 months without new onset of symptoms. Patients were recruited into the cases group if they had discontinued statin therapy because of new muscle symptoms (pain or weakness, with or without elevated CK) which had developed within 6 months of commencement of therapy and which abated on withdrawal but recurred with re-challenge at the same or reduced statin dose. These cases were considered to have statin associated muscle symptoms

(SAMS). Venous blood was collected into tubes containing EDTA as anticoagulant, centrifuged and the plasma aliquoted and stored at -80°C until analysis.

2.10 STATICTICAL ANALYSIS

All statistical analyses were performed using MedCalc for Windows, version 11.2.1.0 (MedCalc Software, Ostend, Belgium).

Data was assessed for normality using the D'Agostino-Pearson omnibus test which calculates a significance value (*p*-value) based on a combination of the coefficients of skewness and kurtosis. Almost all variables failed the test for normality so comparisons were performed using the Mann-Whitney U test for non-parametric data unless otherwise stated. A *p*-value of < 0.05 was taken as significant in all cases. The correlation coefficient (r) between variables were calculated using Spearman rank correlations. Unless stated data is presented as median and 95 % confidence intervals (CI).

2.11 ACKNOWLEDGEMENTS

All analysis performed using the Cobas 8000 Analyser was kindly undertaken by staff in the Core Section based at Blood Sciences, Royal Victoria Infirmary (RVI), Newcastle upon Tyne NHS Trust.

The lipoprotein fractionations were principally performed by Joan Ellis in the Special Endocrine section of Blood Sciences, RVI.

Live cell staining investigations were carried out with the assistance of staff in the Wellcome Centre for Mitochondrial Research, Newcastle University. Particular thanks to Dr Oliver Russell for performing the microscopy analysis.

Chapter Three

Chapter 3. Development of a Method for Ubiquinone Analysis

3.1 INTRODUCTION

3.1.1 High performance liquid chromatography

Chromatographic separation is one of the most powerful analytical techniques available to quantitatively assess many individual components present within a mixture. Technological refinements made over the last half century have seen the introduction of high performance (HPLC) and ultra-high performance (UPLC) liquid chromatography procedures which take advantage of narrow bore columns and small particle size to provide efficient resolution in minimal time.

Separation is based on the polarity and size of the compounds in the mixture and their interaction with the column of choice. Normal phase HPLC employs a polar silica based stationary phase and a non-polar mobile phase. This technique is useful for separating polar compounds as these are retained on the column whilst non-polar analytes elute quickly due to limited column interaction. Strong non-polar solvents, such as hexane or chloroform, are generally required in the mobile phase which increases the risk of toxic exposure.

Reversed phase HPLC uses the opposite effect and is more suitable for separation of hydrophobic molecules. A non-polar stationary phase, usually consisting of C8 or C18 alkyl ligands bonded to silica, binds non-polar compounds which can then be eluted using a polar mobile solvent (Figure 3.1). Water is a common constituent of the mobile phase along with miscible polar solvents such as methanol and acetonitrile. Reversed phase HPLC is nowadays the most commonly utilised of all HPLC applications. It has a broad range of applications and is relatively robust to handle a diverse range of compounds (Kumar and Kumar, 2012).

3.1.2 Mass spectrometry

The origin of mass spectrometry (MS) can be traced to the turn of the 19th century, but the first full functional instrument wasn't built until almost a century later. Significant technological evolution occurred from the 1950s onwards, helping to promote mass spectrometry as a versatile analytical tool capable of measuring characteristics of individual molecules after conversion to ions and manipulation by magnetic and electric fields (Griffiths,

2008). A compound is identified based on its mass to charge ratio (*m/z*) and the system is maintained under vacuum to minimise interfering collisions and facilitate ion transmission. Early instruments were only suitable as detectors for gas chromatography methods but advances in ionisation technologies has enabled coupling to HPLC systems. Mass spectrometry has served to improve the sensitivity and selectivity of a variety of diagnostic methods and is fast becoming a standard analytical tool for screening and routine analysis.

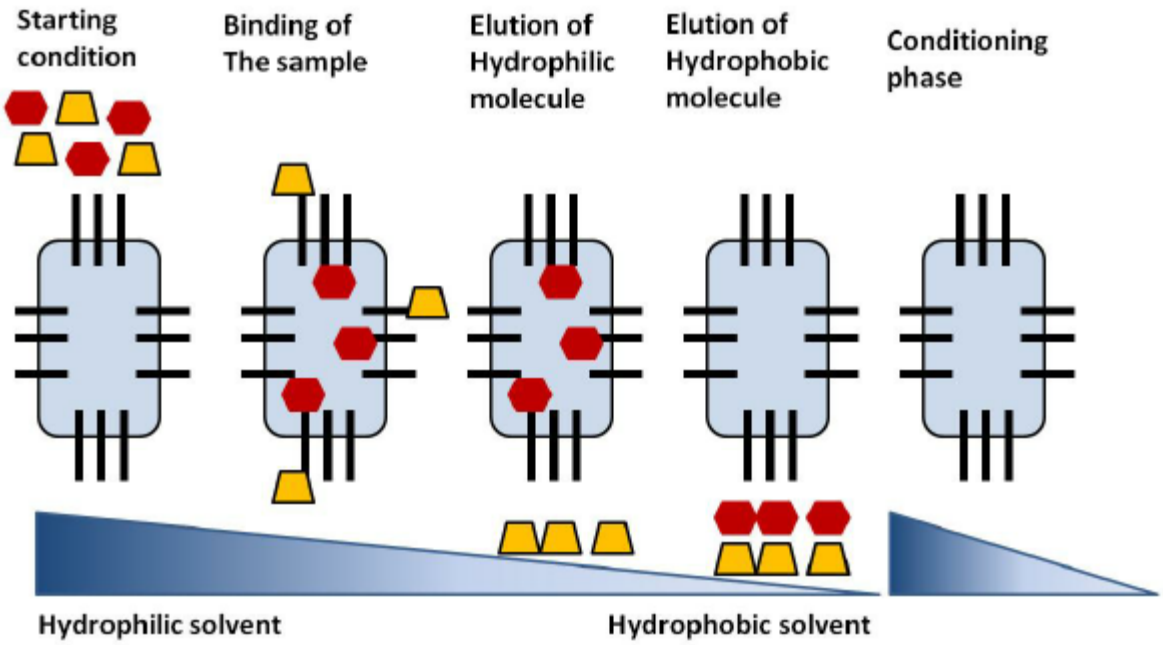


Figure 3.1 Steps of a reversed phase chromatography separation.
Non-polar molecules, such as lipids and proteins, are retained on the column and eluted by increasing the hydrophobicity of the solvent using common solvents such as methanol and acetonitrile. Image taken from (Magdeldin and Moser, 2012)

3.1.2.1 Ionisation methods

Emergence of mass spectrometry in the clinical setting has increased substantially over the last few decades, accredited in part to the development of new ionisation methods. Matrix-assisted laser desorption ionisation (MALDI) and electrospray ionisation (ESI) developed in the 1980s are primarily the choice in clinical laboratories (Blow, 2007; Chen, 2008). MALDI uses a pulsed laser to ionise sample from a solid phase surface (co-crystallized matrix molecules) and generally produces singly charged ions. ESI operates by electrically charging a solvent

dissolved substance and forcing it through a fine capillary with a stream of nebulising gas, usually nitrogen, to produce a spray of charged droplets. The ESI source is maintained at a high temperature and as the solvent progressively evaporates, the Rayleigh limit is reached whereby the surface tension holding the droplet together is exceeded by the electrostatic repulsion of its charges, resulting in an unstable droplet that dissociates, leaving multiply charged gas ions (Figure 3.2) (Bakhtiar and Nelson, 2001).

ESI exhibits superior reproducibility due to difficulty in controlling the crystallisation process in MALDI. Conversely, ESI sensitivity is more attuned to suppression effects of salts, detergents and buffer molecules. This can be overcome by prior coupling to an HPLC system to provide upfront chromatographic separation and purification (LC-MS) or by the use of the relatively new orthogonal ionisation method which prevents large amounts of neutral molecules being admitted into the analyser region (Cristoni and Bernardi, 2003).

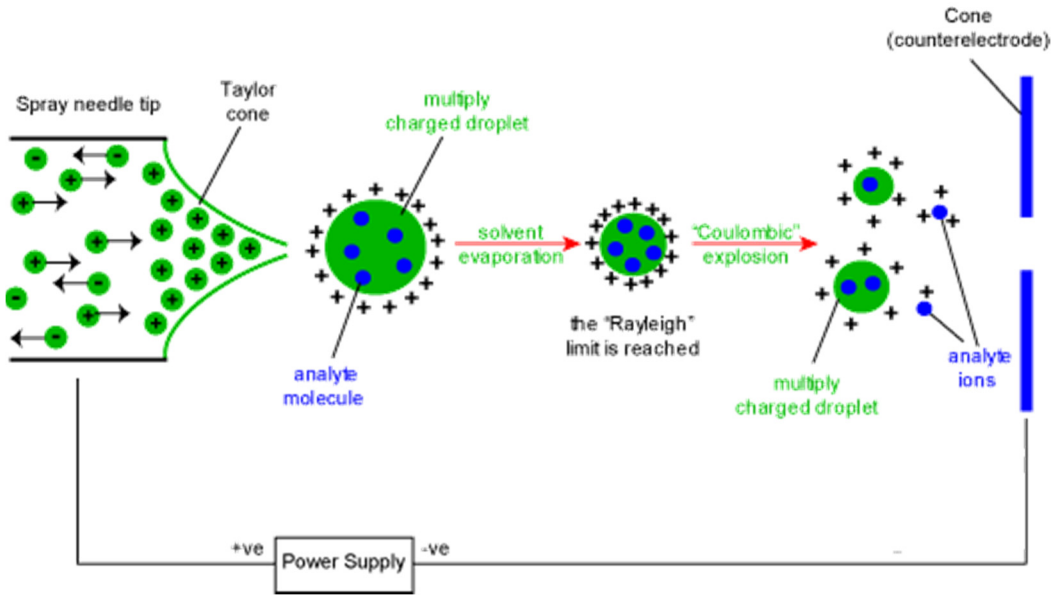


Figure 3.2 Mechanism of ion formation in electrospray ionization (ESI)
High voltage and temperature are applied to a solution passed through a fine capillary tube. Evaporation of the solvent causes an unstable droplet that dissociates, resulting in an aerosol spray of charged analyte ions. Image taken from (Gates, 2005)

3.1.2.2 Mass Analysers

Several mass-analysers with a range of mass accuracy and resolution are currently available, including time-of-flight (TOF) analysers, quadrupoles, ion traps and high performance Fourier transform ion cyclotron resonance MS (FT-ICR). ESI is compatible with all the aforementioned analysers whilst MALDI is more suited to TOF.

TOF measures flight time of an ion from the source to detector down a vacuum flight tube. Lower mass ions (low m/z) traverse quicker and reach the detector first. Using reflective mirrors effectively increases the length of the ion flight path which maximises mass resolution and accuracy, thus TOF are particularly adept for distinguishing compounds that may only differ by a single double bond or carbon atom (Khalsa-Moyers and McDonald, 2006; Dunn, 2008).

Quadrupole mass-analysers are more familiar, particularly in the clinical setting. In these detectors ions travel through an assembly of 4 parallel equidistant metal rods, in an oscillatory motion facilitated by an applied DC/RF voltage. Selected ions are chosen by varying these potentials causing ions outside the required mass range to collide with the rods and become neutralised, thus they do not reach the detector (Ho *et al.*, 2003).

The development of hybrid and tandem (MS/MS) systems has provided greater sensitivity and reduced acquisition times, enabling high mass accuracy accumulation of large volumes of data at high throughput rates. ESI-MS/MS has eliminated the need for laborious sample purification steps by incorporating 3 quadrupoles in linear fashion (Figure 3.3). Whilst the first quadrupole (MS1) serves as a filter to mass select ions of interest ('parent' or precursor ions), the next (MS2) acts as a collision cell, activating and fragmenting the ions by collision induced dissociation (CID) with an inert gas (usually argon). The third quadrupole (MS3) monitors 'daughter' or product ions produced which are related to the molecular structure of the precursor (Ho *et al.*, 2003; Khalsa-Moyers and McDonald, 2006).

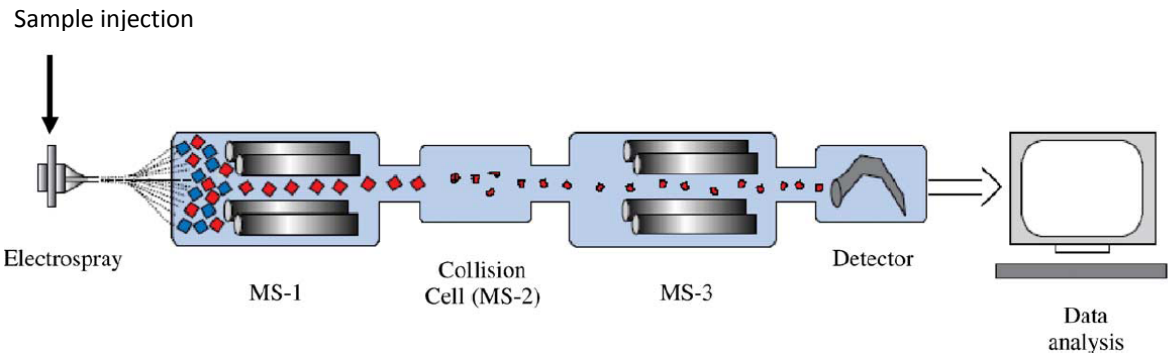


Figure 3.3 Illustration of quadrupole MS/MS analysis.
Ions are separated in MS1 based on their mass to charge ratio. Selected ions enter the collision cell and fragment via collision with an inert gas (collision induced dissociation). MS3 separates the fragment ion and presents them to the detector for quantification and identification. [adapted from (Garg and Dasouki, 2006)]

The mass analysers can be set to enable monitoring of all ions (scan mode) or be static for selected ions (selected reaction monitoring, SRM) thereby providing a range of potential analytical outputs. For quantitative analysis the greatest sensitivity and specificity is achieved using SRM, in which both mass filters (MS1 and MS3) are used to select defined ions for the compound of interest. This does however require prior knowledge of both precursor and fragment ions. Modern day tandem mass spectrometers are capable of monitoring many such SRM transitions simultaneously in a technique known as multiple reaction monitoring (MRM).

3.1.2.3 Ion Suppression

A critical issue associated with mass spectrometry is the matrix effect exhibited by biological specimens. This is often observed as a loss of ionisation signal compared to a pure standard solution of the analyte. Components in the sample matrix, most noticeably salts and lipids, or reagents added to the mobile phase can act as interfering species which reduce the efficiency of the droplet ionisation process and can have a significant impact on method performance (Ho *et al.*, 2003). Ion suppression can be evaluated by monitoring the SRM transitions using a post-column infusion of the analyte of interest with injection of a matrix appropriate sample i.e. blank plasma (Gosetti *et al.*, 2010).

Extensive sample purification methods such as solid phase extraction can be employed to minimise matrix effects but these are time-consuming and can result in poor analyte recoveries. The degree of suppression can be influenced by the choice of HPLC stationary phase, the elution flow rate, and the matrix to analyte concentration ratio, all of which should be considered during method development.

3.1.3 Methods of Analysis of Coenzyme Q10

The hydrophobic nature of COQ10 and the tendency to readily oxidise makes analytical methods technically challenging (Barshop and Gangoiti, 2007). Commonly employed techniques use high performance liquid chromatography (HPLC) with either UV detection at 275 nM (Rousseau and Varin, 1998; Hagerman *et al.*, 2001; Mosca *et al.*, 2002; Jiang *et al.*, 2004), or the more sensitive electrochemical detection (ECD - coloumetry) (Yamamoto and Yamashita, 1997; Menke *et al.*, 2000; Tang *et al.*, 2004; Pastore *et al.*, 2005; Hirayama *et al.*, 2008).

Lang and co-workers described the first simultaneous analysis of both oxidised and reduced CoQ10 and tocopherols using combined UV and ECD (Lang *et al.*, 1986). The weak UV absorbing properties of ubiquinol and tocopherols in contrast to the strongly UV absorbent nature of ubiquinone necessitated the dual detection modes. Menke and colleagues developed an HPLC-ECD method using ammonium formate in place of the standard lithium perchlorate as conductivity salt which is highly corrosive and oxidative and shortens the life span of the detector cells as well as contaminating HPLC columns (Menke *et al.*, 2000).

Recently, liquid chromatography coupled to mass-spectrometry (LC-MS/MS) has become a highly versatile analytical tool. The first use for quantitation of CoQ10 in humans was performed by Hansen and colleagues in 2004 (Hansen *et al.*, 2004). This method relied on formation of anions by negative atmospheric pressure ionisation (APCI-). Subsequent authors have reported using protonated species produced by electrospray ionisation in positive mode (ESI+) (Teshima and Kondo, 2005; Li *et al.*, 2007; Ruiz-Jimenez *et al.*, 2007). Teshima and Kondo enhanced the sensitivity of ESI+ by incorporating methylamine in the mobile phase, forming an 1-alkylammonium adduct $[M+CH_3NH_3]^+$ with 12.5-fold higher signal intensity than the protonated molecule. (Teshima and Kondo, 2005). A common fragment monitored during MS/MS is *m/z* 197 resulting from the loss of the isoprenoid moiety from the benzoquinone

ring (Barshop and Gangoiti, 2007; Ruiz-Jimenez *et al.*, 2007). In 2009, an analytical run time of 2 minutes (compared to standard 12 min runs) was achieved using ultra-HPLC with UV detection (Paliakov *et al.*, 2009). In a different approach, a 14-amino acid chemically synthesised CoQ binding protein was used to immobilize CoQ10 on microfuge tubes thus allowing quantification to be achieved spectrophotometrically (Hagerman, 2003).

Extraction procedures generally employ liquid extraction in hexane, ethanol or 1-propanol, the most lipophilic alcohol miscible with water. Solid phase extraction has proved effective at removing contaminants such as ergosterol (Hagerman *et al.*, 2001). The lack of commercially available non-physiological internal standards means naturally occurring ubiquinone derivatives are often used. CoQ9 is the metabolite of choice in human studies and although small amounts may be endogenously present, levels are likely to be negligible (Ruiz-Jimenez *et al.*, 2007). Duncan and colleagues demonstrated the synthetic derivative di-propoxy-CoQ10 as a suitable alternative for use in CoQ assessment (Duncan *et al.*, 2005).

Total CoQ10 is relatively stable in plasma both pre and post extraction and can withstand up to 7 freeze-thaw cycles (Kaikkonen, 1999). CoQ10 in fresh plasma is almost all in the reduced form which may not be fully oxidised during the extraction procedure. Therefore the inclusion of oxidising agents such as cupric sulphate, iron chloride or 1-4 benzoquinone can be used to ensure complete conversion (Mosca *et al.*, 2002; Hansen *et al.*, 2004; Tang *et al.*, 2004).

Measurement of the redox state can be an indicator of oxidative stress. Altered rates have been detected in several conditions; however, accurate assessment can be problematic. Ubiquinol will readily oxidise within 30 minutes at ambient temperature (Lang *et al.*, 1986), on ice within 4 hours (Rousseau and Varin, 1998) and at -80°C within 48 hrs (Barshop and Gangoiti, 2007). Hexane extracted ubiquinol levels decreased by 43.5 % in 7 hours at room temperature and fluctuate even with storage at -80°C (Yamamoto and Yamashita, 1997; Kaikkonen, 1999). Conversely, plasma samples frozen immediately at -80°C then analysed after 6 months do not show a change in redox levels (Kaikkonen, 1999). Direct injection of the extract is preferential when measurement of the redox capacity is required as drying then dissolving prior to analysis is the main source of oxidation of reduced CoQ10 (Menke *et al.*, 2000; Tang *et al.*, 2004; Pastore *et al.*, 2005; Hirayama *et al.*, 2008). Ultracentrifugation can also increase the oxidation of CoQ10 (Kaikkonen, 1999).

Total CoQ10 concentration in plasma has been reported to range from 0.4 – 2.0 μg/ml (463 – 2316 nmol/L) (Ruiz-Jimenez *et al.*, 2007) and is age and sex independent (Duncan *et al.*, 2005; Barshop and Gangoiti, 2007), although slightly higher values, up to 4.28 μg/ml (4956 nmol/L) have been reported in children (Paliakov *et al.*, 2009). Normalisation to cholesterol or LDL however suggests a decrease with age (Barshop and Gangoiti, 2007). Muscle CoQ10 has been reported ranging from 18.34 ± 5.48 to 33.3 ± 5.3 ρmol/mg wet weight (Pastore *et al.*, 2005) and 140 - 580 ρmol/mg protein (Duncan *et al.*, 2005). Erythrocytes levels are approximately 3 % that in plasma and supplementation with CoQ10 does not appear to influence these levels in healthy patients but does incur an increase in sickle cell anaemia (Niklowitz *et al.*, 2007). In contrast to plasma, mononuclear cells CoQ10 levels correlate well with muscle levels and may be more beneficial in assessing CoQ status (Duncan *et al.*, 2005).

3.1.4 Aim

To develop and optimise an analytical procedure for the measurement of the lipophilic quinone coenzyme Q10 in biological specimens (plasma, muscle, fibroblasts) using liquid chromatography tandem mass spectrometry.

3.2 MATERIALS AND METHODS

3.2.1 Reagents

All solvents used were of HPLC grade or higher. Methanol and formic acid were purchased from VWR International (Leicestershire, UK) and ethanol and 2-propanol were from Rathburn Chemicals (Walkerburn, UK). Methylamine 2M in methanol was purchased from Sigma-Aldrich (Dorset, UK). HPLC grade deionized water obtained from Fisher Scientific (Loughborough, UK) was used for reagent preparation. Sodium borohydride and EDTA disodium salt for ubiquinol synthesis were obtained from VWR Int. and Sigma–Aldrich respectively.

Coenzyme Q10 (CoQ10) and Q9 (CoQ9) were obtained from Sigma-Aldrich. Stock solutions were prepared in ethanol at 500 µg/ml (580 µmol/L). Subsequent dilutions were made using HPLC grade methanol. All standards were stored at -40°C for up to 6 months.

3.2.1.1 Calibrators

Plasma calibration standard Coenzyme Q10 (Chromsystems, Germany) was reconstituted in 2 ml of HPLC grade water to produce 700 µg/L (811 nmol/L). This was used for accuracy checks only.

Influenced by published methods and reported units of concentration, calibration curves were prepared by dilution of the 500 µg/ml CoQ10 stock solution to give the following; 0, 0.01, 0.1, 0.5, 1, 5, 10, 20 µg/ml for quantification of plasma levels and 0, 0.04, 0.1, 0.4, 1, 2 µmol/L for tissue analysis. Linear regression equations were calculated using a weighting scheme by the Xcalibur software (Thermo Electron Corporation, Hertfordshire, UK)

CoQ9 was used as internal standard (IS#) at 0.5 µg/ml, 0.05 µg/ml and 100 nmol/L in methanol for plasma, fibroblasts and tissue extractions respectively.

3.2.1.2 Ubiquinol preparation

Solutions of ubiquinol-9 ($CoQ9H_2$) and ubiquinol-10 ($CoQ10H_2$) were prepared by reduction of the corresponding ubiquinone with sodium borohydride according to the method described by Ruiz-Jimenez *et al (Ruiz-Jimenez et al., 2007).* In brief, stock ubiquinone solution was

extracted with hexane in the presence of 20 mg sodium borohydride and left in the dark at room temperature for 5 minutes. Aqueous EDTA (100 µM) was added, the mixture vortexed and centrifuged and the upper layer, containing the original concentration of the quinone now in the reduced quinol form, was decanted and stored. It should be noted that no checks of the quinol concentrations were performed directly as these solutions were only used to determine retention times for each analyte.

3.2.1.3 Control material

Bi-level CoQ10 Plasma Controls (Chromsystems, Germany) were reconstituted according to manufacturer guidelines, aliquots prepared and stored at -40°C. This gave analyte reference concentrations as shown in table 3.1.

A randomised pooled plasma specimen was prepared using EDTA blood received for analysis at the Blood Sciences Department, NUTH, but which was surplus to requirement. This was analysed for precision studies. Controls were analysed with every batch.

Substance	Unit	Value	Range
CoQ10 – level 1	µg/L	659	527 – 790
	nmol/L	763	610 – 916
CoQ10 – level 2	µg/L	1230	984 – 1476
	nmol/L	1424	1139 – 1709

Table 3.1 Final concentration of CoQ10 in reconstituted control material. Data supplied by Chromsystems.

3.2.2 Equipment

MS/MS was performed using a Finnigan^{TM} TSQ Quantum Ultra Triple Stage Quadrupole Mass Spectrometer (Thermo Electron Corporation, Hertfordshire, UK) with Ion Max source operated in electrospray positive mode (ESI^+). Common parameter settings were as follows: spray voltage 4000 V, sheath gas pressure 25 mTorr, ion sweep pressure 2 mTorr and Q2 collision gas pressure at 1.5 mTorr. Capillary temperature was maintained at 300°C. Dwell time was 0.20 sec and resolution of Q1 and Q3 was set at 0.70 amu. Tube lens settings and collision energy (eV) are analyte specific. A Thermo Scientific Surveyor pump was utilised for

solvent delivery. Injections were performed via a HTC PAL autosampler (CTC Analytics AG, Switzerland) fitted with a 20 µL injection loop and maintained at 5°C. Data were acquired using Xcalibur software version 1.4 (Thermo Scientific, Leicestershire, UK).

Chromatographic separation was performed using a Kinetex core-shell C18 reversed phase column fitted with an HPLC KrudKatcher Ultra Column In-Line Filter (0.5 µm porosity x 0.004 in ID) (Phenomenex, Cheshire, UK). Two column lengths, 150 mm and 50 mm, were tested during method evaluation, both with 2.1 mm I.D and 2.6 µm particle size. Prior to use, columns were allowed to equilibrate with mobile phase reagent for 60 min. After a maximum of 40 injections, re-equilibration was performed. For storage, the column was flushed with 95 % methanol (v/v) followed by 60 % methanol for 30 mins. Column temperature was maintained at 40°C using a Thermo Electron hot pocket (operating range ambient to 85°C).

3.2.3 Optimisation of the mass spectrometry parameters.

Initial infusion experiments confirmed that CoQ10 has poor ionisation properties thus the analytical sensitivity using ESI^+ is very low. Following a review of the literature it was decided to incorporate methylamine as a mobile phase additive as the alkylammonium adduct ions $[M+CH_3NH_3]^+$ have been shown to increase the signal intensity. Therefore, mass spectra were acquired by direct infusion of 1 µg/ml CoQ10 and CoQ9 containing 5 mmol/L methylamine at a flow rate of 5 µl/min. The alkylammonium adduct ions were established and production of the product ions, by collision-induced dissociation (CID) with argon gas, was optimized with respect to tube lens and collision energy using the analyser tuning function. Fragmentation patterns were obtained over the range *m/z* 30 to $[M+CH_3NH_3+5]^+$ by acquisition of 10 scans of 2 sec duration.

3.2.4 Optimisation of the chromatography method

Retention time (RT) investigations were performed by dilution of stock standards using methanol to give a final concentration of 20 µg/ml. Mobile phase conditions were initially tested using 5 mmol/L methylamine in methanol but this failed to elute peaks efficiently. Isopropanol (2-propanol) and formic acid were subsequently included in the mobile phase to give a final working solution of methanol:2-propanol:formic acid (45:55:0.5, v/v/v) containing 5 mmol/L methylamine. Individual standard solutions were injected and adjustments made to the flow rate, scan width and time parameters until satisfactory mass spectral and

chromatographic separation was achieved. The four standard components were then mixed and analysed under the same conditions to identify any analyte interactions. Retention time drift was assessed for within batch variability for both CoQ9 and CoQ10 using a combination of standard and sample matrices.

3.2.4.1 Column dimensions

The majority of the ubiquinone development work was undertaken using 150 mm Kinetex core-shell C18 reversed phase columns. To try to improve on the turnaround times, method development was further explored using a 50 mm column with the same internal dimensions. Extracted samples, containing various biological matrices, were analysed using the established method then immediately reanalysed using the shorter column. Modifications were made to the flow rate and scan programme to account for the shorter column but mobile phase conditions were maintained.

3.2.5 Ion Suppression

Authentic standard solution of CoQ10 (10 µg/ml) was directly infused into the mass spectrometer at 10 µl/min flow using a T-junction fitted post-column. A plasma extract, without added IS#, or a blank sample i.e. mobile phase, was simultaneously injected via the autosampler and the SRM transitions monitored. Ion suppression was monitored over 10 minutes. The experiment was repeated using CoQ9 infusion.

3.2.6 Sample extraction

Extraction was optimised suitable for analysis of CoQ10 in a variety of sample types, primarily plasma, muscle tissue and skin fibroblast cells. It was further decided to examine the CoQ10 content in relation to lipoprotein fractions (HDL, LDL and VLDL). These fractions were prepared by the Blood Sciences department, NUTH and had been requested for beta-quantification assessment in hyperlipidaemic patients (refer to section 2.7 for preparation). Additionally the feasibility of analysis using dried blood spots was briefly investigated which has the advantage of minimal sample volume, ease of transport and potentially improved stability.

3.2.6.1 Plasma or serum

Analysis of CoQ10 in plasma and serum was based on the method of Ruiz-Jimenez *et al* (Ruiz-Jimenez *et al.*, 2007). One hundred microliters of sample was mixed with an equal volume of internal standard (0.5 µg/ml CoQ9 in methanol) in an Eppendorf microtube. The polarity of CoQ10 necessities the use of a non-polar organic solvent therefore following deproteinisation the sample was centrifuged at 1500g for 5 min and the supernatant extracted into hexane. Different volumes of hexane and number of extractions were tested. Optimal conditions involved 500 µl hexane, vortex for 1 min then centrifuge at 900g for 1 min. The upper phase was transferred to a clean tube, a further 500 µl hexane added to the original tube and the extraction repeated. The upper phases were combined and evaporated to dryness using nitrogen gas in a Techne sample concentrator set to 40°C. The dried extract was reconstituted with 100 µl methanol prior to analyse and transferred to a 96-well microplate. It should be noted that direct analyse of the hexane phase was investigated but this failed to produce a usable profile.

3.2.6.2 Muscle biopsy

According to the literature, CoQ10 in muscle may be expressed per wet weight of tissue or correlated to protein content. Initial experiments were designed to measure CoQ10 per weight of tissue and based on the methods of Pastore and colleagues (Pastore *et al.*, 2005) and Teshima and Kondo (Teshima and Kondo, 2005). Approximately 5 - 10 mgs of tissue, cut in a Petri dish maintained over dry ice to preserve tissue in the frozen state, was weighed directly into a 1.5 ml microtube. Methanol (250 µl) was added and the sample homogenised

using a hand held motor driven pellet pestle (Sigma-Aldrich). IS# CoQ9 prepared in hexane (25 ρmols per sample) was added then the sample sonicated for 30 sec and vortex mixed for 2 min to ensure maximal extraction. Following centrifugation at 16,000*g* for 5 min the upper phase was transferred to a clean microtube, evaporated to dryness using nitrogen then reconstituted in 100 µl methanol prior to analysis.

To improve reproducibility two modifications were subsequently made. First, to measure total CoQ10 by complete oxidation of the quinol using ferric chloride ($FeCl_3$) and secondly to enable correlation to protein content. Tissue was homogenised in 250 µl TRIS Buffer (100 mmol/L, pH 8) and centrifuged at 16,000*g* for 5 min. An aliquot of supernatant was analysed for total protein on the Roche modular analyser (section 2.4). $FeCl_3$ (50 µl, 10 mmol/L in methanol) and 25 ρmols IS# CoQ9 (100 nmol/L in methanol) were added to the remaining supernatant and mixed at room temperature for 15 min to allow total quinol oxidation. The quinones were then extracted into hexane, dried and reconstituted as stated previously.

3.2.6.3 Fibroblast cell lines

Refer to the general methods chapter for further details regarding routine tissue culture procedures (section 2.2). Fibroblast cells were incubated at 37°C in 25 cm^2 culture flasks with 10 % FBS supplemented MEM. Cells were harvested at 70 % confluency using trypsin, washed with PBS and the final pellet resuspended and sonicated for 30 sec in 0.5 ml PBS. All cells then underwent one freeze-thaw cycle and were homogenised using a hand held micro homogeniser (Sigma-Aldrich). An aliquot of supernatant was used for protein measurement as stated previously and CoQ10 was extracted as per the plasma method using a diluted internal standard at 0.05 µg/ml.

3.2.6.4 Dried Blood Spots

EDTA blood was applied to Whatman® 903 filter paper (Scientific Laboratory Supplies, Nottingham, UK) as a single application and air dried for 4 hours. A 6 mm disk (comprising 12 µl blood) was punched from the card, placed in a 1.5 ml microtube and 100 µl IS# containing 0.05 µg/ml CoQ9 in methanol added. The sample was extracted into 0.5 ml hexane by shaking for 1 hr at room temperature using a multitube vortexer followed by 30 min sonication. The upper phase was transferred to a clean microtube and the extraction repeated using 2 min vortex on maximum setting. The upper phases were combined, evaporated to dryness and

reconstituted in 100 µl methanol. An aliquot of plasma from the same sample was also tested for comparison.

3.2.7 Method validation

The linearity was determined by sequential dilution of 20 µg/ml CoQ10 standard and by reference of the peak area intensity to CoQ9 used as internal standard. This corresponds to a maximal limit one order of magnitude greater than the reported plasma values in a normal population.

Limits of detection (LOD) and quantification (LOQ) were determined by analysis of standard CoQ10 ranging from 1 – 10 nmol/L (0.86 – 8.63 ng/ml). Accepted LOD and LOQ were the concentrations at which a peak is detectable from background noise with signal-to-noise ratio, S/N, ≥ 3 and ≥10 respectively. Sequential dilutions of the level 1 control with isotonic saline were also examined for matrix influence at low concentrations. Each level was analysed as four times.

Intra-day precision was evaluated at 3 levels (n=5) by analysis of quality control material and pooled plasma. Reproducibility of injections was determined by replicate analysis of the same extract (n=5). Inter-day precision was determined by multiple sample extraction over a period of 12 weeks (n=20). Re-injection of samples was performed after a short interval (2 days) to consider CoQ extract stability.

Recovery experiments were performed by spiking either pooled plasma or muscle homogenate with authentic standards prior to the extraction procedure. A standard:sample ratio of 1:25 (v/v) was used. All extractions were performed in triplicate.

Spiked dried blood spots were prepared by mixing 250 µl whole blood with 10 µl of known concentrations of CoQ10 standard, vortexed hard for 15 min then spotted onto Whatman® 903 filter paper in a single application. The concentrations added were in the range 10 – 2000 nmol/L

3.2.8 Direct MS/MS analysis

An investigation was performed to see if it was possible to analyse CoQ10 using direct (flow injection) MS/MS without prior chromatographic separation. Direct MS/MS may lack specificity as analytes with equal mass transitions are not separated by prior chromatography. However, if the transitions chosen are unique to the compound of interest then MS/MS may be usable as a quick procedure to enable high throughput of samples. As both CoQ9 and CoQ10 produced *m/z* 197 daughter ions an acquisition method was defined with Q1 set to scan range *m/z* 800 – 920 and Q3 fixed for *m/z* 197 (precursor scan). A second SRM scan function was also included to monitor the same ion transitions used for LC-MS/MS method. As no prior chromatography is required the analysis time was reduced to 3 min per sample. Analyte parameters and mobile phase remained unchanged. To allow comparison with the LC-MS/MS method a limited number of plasma sample extracts were re-injected using the modified acquisition parameters.

3.3 RESULTS

3.3.1 Mass spectrometry parameters

For both oxidised analytes the most abundant product ion was *m/z* 197 which corresponds to loss of the isoprenoid chain (Figure 3.4). It was therefore inferred the same product ion would occur during analysis of the reduced forms of CoQ with a respective *m/z* +2 parent ion. The optimised instrument parameters were selected for creation of the acquisition scan in selected reaction monitoring (SRM) mode (Table 3.2).

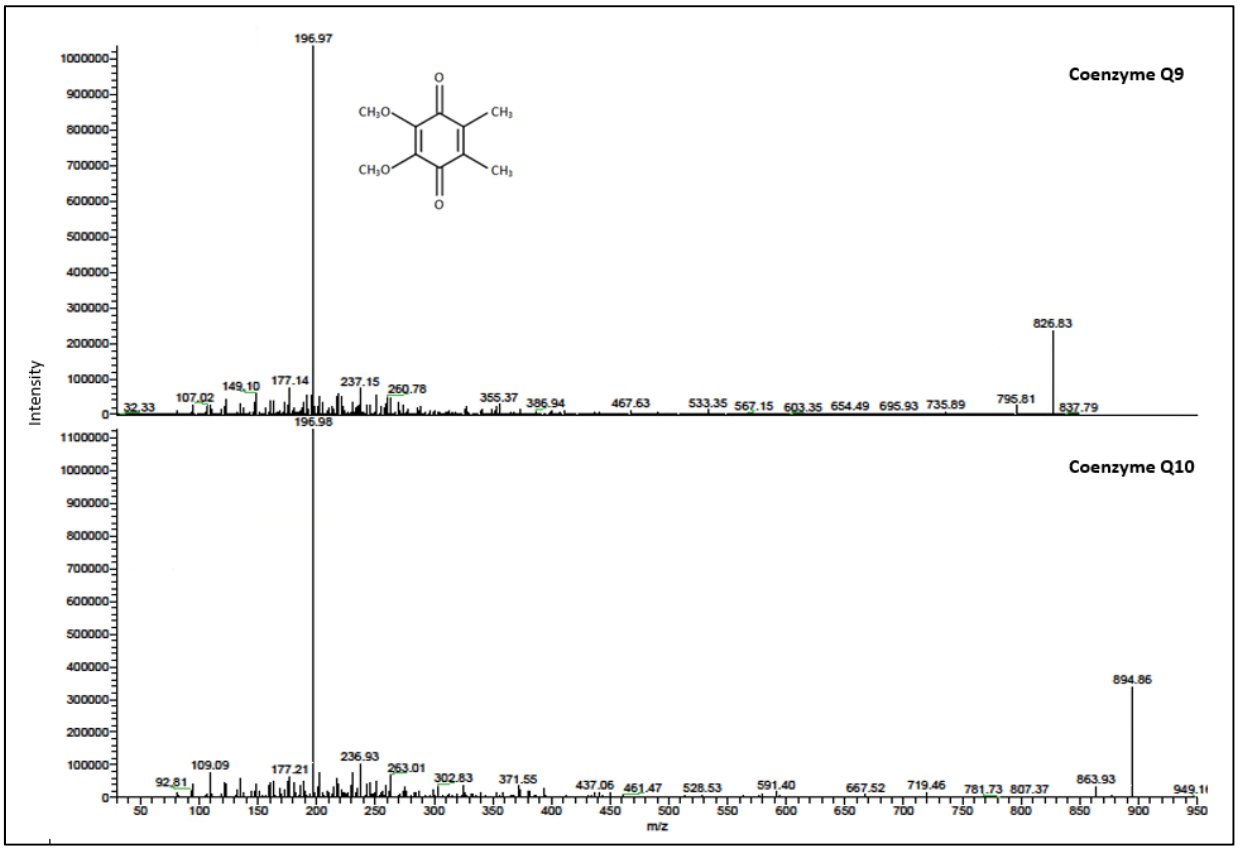


Figure 3.4 Mass spectra for Coenzyme Q (methylamine adducts) obtained by infusion of 1 µg/ml standard at a flow rate of 5 µl/min.
Collision energy was 22(eV). Structure of the predominant product ion (*m/z* 197) for both analytes is shown.

3.3.2 Chromatographic Method

Using the mobile phase solvent as defined above and a 150 mm Kinetex core-shell C18 column (2.1 mm I.D, 2.6 µm particle size), maintained at 40°C, LC-MS/MS analysis was initially achieved using 10 µl sample injections and an isocratic flow rate of 200 µl/min. Elution of all analytes occurred within 6 min. However, the LC pump pressure was approaching the maximum limits of the instrument (4000 psi) which has the risk of causing the system to fail mid run. Therefore, the flow rate was reduced to 100 µl/min, producing approximately 2000 psi of back pressure which allowed scope for partial blockages and pressure fluctuations. Using these conditions baseline resolution of all analytes was achieved in a total run time of 10 min (Figure 3.5). No extra time was needed for equilibrating the column. Signal intensity in the full mass spectrum was excellent (> $1e^5$) indicating the methylamine adduct has a good ionisation efficiency. Chromatographic resolution displayed minimal RT drift allowing consecutive analysis of over 40 samples before column reconditioning is required (Figure 3.6).

Analyte		**$[M+CH_3NH_3]^+$ (*m/z*)**	**Product ion (*m/z*)**	**Collision energy (eV)**	**Tube lens**	**Approx. Retention Time (min)**
Coenzyme Q9 (oxidised)	CoQ9	**827**	197	23	97	6.5
Coenzyme Q9 (reduced)	$CoQ9H_2$	**829**	197	23	97	5.1
Coenzyme Q10 (oxidised)	CoQ10	**895**	197	22	104	7.5
Coenzyme Q10 (reduced)	$CoQ10H_2$	**897**	197	22	104	5.8

Table 3.2 SRM parameters for coenzyme Q by ESI^+-LC-MS/MS.
Dwell time for each transition was 0.2 seconds. Capillary spray voltage was 4000 V.

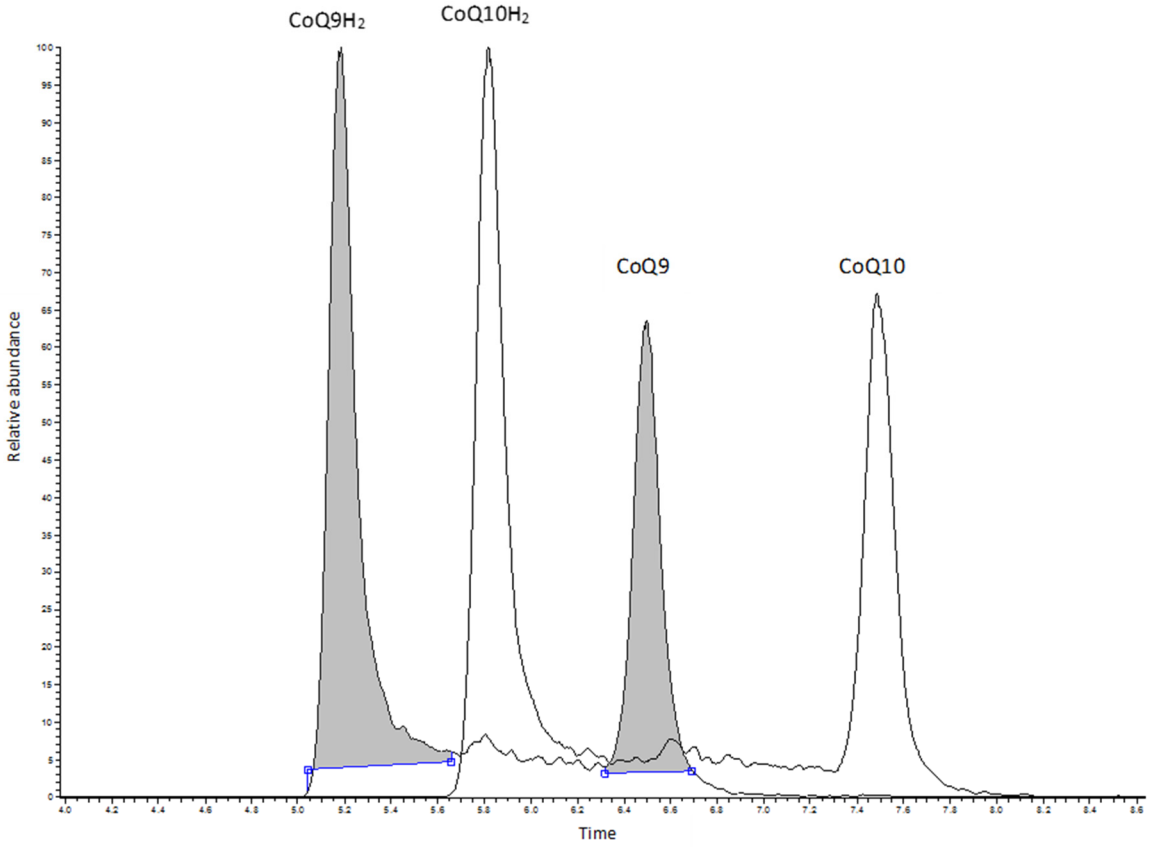


Figure 3.5 Chromatograms obtained with standard solutions 20 µg/ml for coenzyme Q9 and Q10 (reduced and oxidised forms) acquired by LC-MS/MS in SRM mode.
A 150 mm Kinetex core-shell 2.6 µm partical size column was used with an isocratic flow rate of 100µl/min. Time is measured in minutes. Transition used are as follows: CoQ9 827>197, CoQ10 895>197, $CoQ9H_2$ 829>197, $CoQ10H_2$ 897>197.

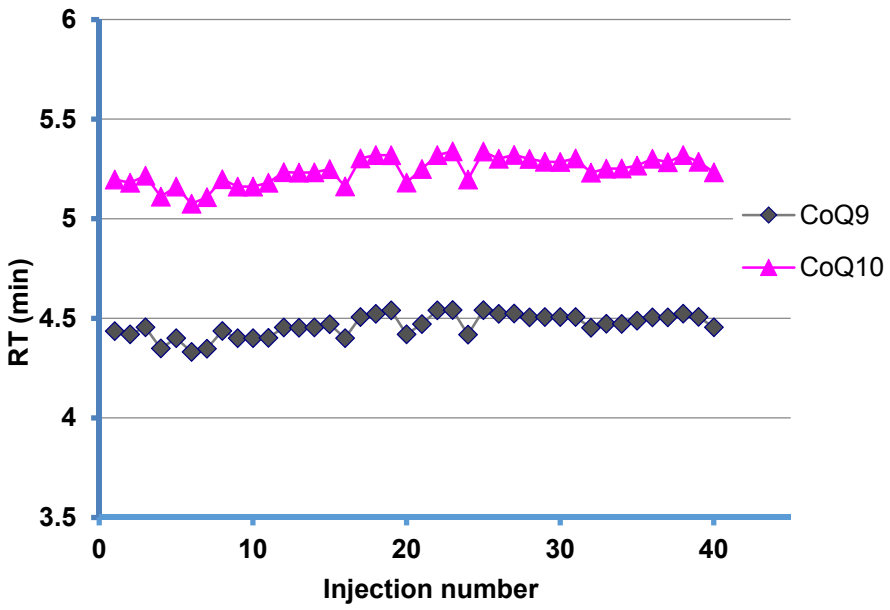


Figure 3.6 Within-batch retention time (RT) stability for LC-MS/MS analysis of CoQ9 and CoQ10.
Consecutive analysis of 40 individual samples, comprising standard and plasma matrices, was performed.

3.3.2.1 Column dimensions

The 150 mm Kinetex core-shell C18 reversed phase column provided exceptional base line resolution of CoQ10 and CoQ9 in both reduced and oxidised forms in less than 10 mins. Using a flow rate of 200 µl/min, comparable results in a total run time of just 3 min was achieved using the shorter 50 mm column.

Calibration curves were constructed using the 50 mm column and results from sample analysis compared with the original concentrations calculated using the 150 mm column (Figure 3.7). Good accordance between methods was observed, although fibroblasts tend to show slightly more variation. This may be due to lower sensitivity observed with cell matrices or reflect the increased comparisons performed.

Allowing for pre and post injection cleaning, use of the 50 mm column would permit analysis of 15 samples per hour instead of the current 6 enabling more efficient sample throughput for ubiquinone analysis. However, further in-depth evaluation is needed and was not performed as part of this study.

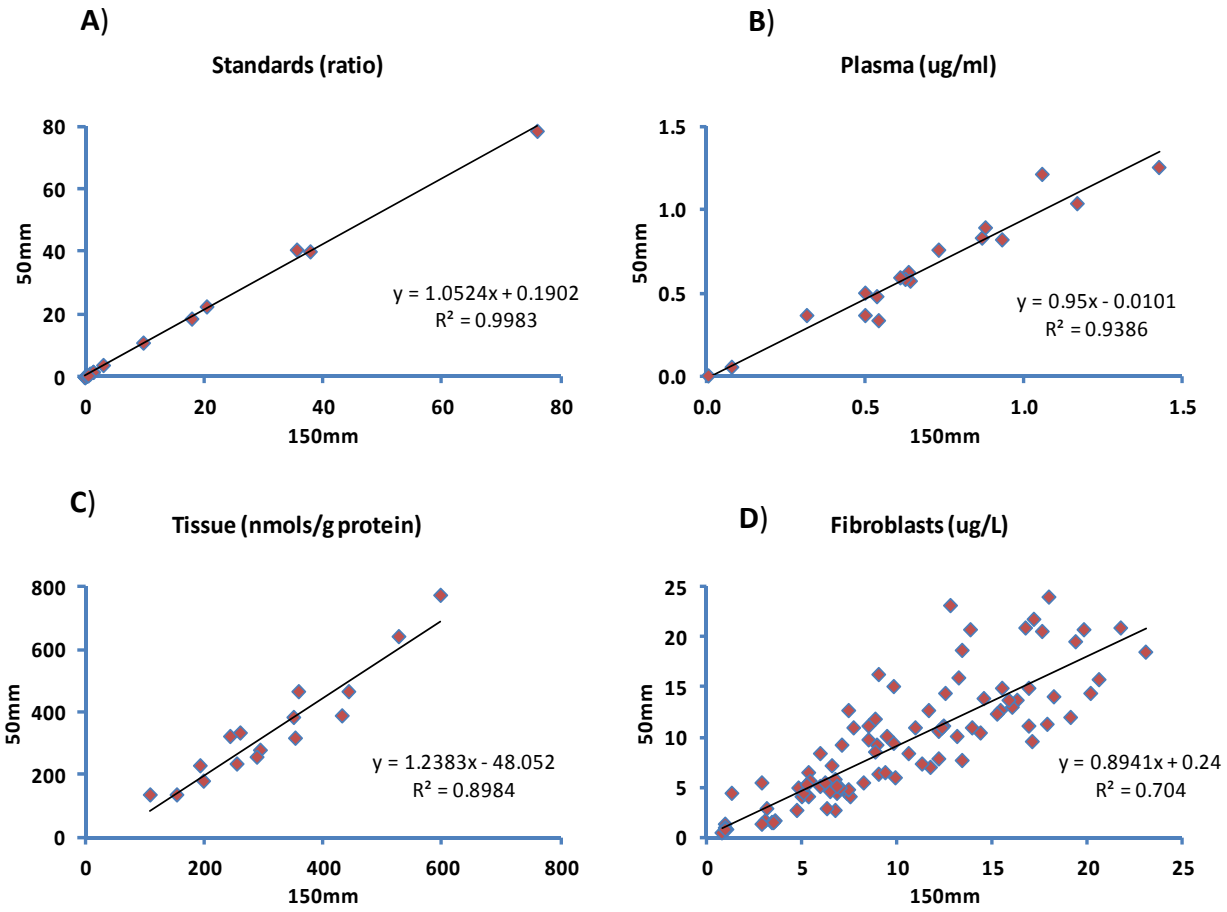


Figure 3.7 Comparison of CoQ10 concentrations determined using 2 different length Kinetex core-shell C18 reversed phase columns (2.1mm I.D, 2.6µm particle size).

Graphs represent the the effect of different samples matrices **A)** standard solutions, **B)** plasma samples, **C)** muscle tissue and **D)** fibroblast cells. All data points were the result of single determinations. P-value, calculated using Spearman rank correlation was >0.05 in all cases.

3.3.2.2 Ubiquinol

Analysis of freshly prepared ubiquinol standards, subjected to extraction conditions, produced significant peaks consistent with the reduced ion pair transitions (897 > 197 & 829 > 197 for CoQ10 and CoQ9 respectively). However, a second significant peak was also consistently present corresponding to the oxidised form. This suggests either incomplete reduction during preparation or instability of the quinols during the work-up procedure. Re-evaluation of $CoQ10H_2$ after 24 hrs at 4°C showed further oxidation had occurred, suggesting fresh standard would need to be prepared daily. Imprecision in the analysis of $CoQ10H_2$ and stringent collection and procedural requirements has been reported by several authors so the decision was made to measure total CoQ10 only in all samples (Yamamoto and Yamashita, 1997; Kaikkonen, 1999; Tang *et al.*, 2001). The quinol ion transitions were still monitored to confirm complete conversion. If warranted, quinol levels could be estimated by sample extraction in the presence and absence of $FeCl_3$ and calculated based on the following equation:

$$[CoQ10H_2] = [\text{total CoQ10}] - [CoQ10]$$

It is worth noting that ubiquinol was not detected in any of the plasma samples tested even in the absence of an oxidising agent. A small peak was detectable in muscle samples. Results did suggest a degree of reduction was occurring within the ESI source as a peak corresponding to the adducts of the ubiquinols was detected with the same retention time as the ubiquinones. However this was often an inconsequential peak and was at least a factor of 10 lower than the ubiquinone transition. Modifications to the scan variables did not prevent this occurring.

3.3.3 Ion Suppression

The infusion experiments with neither CoQ10 nor CoQ9 showed any evidence of significant ion suppression. Injection of plasma extract did produce an area of suppression between 3.4 and 4.5 mins. However this was well outside the expected elution times of the ubiquinone compounds which elute after 7 mins (Figure 3.8).

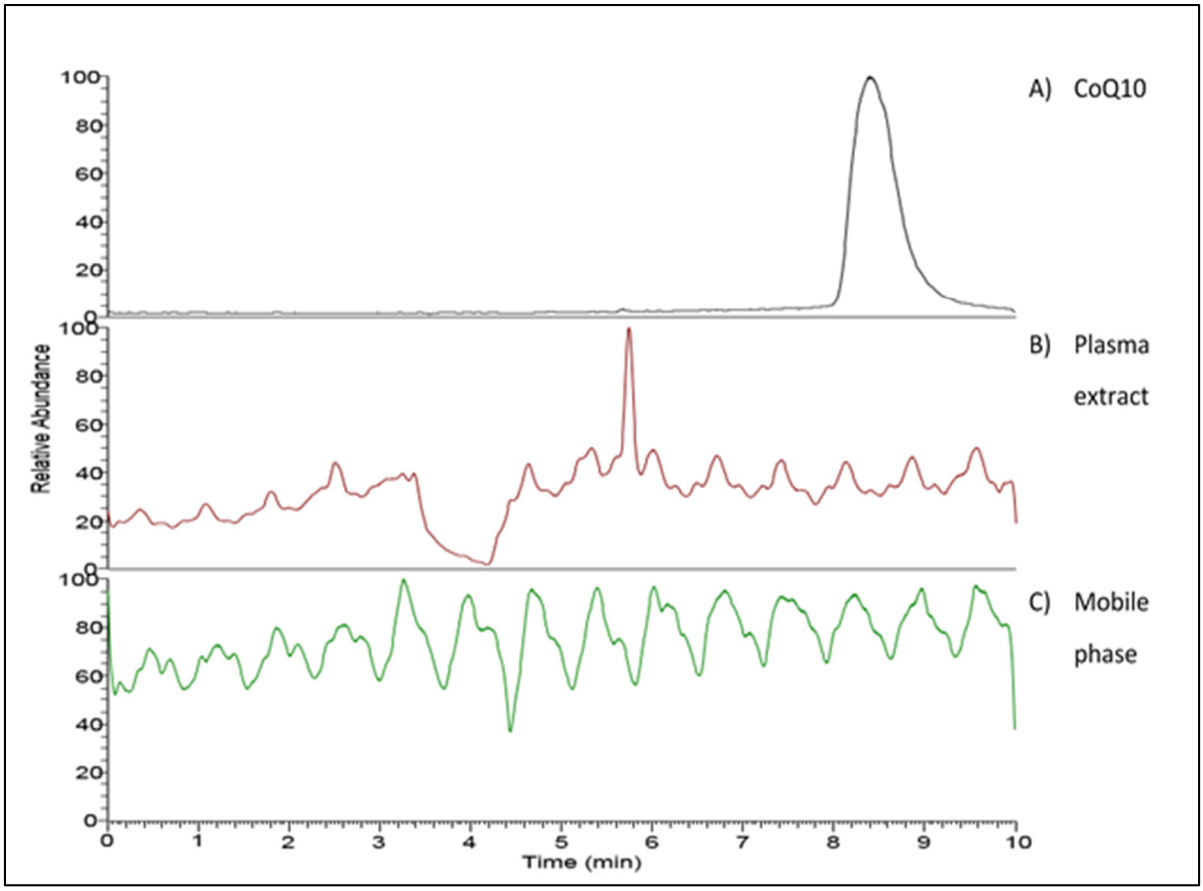


Figure 3.8 Ion suppression infusion experiments for CoQ10.
A) Injection of 10 µg/ml solution of CoQ10, **B)** Infusion of CoQ10 and injection of a plasma extract showing an area of suppression occurring at approximately 4 mins, **C)** Infusion of CoQ10 and injection of mobile phase. Different coloured lines are used to signify different experiments only. The retention time for CoQ10 in this experiment was 8.5 min. Transitions monitored were 827>197, 829>197, 895>197 & 897>197

3.3.4 Linearity and detection limits

Calibration plots were determined using least square regression analysis for CoQ10 by reference of the peak area intensity to CoQ9. The regression statistics and dynamic ranges, which are dependent on the tissue type, are shown in table 3.3.

Limits of detection (LOD) and quantification (LOQ) were estimated as the lowest concentration at which a peak was distinguishable from background noise at the accepted value of S/N ratios. To improve accuracy of measurement at very low concentrations, a calibration plot was prepared using standards with an upper limit of 8.63 ng/ml (10 nmol/L). LOD and LOQ obtained by this method are 1.6 ng/ml and 3.3 ng/ml respectively. These are significantly below the reported normal values obtained from plasma (400 – 2000 ng/ml).

Tissue	Range	unit	Slope	Intercept	Correlation coefficient (R2)	LOD (ng/ml)	LOQ (ng/ml)
Plasma	0 - 20	µg/ml	1.197	4.668e-4	0.9927		
Plasma – low limit	0 - 8.63	ng/ml	1.187e-3	5.365e-4	0.9757	1.6	3.3
Muscle	0 - 2	µmol/L	5.05e-2	3.984e-1	0.9915		

Table 3.3 Regression parameters for the determination of CoQ10.
Curve parameter results are the mean of 3 determinants.

3.3.5 Precision and accuracy

Precision and accuracy were evaluated by relative standard deviation (RSD) and relative error (RE) and are shown in Table 3.4. Reproducibility for peak area intensity of 5 repeat injections was 7.7 % variation for CoQ9 and 5.7 % for CoQ10. Sample extracts stored at 4°C for 48 hrs before re-analysis remained relatively stable with an average change of 1.65 % (SD 6.95, p = 0.9, n = 40) and 72 % of extracts showed less than 5 % variation.

	Intra- day (n=5)				Inter- day (n=20)			
	Mean CoQ10 (ug/L)	sd	RSD %	RE %	Mean CoQ10 (ug/L)	sd	RSD %	RE %
Sample 1	701	33	4.7	6.9	719	38	5.3	9.0
Sample 2	1415	95	6.7	15	1312	113	8.6	6.6
Sample 3	1175	72	6.1		952	106	11.1	

Table 3.4 Precision and accuracy of the proposed method for CoQ10 quantitation in plasma.

3.3.5.1 Calibrator Standard

The Chromsystem Calibration Standard (700 µg/L) initially failed to produce a usable chromatographic profile for either analyte when extracted using the sample procedure given in section 3.2.6.1. Results were eventually obtained if the hexane extraction was performed without first removing any precipitate. No significant difference was seen when this modification was applied to plasma samples. This suggests a matrix effect unique to this product which binds both the CoQ10 and the supplementary CoQ9 and removes them from solution. Removal of the pellet did aid in transfer of the organic phase therefore this step was retained for patient extracts but the calibrator was extracted using this method modification. The mean value obtained was 705.98 µg/L (n = 10) and validates the accuracy of the assay.

3.3.6 Recovery

A plasma sample analysed following saline dilution, and muscle homogenate spiked with authentic CoQ10 demonstrated excellent linear correlation (Figure 3.9). Recovery rates were 87 – 114 % for plasma and 73 – 91 % in the muscle extracts.

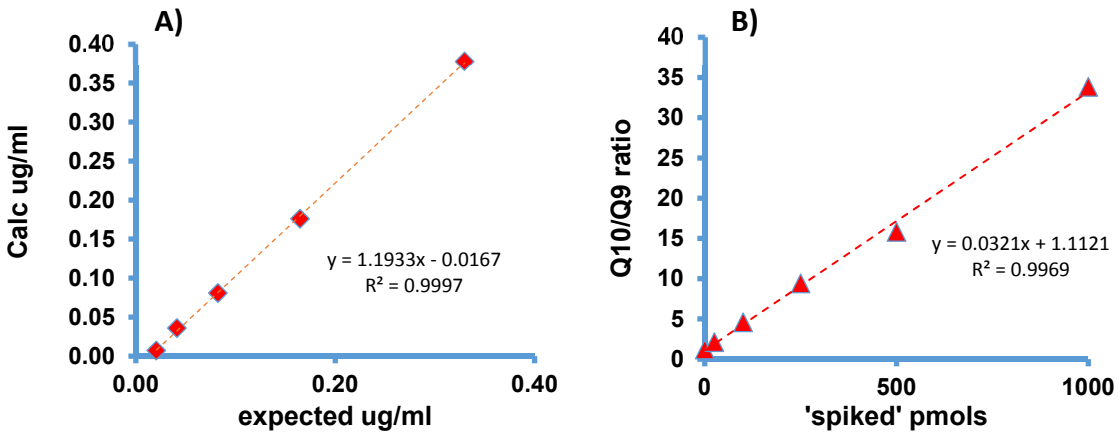


Figure 3.9 Effect of sample dilution on CoQ10 levels in serum (A) and spiking experiments in muscle homogenate (B). Results are the mean of 3 replicates.

Recovery of known concentrations of CoQ10 spiked into a pooled plasma sample and extracted in triplicate are shown in table 3.5.

Concentration added	Measured concentration (n = 3)	% Recovery
0	953.0	109.7
200	1172.4	109.7
500	1430.1	95.4
1000	1901.0	94.8

Table 3.5 Recovery of CoQ10 in spiked plasma. Concentrations are reported in µg/L.

3.3.7 Sample extraction validation

3.3.7.1 Plasma/serum

Anonymised plasma or serum samples from 44 individuals (male:female ratio 28:16) were analysed by the proposed method. The mean CoQ10 concentration was 0.808 µg/ml and the range 0.28 – 2.23 µg/ml (95 % CI), which is in good accordance with other authors. No correlation between patient age or sex (p = 0.8262) was detected (Figure 3.10).

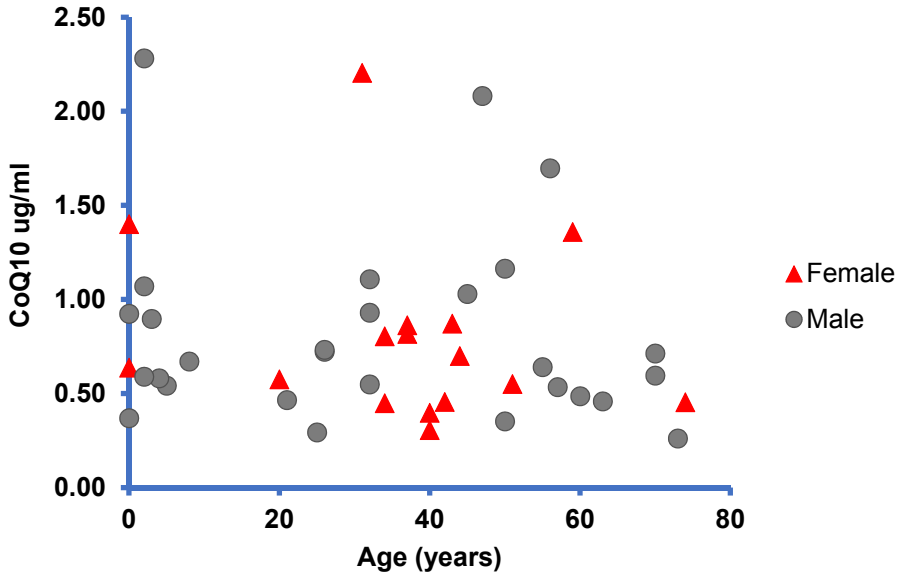


Figure 3.10 Age and sex related correlation of CoQ10 concentration in plasma and serum.
(n = 44, Female = 36.3%).

3.3.7.2 Muscle

Early experiments with muscle tissue gave highly variable quantitative results (mean 18.4 ρmol/mg tissue, RSD 30.3 %). It was possible this was due to the instability of ubiquinol and the extent of conversion to ubiquinone. Modifications aimed at measuring total CoQ10 by ensuring complete oxidation of the quinols (Figure 3.11) did show improvement but RSD was still 23.48 % (mean 27.48 ρmol/mg tissue). To try to improve these statistics further it was decided to correlate the total CoQ10 to protein content. This involved homogenising the sample in aqueous solution prior to the organic extraction phase. This significantly improved reproducibility with RSD for 20 extractions recorded at 12.5 % (mean 243.4 ρmol/mg protein).

A reference range calculated using biopsy samples from twenty patients with no diagnosis of mitochondrial disease was 283.69 (121.9 – 519.1) ρmols/mg protein (mean, 95 % CI). Ubiquinone deficiency is rare so it was not possible to obtain muscle tissue with a known low level. However the assay was validated using an anonymised sample received for diagnostic analysis. Under the conditions described, this sample gave a significantly low CoQ10 concentration (17 ρmols/mg protein) which was confirmed by results obtained from the current referral laboratory, the Neurometabolic Unit, University College Hospital NHS Foundation Trust, Queens Square, London (5 ρmols/mg, reference range 140 – 580 ρmols/mg).

3.3.7.3 Fibroblast cell lines

Authors have reported normal values, in nmol/g protein, of 120 ± 32 (Mollet *et al.*, 2007), 42 - 108 (Haas *et al.*, 2009), and 107 – 145 (Montero *et al.*, 2008). Control cell lines harvested and analysed by the proposed method suggest a reference range 73 – 190 nmol/g protein (n = 10). Between batch precision was 13.03 % (n = 6). Extracting the cells without addition of CoQ9 estimated the contribution of endogenous CoQ9 to be less than 2 % of CoQ10 suggesting this would have a negligible impact on quantitation. Thus CoQ9 can be used as a suitable internal standard.

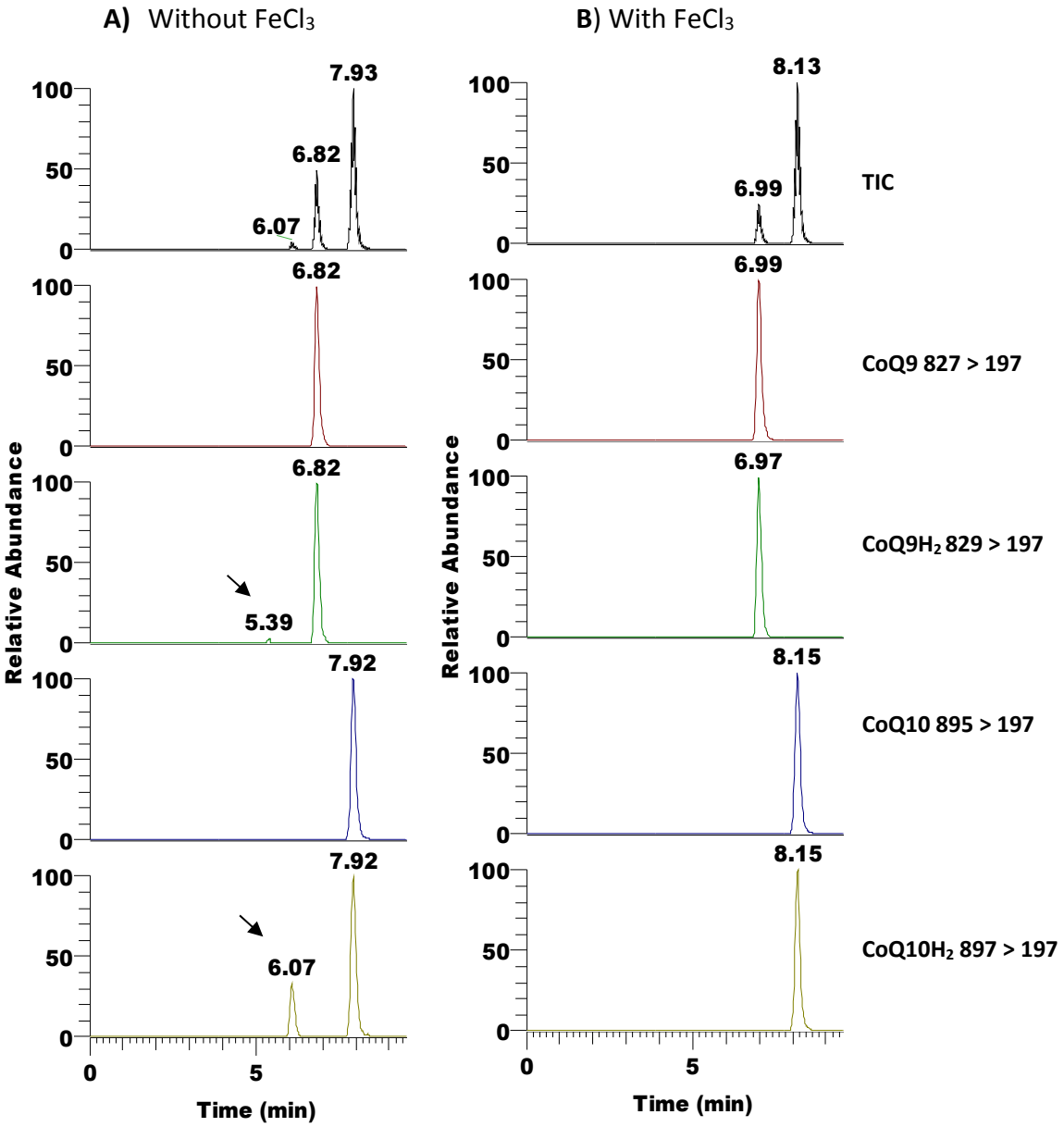


Figure 3.11 LC-MS/MS chromatograms (TIC and extracted ions) for coenzyme Q extracted from muscle tissue in A) the absence and B) presence $FeCl_3$.
The peaks at 5.39 and 6.07 in panel **A** represent the quinols which are completely oxidised during the extraction in the presence of $FeCl_3$ as shown in panel **B**.

3.3.7.4 Blood spots

Results for CoQ10 recovery from dried blood spots, calculated using a calibration curve with the range 0 – 2500 nmol/L are shown in table 3.6. The calculated CoQ10 concentration in unspiked blood was 12.4 % lower compared to the corresponding plasma concentration of (639.9 vs 730 nmol/L).

Added nmol/L	Expected nmol/L	Measured nmol/L[a]	Difference between replicates %	RE %
0	639.97	639.97	6.64	
10	649.97	619.68	5.13	-4.66
50	689.97	671.40	3.29	-2.69
100	739.97	860.56	11.88	16.3
250	889.97	939.53	1.84	5.57
500	1139.97	966.75	1.80	-15.2
1000	1639.97	1529.14	5.96	-6.76
2000	2639.97	2592.38	4.04	-1.8

[a]mean of 2 determinations

Table 3.6 Analysis of CoQ10 in spiked dried blood spots. Duplicate extractions at each level were performed.

Twenty nine independent comparisons were made between CoQ10 concentration in plasma or dried blood spot. In general, results in plasma, (616.6 nmol/L [507.7 – 725.5 nmol/L], mean [95 % CI]) were higher than in whole blood (493.2 nmol/L [391.7 – 594.7 nmol/L]) but this did not reach statistical significance (p = 0.065) (Figure 3.12).

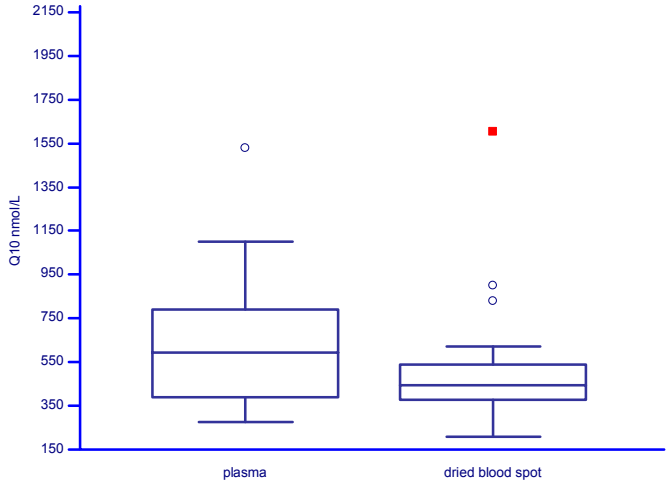


Figure 3.12 Comparison of CoQ10 concentrations analysed in plasma versus dried blood spot samples.
The horizontal bar in the box and whisker plots represents the median and the box the interquartile range (IQR). Circles and red squares represent results which exceed plus/minus 1.5 and 3 times the IQR respectively. n=29

3.3.8 Direct MS/MS analysis

Calibration curves, determined in triplicate, for standard CoQ10 were linear over the same range as measured using LC-MS/MS (data not shown). The profiles produced were clean and peaks easily identifiable. Plasma samples however, analysed under the same conditions had a pronounced matrix effect with significantly lower intensities (factor X20) and higher background. Although it is still possible to identify relevant peaks, using precursor or SRM scans, the S/N ratio is low which would infer higher detection limits (Figure 3.13). Results suggest LOD to be about 2 µg/L using SRM and 5 µg/L in scan mode.

The LC-MS/MS results suggested a small amount of reduction may be occurring at the ESI source and this is confirmed by the parent scan (*m/z* 197) which indicates the main peaks corresponding to the methylamine adduct of ubiquinone but also peaks at M+1 and M+2 which are consistent with the mass of the semiquinone and ubiquinol. This was seen during analysis of standard compounds and plasma samples.

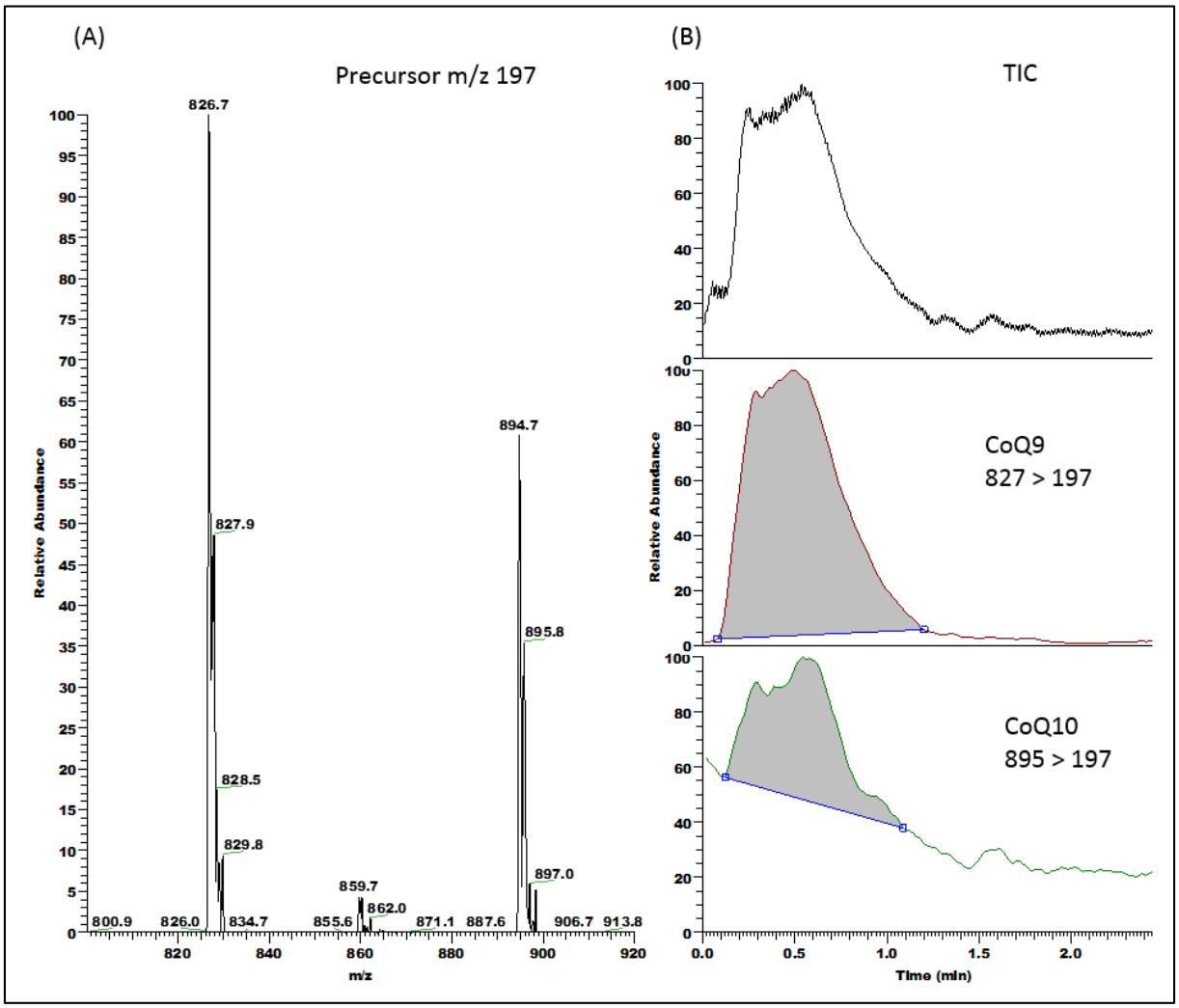


Figure 3.13 Analysis of coenzyme Q in plasma using flow injection MSMS analysis acquired using (A) precursor ions of *m/z* 197 and (B) SRM scan modes.
The precursor scan shows the detection of M+1 and M+2 ions, representing the methylamine adducts of semiquinone and ubiquinol respectively. Poor peak profiles in SRM mode for CoQ10 suggest reduced sensitivity and a high detection limit.

Plasma samples prepared and analysed by LC-MS/MS were subsequently re-analysed by direct MS/MS. The method correlation and Bland-Altman plots are shown in figure 3.14. In general, low concentrations were slightly higher using MS/MS and the reverse true for high concentrations. Mean difference between methods was 8.3 % (range -24 – 38 %) although this was not statistically significant (*p* = 0.6127). The preliminary results suggest MS/MS analysis without prior chromatographic separation is feasible for quantification of CoQ10 for

samples within the normal physiological range. However, accuracy may decrease at very low levels seen in deficient samples. Further evaluation is needed and was outside the scope of this project.

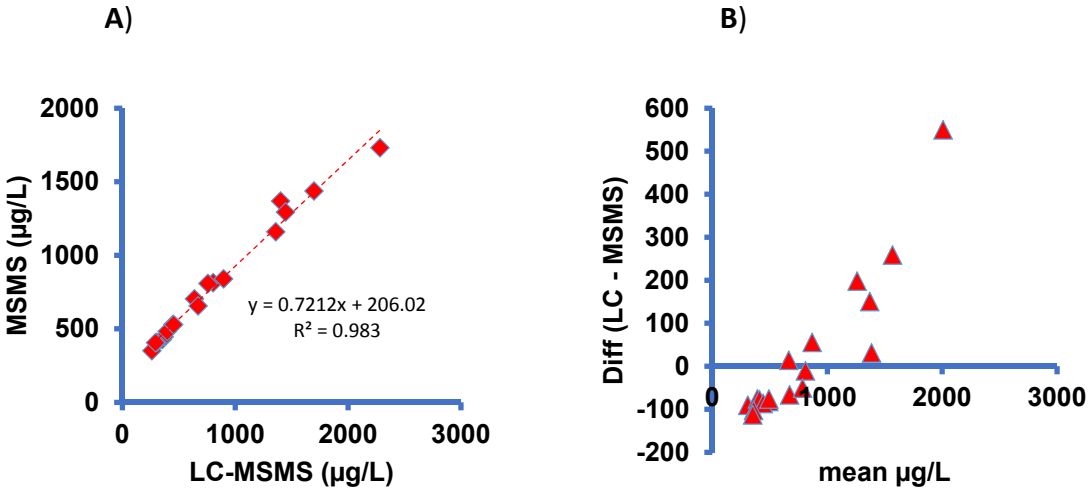


Figure 3.14 Comparison of CoQ10 values in plasma samples analysed by LC-MS/MS and direct MS/MS acquisition (n = 18).
Although both methods show good correlation (graph **A**) the Bland-Altman plot (graph **B**) indicates low concentrations of CoQ10 are potentially overestimated when analysed by direct MSMS scanning and high concentrations are underestimated compared to analysis using LC-MS/MS.

3.4 DISCUSSION

3.4.1 Development of a tandem mass spectrometry method for analysis of CoQ10

Tandem mass spectrometry enables high mass accuracy accumulation of data with great sensitivity and high specificity. The use of a chromatographic column coupled to the mass analyser enhances the specificity of analytes of interest. The data presented here demonstrates a suitable electrospray ionisation LC-MS/MS method has been established to quantitate CoQ10 in a variety of biological samples. Further specificity is obtained by using the analyser in MRM mode which measures the production of specific fragment ions produced by collision induced dissociation of the precursor ion. In order to maximize the ionisation efficiency of CoQ, an alkylammonium adduct ion was produced by incorporation of methylamine into the mobile phase reagents. The [M+CH3NH3]+ ion created provided ample signal intensity even at very low concentrations and enabled a detection limit of 1.6 ng/ml, a hundred fold below physiological limits in plasma.

The MS/MS instrument used in this assay is used for other clinical applications so it was important to use mobile phase solvents that do not affect other procedures. The lipophilic nature of CoQ10 enabled the use of reverse phase chromatography with organic solvents such as methanol and isopropanol in the mobile phase. These have not been shown to cause any adverse effects with existing applications. An attempt was made to use a standard C18 fully porous column as these tend to be cheaper (data not shown). However the Kinetex core-shell technology, which consists of a homogenous porous shell structure, provided significantly improved peak profiles with reduced band broadening, better resolution, a shorter run time and moderate backpressure using a low flow rate of 100 µl/min. Therefore this column was considered ideal for use in this application and proved to be very robust.

Although method development was primarily performed using a 150 mm column, limited work was undertaken using a short 50 mm column with identical internal dimensions. Acceptable chromatography was still achieved in a run time of under 3 min, compared to 10 minutes using a 150 mm column. This use of this column would be hugely advantageous to reduce analysis time and maximising efficiency. However, the robustness of this short column for the application described has yet to be explored.

Owing to the exceptional ionization properties and unique ion transitions it was possible, with this instrument, to develop a scan function using direct flow injection MS/MS analysis. Limited comparisons between the methods have been performed but data is encouraging although it remains to be seen if low concentrations are determined with the same level of accuracy. Direct MS/MS analysis would greatly simplify analytical procedures, reduce column expense, and significantly improve sample throughput. It would also enable the analysis to be streamlined into the existing instrument workflow. To my knowledge, this method has not been reported in the literature.

3.4.2 Sample preparation

The method was developed to enable quantitation of CoQ10 in a variety of biological samples. The extraction procedures employed were a modification of the Bligh and Dyer method for lipid extraction. Hexane was used to eliminate the use of the more toxic chloroform and to simplify partitioning and transfer of the organic layer from the aqueous fraction. The method is simple yet reproducible and analyte recovery was within 10 % of the nomimal value in plasma matrix. Small sample volumes were used; 100 µl plasma and approximately 10 mg tissue, and detection limits were significantly below physiological values indicating the assay has sufficient sensitivity to detect deficient samples. Minimal peak drift was detected over 40 continuous injections. However, variation was more pronounced between analyses performed on different days. This did not significantly affect quantitation.

Coenzyme Q10 is a major constituent in plasma membranes. Homogenisation and a freeze/thaw cycle were used during extraction from tissue and fibroblasts to cause maximal disruption of the membranes and release of the CoQ10. Cell debris was removed by a short centrifugation step before liquid-liquid extraction of the lipids. Correlating the CoQ10 concentration to the protein content of the extract dramatically improved the precision. It was suggested to try using the cholesterol content analysed simultaneously in the extract but under the experiment protocols described here, cholesterol failed to produce any significant parent or daughter ions and would thus probably necessitate the use of a derivatisation stage, which would add time and complexity to the assay.

In any mass spectrometry application, a good mix of injected sample and mobile phase is required to obtain good peak shapes. Direct injection of the hexane extraction phase was not

possible as this proved incompatible with the methanol based mobile solvents. Evaporating the extract to dryness and reconstituting in 100 % methanol ensured good recovery from the sample tube and adequate column retention and chromatographic profiles. Only 10 µl of sample was injected on to the column, at a flow rate of 100 µl/min, thus further minimising any incompatibility of phases.

Analysis of control plasma samples (range 0.28 – 2.23 µg/ml) did not show any age related correlation although it is acknowledged the oldest person on this group was 74 years old. A potential change in CoQ10 concentration beyond this age cannot be inferred from this cohort.

Endogenous CoQ9 accounted for a maximum of 3 % of the CoQ10. Thus in accordance with other authors and due to the lack of commercially available standards it is appropriate to use CoQ9 for internal standardization. With the exception of the muscle biopsies, which had been cryopreserved immediately after being taken, reduced CoQ10 was not detectable in the other sample types. This implies oxidation had either occurred prior to separation and storage (all samples were processed for storage within 12 hrs of receipt in the lab) or that the sample work-up, which involved an evaporation stage, promoted complete conversion of the quinol. For the purpose of this research measurement of the redox state was not considered to be essential so all measurements were on based on total CoQ10.

Analysis of CoQ10 using dried blood spots has the advantage of minimal sample requirement and ease of transport. It also has potentially improved long term stability although this has not yet been determined. The assay developed here produced good recovery of analyte from the filter paper disk using methanol extraction followed by liquid-liquid extraction in hexane. Although only limited investigations have been performed the results obtained were comparable to those detected in plasma samples which suggests dried blood analysis of CoQ10 may be a viable alternative to liquid sample analysis.

It is acknowledged that CoQ10 in white blood cells is considered a more reliable measure of endogenous CoQ10 availability for diagnosis of ubiquinone deficiency than plasma. Analysis in a limited number of leucocytes has been investigated but the data is not presented as part of this project.

3.4.3 Validation of method

Ion suppression was examined and no evidence of interference was seen. The assay was linear to at least one order of magnitude greater than the expected physiological range in human plasma and the limits of detection, at 1.6 ng/ml, were significantly below endogenous concentrations, estimated to be > 280 ng/ml.

The method developed showed good recovery of analytes and reproducibility of injections and quantitation were excellent. Furthermore, the values obtained here are in good agreement with published reports (Duncan *et al.*, 2005; Paliakov *et al.*, 2009) and the assay was validated for use in muscle tissue by the detection of a deficient sample which was in good accordance with the reference laboratory.

3.4.4 Conclusion

Extraction procedures have been established for the quantification of CoQ10 in a variety of tissue types, including plasma, muscle biopsy, fibroblast cells and dried blood spots. With the exception of blood spots, in which, reference values have not previously been reported, all assays produced normal values comparable to those reported in the literature. Owing to the high degree of variability in tissue extracts, it is recommended to correlate the CoQ10 concentration to another endogenous analyte such as protein. The LC-MSMS assay developed in this study is reliable and suitable for the quantitation of total coenzyme Q10 in a variety of biological specimens.

Chapter Four

Chapter 4. Distribution of Coenzyme Q10 in Lipoprotein Particles

4.1 INTRODUCTION

The lipophilic nature of CoQ10 constrains its transport via the aqueous environment of the blood stream. Consequently, lipoproteins are required to emulsify CoQ10 and other lipid molecules, including cholesterol and triglycerides, to allow movement in and out of cells. The biochemical assembly of lipoproteins consists of phospholipids and apolipoproteins, with the hydrophilic head groups orientated outwards and the hydrophobic tail regions towards the core. This structure allows the lipoprotein particle to be water soluble whilst shielding the lipophilic contents from the water (Figure 4.1).

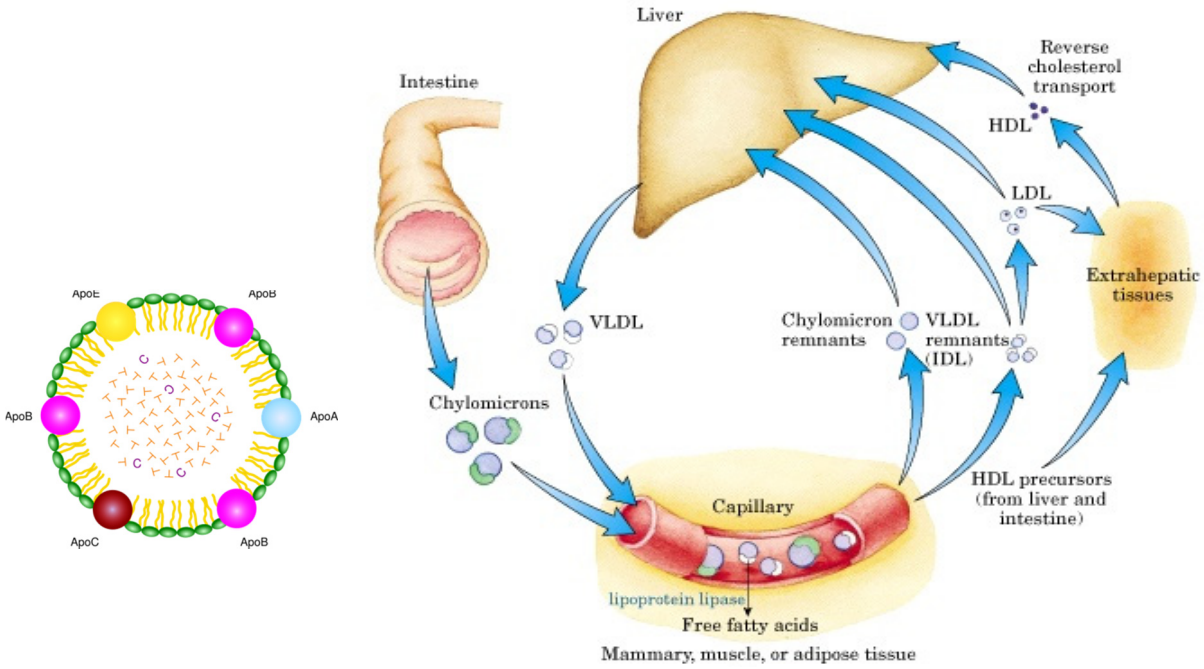


Figure 4.1 Generalized structure of a lipoprotein molecule and the transport mechanism for lipids.
The exogenous pathway transports fats from the diet via chylomicrons whilst the liver provides the central platform for the endogenous pathway. T=triglyceride C=cholesterol (taken from (Nelson *et al.*, 2008)

The size and density of lipoprotein particles, which are inversely related, correlates with their lipid to protein ratio and is used as a basis for classification (Figure 4.2). Chylomicrons are predominately composed of triglycerides and carry fats from the intestine into the

bloodstream, releasing fatty acids and glycerol for energy or storage, before ultimately being hydrolysed by the liver. Endogenously synthesized lipids are released from the liver packaged as VLDL particles. VLDL are rich in triglycerides and, like chylomicrons, circulate in the blood releasing fatty acids via the action of lipoprotein lipase (LPL). The delipidated particle, known as LDL, contains a relatively high cholesterol content and increased protein composition. These latter particles bind to LDL receptors on the target tissues, become endocytosed into the cell and release their contents, chiefly cholesterol esters, for use by the cell. Alternatively, they may be absorbed by the liver and recycled. Small, dense HDL takes part in reverse cholesterol transport, picking up excess cholesterol from tissue for transport back to the liver for degradation (Figure 4.1).

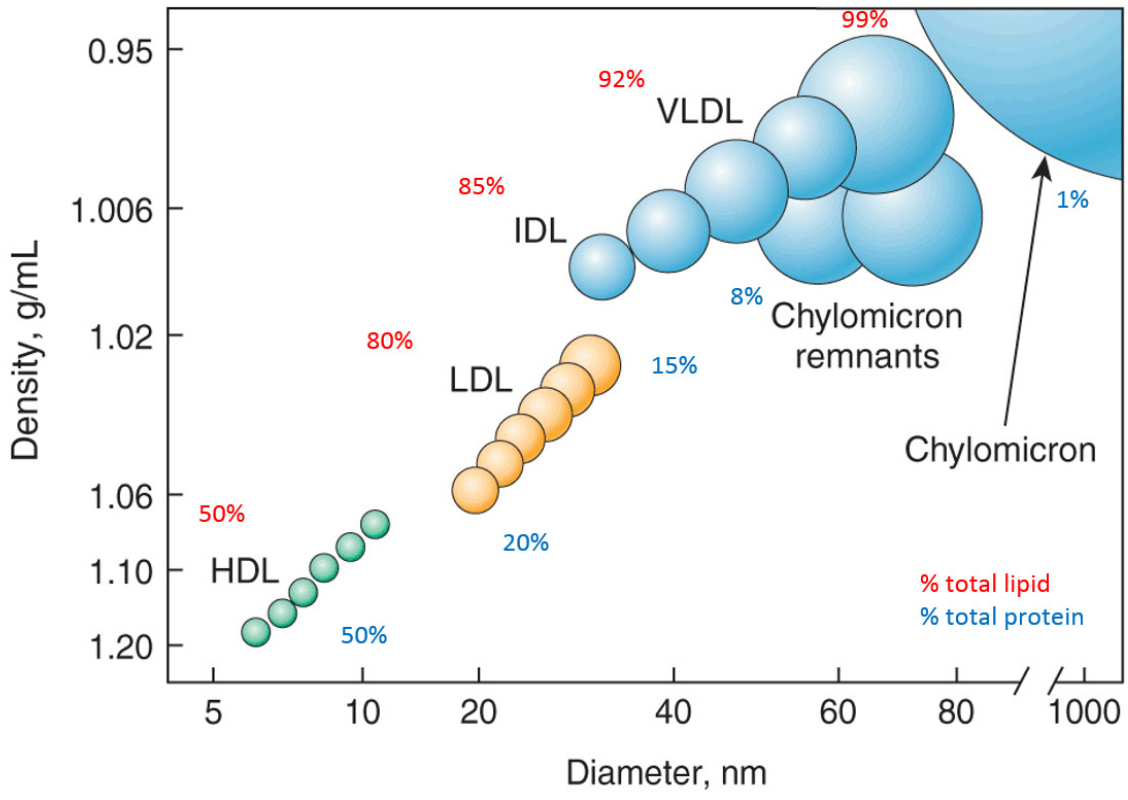


Figure 4.2 Density and size distribution of lipoprotein particles.
Density is determined by ultracentrifugation and is underpinned by the ratio of lipid to protein within each molecule. Adapted from (Rader and Hobbs, 2005).

High levels of LDL-C, the amount of cholesterol within LDL, are causally associated with an increased risk of atherosclerosis and cardiovascular disease, and a key step in this development is LDL-C oxidation (Jialal and Devaraj, 1996). A disturbance between an oxidant challenge and the antioxidant protection results in oxidative stress and an enhanced rate of LDL uptake by macrophages, the formation of foam cells, and recruitment of monocytes to the area leading to deposition in the arterial wall and plaque formation which can trigger a myocardial infarction. Concomitant with the transport of cholesterol and triglycerides, endogenously synthesized ubiquinone, or more importantly ubiquinol, is also released from the liver in VLDL and predominately transported in LDL (Perugini *et al.*, 2000). An important lipid-soluble antioxidant, it has in fact been shown that CoQ10 is more active and is preferential used over the principle plasma antioxidant α-tocopherol during the early stages of LDL peroxidation (Stocker *et al.*, 1991).

Furthermore, in addition to providing defence for circulating lipoproteins, CoQ10 aids in maintaining normal endothelial processes in the arteries, such as immune functions, coagulation, adhesion and electrolyte content of the intravascular space (Tiano *et al.*, 2007). Research has linked endothelial dysfunction to postprandial triglyceride levels (Gaenzer *et al.*, 2001). Decreased bioavailability of nitric oxide (NO) due to an increase release of ROS is considered to be a hallmark of endothelial dysfunction and results in the inability of arteries to fully dilate in response to stimulus. CoQ10 counteracts the oxidation of nitric oxide by ROS to maintain a balance between vasodilation and vasoconstriction. Support for this is demonstrated by supplementation of CoQ10 which has been shown to be effective in ameliorating endothelial dysfunction (Belardinelli *et al.*, 2006).

4.1.1 Hyperlipidaemia

Primary, or familial, hyperlipidaemias are defined by elevated levels of lipids in the blood due to a genetic cause. Whilst numerous genetic mutations are known, many patients exist without a molecular diagnosis (García-Otín *et al.*, 2007). Classification is based on the Fredrickson classification (Beaumont *et al.*, 1970) according to the phenotypes of lipids elevated and the pattern of lipoproteins and can be broadly defined as shown in table 4.1. Type II is the most prevalent (approx. 1 in 250) with type III and I having relatively low incidence in the population of between 1 in 5,000 and 1 in 1,000,000 respectively (Marshall and Bangert, 2008).

Hyperlipidaemia	Synonym	Increased lipoprotein	Increase lipid
Type I	Familial chylomicronaemia	**Chylomicrons**	Triglycerides
Type IIa	Familial hypercholesterolaemia (FH)	**LDL**	Cholesterol
Type IIb	Familial combined hyperlipidaemia (FCH)	**LDL & VLDL**	Cholesterol & triglycerides
Type III	Familial dysbetalipoproteinemia	**IDL (floating beta VLDL)**	Cholesterol & triglycerides
Type IV	Familial hypertriglyceridaemia	**VLDL**	Triglycerides
Type V		**VLDL & Chylomicrons**	Triglycerides

Table 4.1 Fredrickson classification of hyperlipidaemias.
Data taken from (Beaumont *et al.*, 1970).

Hyperlipidaemias carry an increased risk for cardiovascular disease and endothelial dysfunction. Specifically, LDL-C or the ratio of total cholesterol to HDL-cholesterol (TC/HDL-C) are positively correlated with increased risk of atherogenicity (Tomasetti *et al.*, 1999). Reducing dietary intake of fat is recommended to decrease both blood cholesterol and triglyceride levels but this is often insufficient and pharmacological intervention is required. Statin drugs are commonly prescribed to reduce LDL-C levels whilst fibrates are employed to decrease triglyceride production. A combination approach may be undertaken in mixed hyperlipidaemias with close medical supervision due to an increase risk of side effects such as myopathy (Seehusen *et al.*, 2006).

Although the distribution of CoQ10 has been defined in healthy individuals and suggests approximately 60 % is located in the LDL fraction (Tomasetti *et al.*, 1999; Perugini *et al.*, 2000), little evidence exists regarding this important antioxidant in dyslipidaemic patients. The goal of lipid reduction therapies is to reduce LDL cholesterol levels thus in effect the carrying capacity of CoQ10, and hence the tissue availability may also be reduced. Dyslipidaemia, in particular hypertriglyceridaemia, is an important predisposing factor to a number of conditions including endothelial dysfunction, diabetes mellitus and pancreatitis mediated by ROS (Bae *et al.*, 2001; Hegele *et al.*, 2014). Consequently, the distribution of CoQ10 in the lipoprotein fractions in these patients may be a contributing factor to the pathogenesis of disease.

4.1.2 Aim

To determine the lipid composition in lipoprotein fractions and examine the distribution of coenzyme Q10 in dyslipidaemic patients to determine if there are differences which might contribute to pathogenesis mediated by free radicals.

4.1.3 Acknowledgement

Lipoprotein fractionation was performed by Joan Ellis, Blood Sciences Department, Special Endocrine Section, Royal Victoria Infirmary, Newcastle upon Tyne NHS Foundation Trust.

4.2 MATERIALS AND METHODS

4.2.1 Specimens

Samples were available from 51 patients (22 females and 29 males), age range from 18 – 84 years (mean 52 years). All samples were received at the Blood Sciences department RVI, Newcastle, from patients attending a lipid clinic under the care of Dr Dermot Neely for investigation of hyperlipidaemia and were classified using the Fredrickson classification as stated in table 4.2. For the purpose of this study all samples were anonymised.

	Hypercholesterolaemia		**Hypertriglyceridaemia**	
Classification	**IIa**	**IIb**	**IV**	**V**
Subjects	12	24	5	10

Table 4.2 Hyperlipidaemic class of study samples

4.2.2 Lipoprotein isolation

Lipoprotein fractions were isolated from either 0.5 ml or 1 ml EDTA blood using the betaquantification procedure as described in chapter 2, section 2.7. Four fractions; plasma, infranatant d > 1.006 g/ml [LDL + HDL], VLDL d < 1.006 g/ml and HDL supernatant, were isolated for each sample and stored at -40°C until analysis.

4.2.3 Methods

Cholesterol and triglycerides were measured in each fraction using the enzymatic procedures and Roche modular analyser as described in section 2.5.

CoQ10 was analysed using the LC-MSMS method developed and described in chapter 3. In brief, 100 µl each of sample and internal standard (CoQ9 0.5 µg/ml) plus 200 µl methanol were vortex mixed in a microtube. The CoQ10 was double extracted using 500 µl hexane, the organic phase evaporated to dryness using nitrogen then reconstituted in 100 µl methanol and transferred to 96-well microplate for analysis. A 150 mm Kinetex core-shell C18 column was used to provide the chromatographic separation. Formulae were applied to calculate the true values in each fraction as described in section 2.7.

4.3 RESULTS

4.3.1 Analyte recovery

The recovery rate of cholesterol in lipoprotein fractions was between 93 - 103 %. Triglyceride recovery was 90 - 110 %.

Using the method as described for extraction of CoQ10 in plasma, recovery rates from the lipoprotein fractions were highly variable (range 46 - 124 %). Inclusion of an additional 200 µl methanol added prior to hexane extraction to aid delipidation of the lipoproteins significantly improved recovery rates. All extracts used in the calculations had recovery of CoQ10 between 87 - 113 %.

4.3.2 Lipid profiles in plasma and lipoproteins

The absolute concentrations of cholesterol and triglycerides detected in plasma and within each lipoprotein fraction are reported in table 4.3. No significant differences were observed between the two groups for total cholesterol or LDL-triglycerides. As expected, type IV and V had significantly higher total triglyceride levels than type II (mean 12.33 vs 3.32 mmol/L) and lower LDL-C (1.71 vs 5.12 mmol/L), consistent with impaired VLDL processing. The variation between the two populations is depicted in figure 4.3.

Fraction		All (n=51)		Type II (n=36)		Type IV + V (n=15)		P-value*
Cholesterol	Plasma	**7.77**	(4.64-12.01)	**7.86**	(5.18-12.02)	**7.21**	(4.09-11.66)	0.0898
	LDL	**4.14**	(0.54-9.15)	**5.12**	(1.13-9.86)	**1.71**	(0.41-3.33)	<0.0001
	VLDL	**1.2**	(0.10-9.92)	**0.76**	(0.10-3.68)	**4.66**	(1.62-10.40)	<0.0001
	HDL	**1.22**	(0.56-3.56)	**1.45**	(0.82-3.82)	**0.84**	(0.33-1.23)	<0.0001
Triglycerides	Plasma	**3.89**	(0.71-30.41)	**3.32**	(0.69-7.57)	**12.33**	(3.63-34.47)	<0.0001
	LDL	**0.33**	(0.07-0.98)	**0.40**	(0.063-0.94)	**0.32**	(0.16-1.07)	0.4609
	VLDL	**2.7**	(0.19-28.63)	**2.48**	(0.17-6.81)	**11.30**	(2.59-33.03)	<0.0001
	HDL	**0.47**	(0.24-1.17)	**0.44**	(0.23-0.70)	**0.67**	(0.35-1.32)	0.004

Values are geometric mean (95% CI) in mmol/L. *Significance between Type II and Type IV+V calculated using Mann-Whitney U test

Table 4.3 Concentration of major lipids in plasma and lipoprotein fractions isolated from dyslipidaemic subjects.

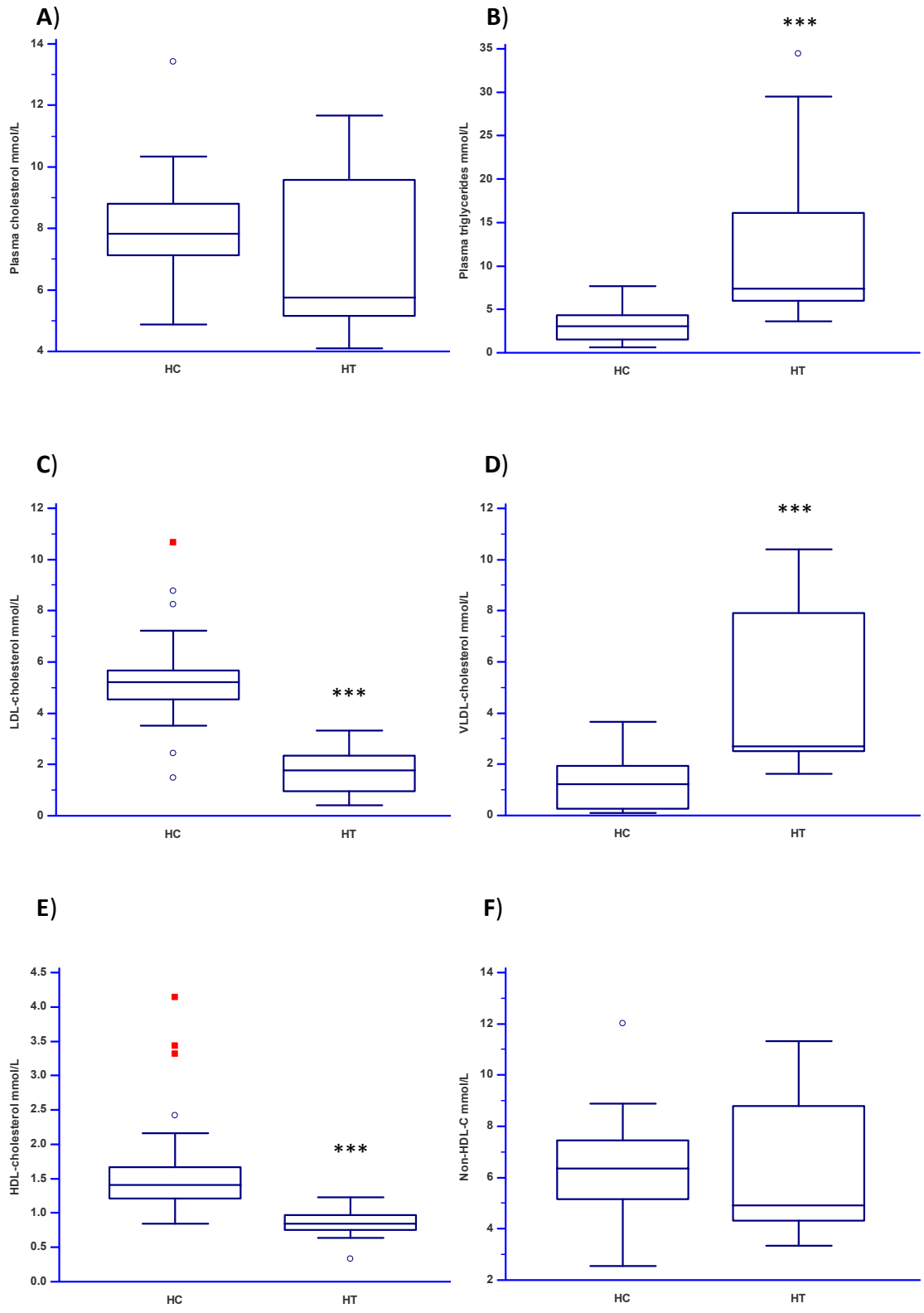


Figure 4.3 Comparisons of lipid content in plasma and lipoprotein fractions between dyslipidaemic subjects. A) Total cholesterol, **B**) total triglycerides, **C)** LDL-cholesterol, **D**) VLDL-cholesterol, **E)** HDL-cholesterol, and **F)** non-HDL-cholesterol. HC- hypercholesterolaemic (Fredrickson classification type IIa & IIb n=36), HT – hypertriglceridaemic (type IV & V n=15). ***p < 0.0001 calculated using Mann-Whitmey U test Circles and red squares represent results which exceed plus/minus 1.5 and 3 times the IQR respectively.

Type II subclasses differed in triglyceride (1.58 [0.72 – 3.7] vs 4.01 [0.78 – 7.65]) and VLDL-C (0.39 [0.1 – 1.39] vs 1.62 [0.11 – 3.61]) concentrations in IIa (FH) and IIb (FCH) subjects respectively ($p < 0.001$). Total cholesterol and LDL-C were comparable but HDL-C was higher in the IIa subjects (2.15 [1.23 – 4.14] vs 1.3 [0.84 – 2.35]) ($p = 0.0011$).

Plasma cholesterol significantly correlated with LDL-C ($r = 0.411$, $p = 0.003$) but not VLDL-C or HDL-C. The correlation was stronger between non-HDL cholesterol and plasma cholesterol ($r = 0.924$, $p < 0.001$) but only weakly correlated with total triglycerides ($r = 0.316$, $p = 0.023$) (Figure 4.4).

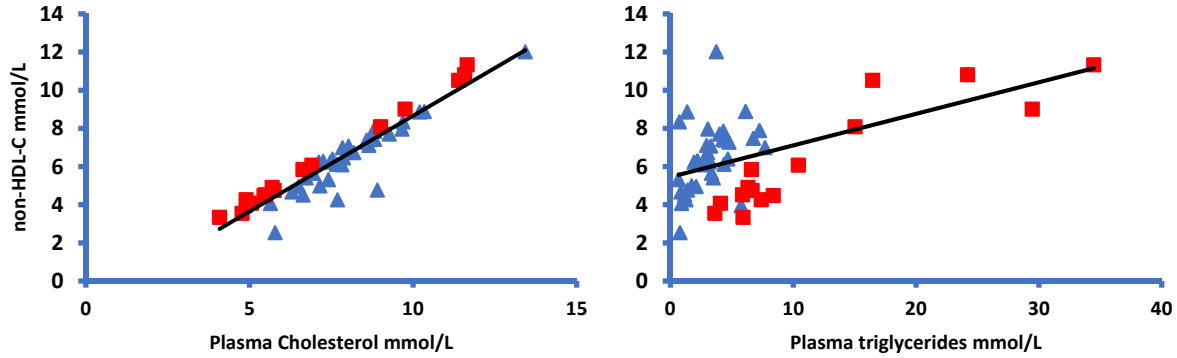


Figure 4.4 Correlation between plasma lipids and non-HDL cholesterol (plasma-C minus HDL-C).
Red squares represent the hypertriglyceridaemic subjects (n=15) and blue triangles represent type II (n=36) subjects. The correlation coefficients are r=0.924, p<0.001 for cholesterol and r=0.316, p=0.023 for triglycerides.

4.3.3 CoQ10 in lipoprotein fractions

No difference was observed in the absolute concentration of the lipid soluble antioxidant CoQ10 in plasma between hypertriglyceridaemic and other dyslipidaemic subjects. Moreover, this relationship was maintained when CoQ10 is expressed normalised to total plasma cholesterol. However, concomitant with the dissimilar levels of LDL between different groups of patients, the ratio of plasma CoQ10 to LDL-C was highly significant (Table 4.4). The variation in CoQ10 distribution is depicted in figure 4.5. CoQ10 content between type II subsets did not show any significant difference (mean 8.134 and 7.951 $p = 0.8015$ for type IIa and IIb respectively).

Statistical significance was observed between the two groups for absolute CoQ10 concentration associated with both LDL and VLDL but not with HDL. Comparable differences also exist when normalised for total cholesterol with a higher CoQ10 concentration in VLDL, but lower in LDL fractions in hypertriglyceridaemic subjects. Conversely, this relationship was reversed when normalised to the cholesterol content of the lipoprotein fractions (Table 4.4).

CoQ10 parameter		All		Type II		Type IV + V		*p*-value*
Plasma	µmol/L	**1.34**	(0.6-3.08)	**1.39**	(0.59-3.42)	**1.31**	(0.62-2.07)	0.7696
	µmol/mmol chol	**0.178**	(0.075-0.413)	**0.177**	(0.083-0.447)	**0.199**	(0.069-0.321)	0.3077
	µmol/mmol LDL-C	**0.558**	(0.141-3.373)	**0.286**	(0.133-0.731)	**0.867**	(0.360-3.638)	<0.0001
LDL	µmol/L	**0.78**	(0.19-0.41)	**0.97**	(0.25-2.93)	**0.57**	(0.13-1.19)	0.0014
	µmol/mmol chol	**0.104**	(0.024-0.361)	**0.123**	(0.039-0.395)	**0.071**	(0.022-0.234)	0.0213
	µmol/mmol LDL-C	**0.23**	(0.055-1.367)	**0.196**	(0.062-0.568)	**0.342**	(0.058-1.564)	0.0029
VLDL	µmol/L	**0.43**	(0.01-1.47)	**0.33**	(0-1.39)	**0.66**	(0.12-1.49)	0.0024
	µmol/mmol chol	**0.058**	(0.016-0.228)	**0.04**	(0-0.157)	**0.099**	(0.022-0.23)	0.0005
	µmol/mmol VLDL-C	**0.427**	(0.001-2.925)	**0.518**	(0.001-2.931)	**0.201**	(0.026-0.590)	0.0956
HDL	µmol/L	**0.063**	(0.012-0.37)	**0.06**	(0.01-0.45)	**0.08**	(0.01-0.23)	0.9436
	µmol/mmol chol	**0.008**	(0.002-0.049)	**0.008**	(0.002-0.059)	**0.013**	(0.003-0.041)	0.6496
	µmol/mmol HDL-C	**0.051**	(0.008-0.289)	**0.044**	(0.008-0.319)	**0.099**	(0.019-0.231)	0.0424

Values are geometric mean (95% CI)
*Significance between Type II and Type IV+V

Table 4.4 Concentration of CoQ10 in plasma and lipoprotein fractions isolated from dyslipidaemic subjects. Data represents absolute and cholesterol normalized (total and lipoprotein content) concentrations

Plasma CoQ10 positively correlated with total cholesterol (r = 0.319, p = 0.021) and LDL-CoQ10 (r = 0.647, p = <0.001) but no correlation to triglycerides was observed. This was maintained when the groups were considered independently. Additionally, correlations were also observed between plasma CoQ10 and its contents, normalised for cholesterol, in all lipoprotein fractions, although it was weaker for HDL and VLDL (r = 0.324, p = 0.019 and r = 0.31, p = 0.026 respectively) compared with LDL (r = 0.492, p <0.001). No correlation was seen between plasma CoQ10 and non-HDL-C (r = 0.196, p = 0.164) (Figure 4.6).

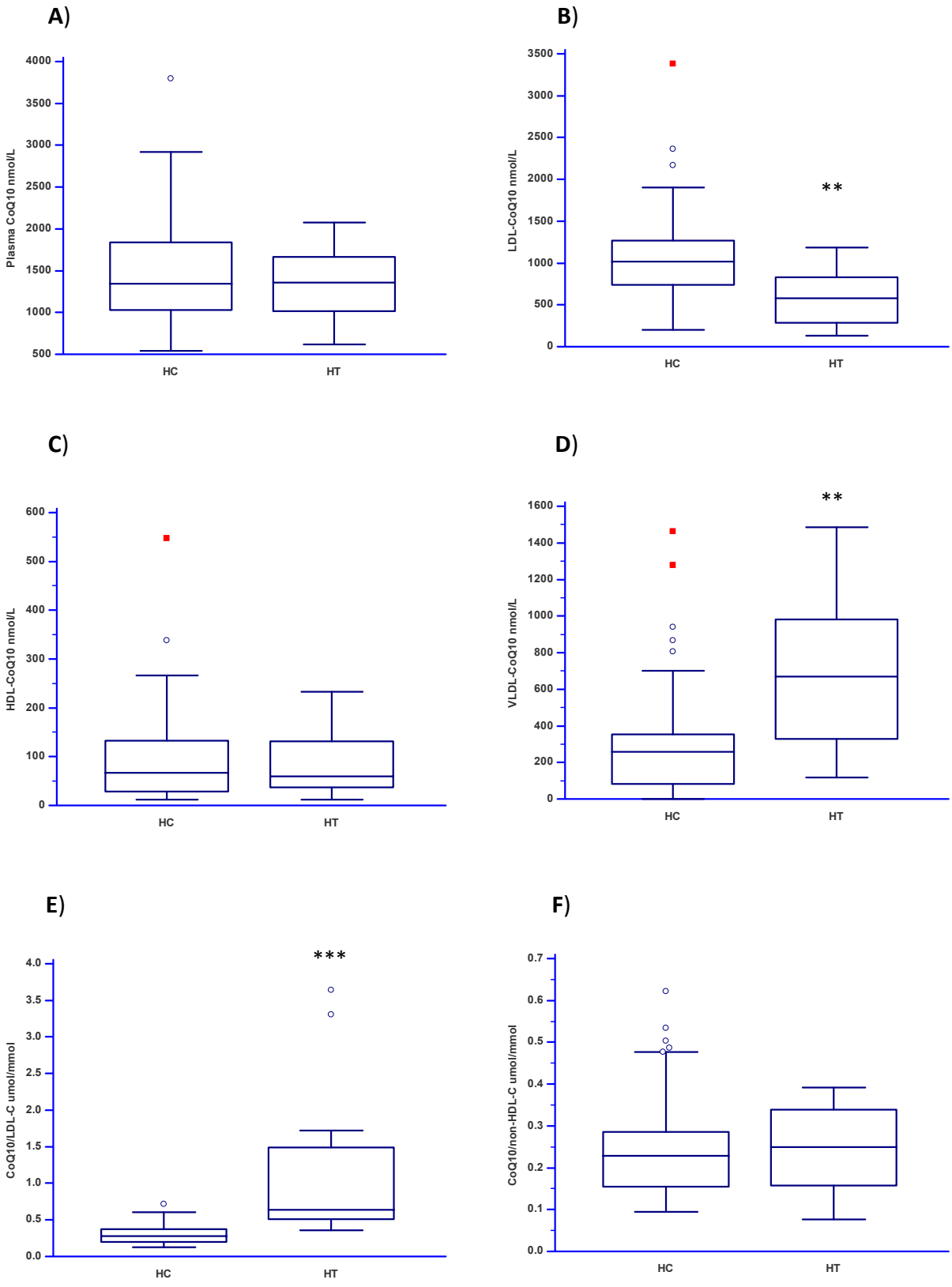


Figure 4.5 Comparisons of CoQ10 content in plasma and lipoprotein fractions between dyslipidaemic subjects. **A)** total plasma CoQ10, **B)** LDL-CoQ10**, C)** HDL-CoQ10, **D)** VLDL-CoQ10, **E)** total CoQ10 to LDL-cholesterol ratio, and **F)** total CoQ10 to non-HDL-cholesterol ratio. HC- hypercholesterolaemic (Fredrickson classification type IIa & IIb n=36), HT – hypertriglceridaemic (type IV & V n=15). P-values calculated using Mann-Whitney U test ***p < 0.0001, ** <0.005. Circles and red squares represent results which exceed plus/minus 1.5 and 3 times the IQR respectively.

LDL-CoQ10 positively correlated with both HDL and LDL cholesterol (r = 0.567 and 0.464, respectively, p <0.001) but was inversely related to VLDL cholesterol (r = -0.512, p <0.001) and total triglycerides (r = -0.505, p <0.001). However, this latter finding was invalidated when triglyceride concentrations exceeding 5 mmol/L were evaluated.

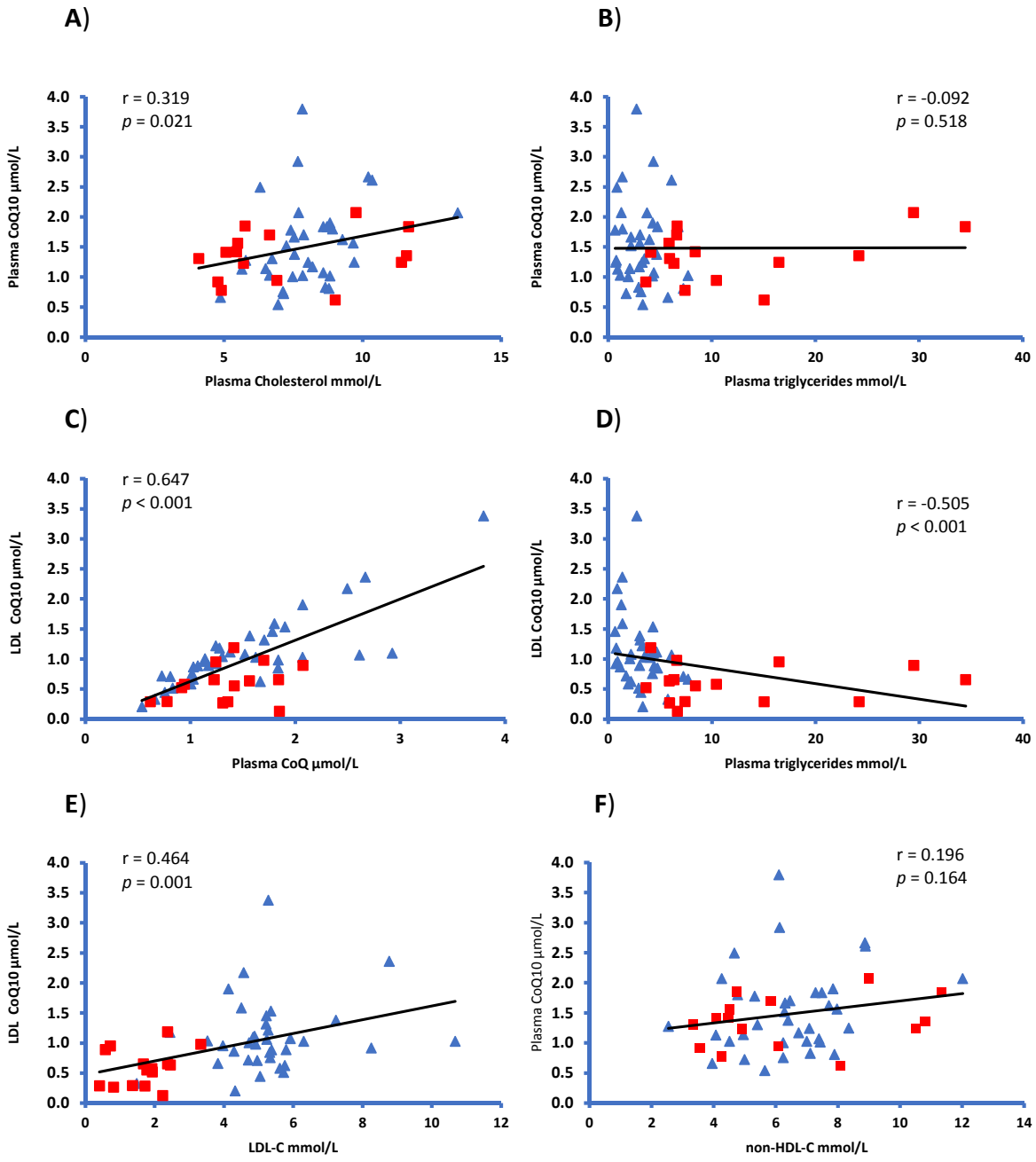


Figure 4.6 Correlation between CoQ10 and lipids in plasma or content in lipoproteins in dyslipidaemic subjects. **A)** total plasma CoQ10 and cholesterol, **B)** total plasma CoQ10 and triglycerides, **C)** total CoQ10 and CoQ10 in LDL, **D)** CoQ10 in LDL and triglycerides, **E)** CoQ10 and cholesterol content in LDL and **F)** plasma CoQ10 and non-HDL cholesterol. Red squares represent the hypertriglyceridaemic subjects (n=15 vs n=36). Correlation statistics calculated using Spearman Rank Sum test.

4.3.4 Distribution of CoQ10 and lipids among lipoproteins

The proportion of CoQ10, cholesterol and triglyceride carried by each lipoprotein class was calculated as a percentage of the plasma concentration. The majority of CoQ10 was found in LDL (63.8 ± 22.7 %; mean ± sd), but as expected, this showed wide variation among dyslipidaemic patients (range 7 – 98 %). Overall, VLDL carried 29.7 ± 21.5 % (range 0 – 80 %) and the remainder was located in HDL (6.5 ± 5.86 %, 0.7 – 27 %). This pattern mirrored the cholesterol distribution and was inversely related to triglycerides (Figure 4.7).

When evaluated based on lipidaemic classification, CoQ10 in hypertriglyceridaemic patients (mean triglyceride 12.3 mmol/L vs 3.3 mmol/L) was roughly equally distributed between VLDL and LDL. The percentage VLDL-CoQ10 was significantly increased compared to other dyslipidaemia types (48 % vs 22.3 %) and LDL-CoQ10 was reduced (45.3 % vs 71.4 %) (Table 4.5 & Figure 4.8). CoQ10 content in HDL was similar in both groups.

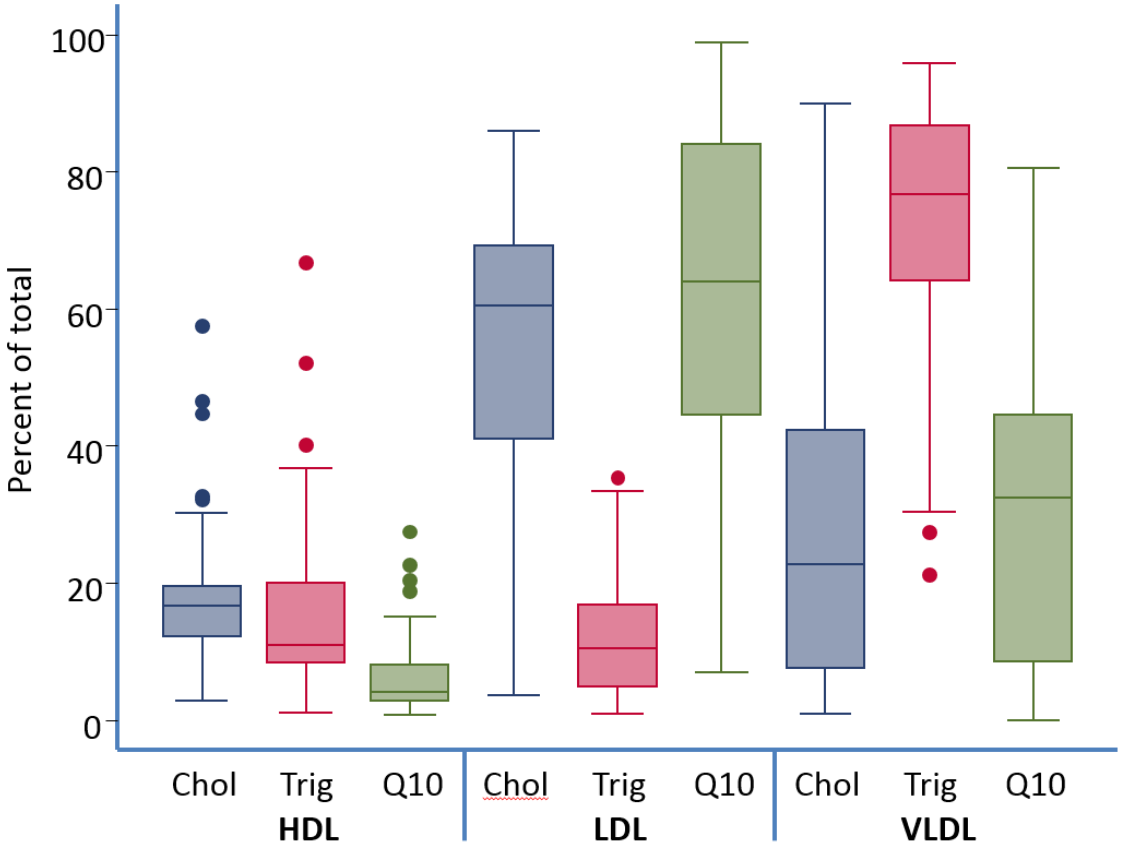


Figure 4.7 Distribution of major lipids and antioxidant CoQ10 in lipoprotein fractions in dyslipidaemic subjects. The content in each fraction was calculated as a percent of the total concentration measured in plasma. The box represents the interquartile range and the bars the range. (n = 51). Cholesterol (Chol); triglycerides (Trig); CoQ10 (Q10).

Type	HDL			LDL			VLDL		
	Chol	Trig	CoQ10	Chol	Trig	CoQ10	Chol	Trig	CoQ10
IV & V	**13.5** (3-26)	**7.6** (1-23)	**6.7** (1.5-22.5)	**27.9** (3.5-50)	**3.8** (1-7)	**45.3** (7-84)	**58.5** (34-90)	**88.5** (71-96)	**48** (8-80)
IIa & IIb	**20.4** (10-28)	**19.0** (6-67)	**6.4** (1-27)	**63.2** (12-86)	**14.9** (1-35)	**71.4** (38-99)	**16.8** (1-77)	**66** (22-90)	**22.3** (0-57)
Control*			**26** (10-46)			**58** (47-76)			**16** (8-35)
P-value	0.023	0.0029	0.8587	<0.0001	<0.001	0.0001	<0.0001	<0.0001	<0.0001

Table 4.5 Lipoprotein composition in dyslipidaemic subjects.
Values represent the mean (range) as a percent of the plasma concentration. *data taken from Tomasetti *et al* 1999. *P*-value is significance between type IV & V (n=15) and IIa & Iib (n=36)

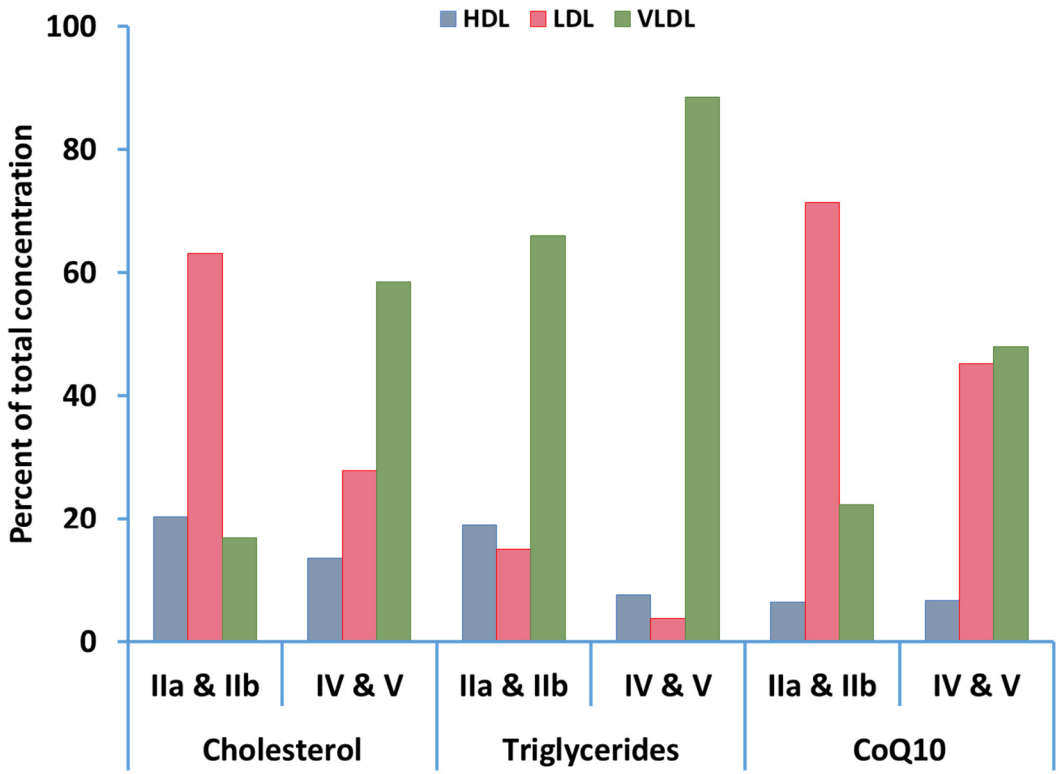


Figure 4.8 Lipoprotein content in hypertriglyceridaemia (n=15) and other dyslipidaemia (n=36) classes.
Bars represent the mean percentage in each group.

4.4 DISCUSSION

Increased concentrations of plasma LDL cholesterol constitute a major risk factor for cardiovascular disease. Existing guidelines also recognise the importance of non-HDL cholesterol, which represents the amount of cholesterol carried on all potential atherogenic particles (LDL, VLDL, IDL and chylomicrons) as a risk factor, particularly in combined dyslipidaemias (Hsia, 2003). The ratio of total cholesterol to HDL cholesterol is commonly used in conjunction with other variables, such as age, sex, blood pressure and smoking status, to predict the risk factor for development of atherosclerosis (2014).

4.4.1 Study subjects

The data presented here was obtained from subjects diagnosed with familial hyperlipidaemia categorised to four different sub-classes according to lipid levels and elevations in specific lipoprotein fractions determined by ultracentrifugation. The highest proportion of subjects represented combined hyperlipidaemia (type IIb), followed by familial hypercholesterolaemia (type IIa), type V and lastly familial hypertriglyceridaemia (type IV). This is in keeping with the estimated prevalence of each classification in the population. No samples were available from patients diagnosed with the rare forms of type I and type III hyperlipidaemias. Data was evaluated by grouping subjects according to those with predominately raised LDL-C (hypercholesterolaemic), and those with high VLDL-C and extreme triglyceride levels (hypertriglyceridaemic).

4.4.2 Distribution of major lipids

Total cholesterol concentration was not significantly different between the groups and although cholesterol concentrations alone are not sufficient to assess cardiovascular risk, the levels observed in these subjects were considered to be above the desirable level (mean 7.77 mmol/L, 90th centile in adults 7.2 mmol/L). Higher than normal levels were also seen for triglycerides (mean 3.89 mmol/L, compared to 90th centile in adults 3.1 mmol/L). Lipid age and gender distributions were derived from data obtained from a Health Survey Study undertaken by NUTH NHS trust (personal communication from Dr RDG Neely).

4.4.3 Distribution of Coenzyme Q10

Previous authors have reported results from control subjects indicate CoQ10 is strictly related to plasma cholesterol and also correlates to plasma triglyceride levels. These facts support the view that VLDL carries CoQ10 secreted by the liver which is then transferred in a similar fashion to cholesterol and apoprotein B during metabolic conversion of VLDL to LDL. Approximately 60 % of the total CoQ10 content is estimated to be associated with LDL particles in healthy subjects (Elmberger *et al.*, 1989; Tomasetti *et al.*, 1999).

Overall, in the hyperlipidaemic patients investigated in this study, the content of CoQ10 in LDL was higher than in VLDL particles which concurs with the current data that CoQ10 is preferentially sequestered in LDL and mirrors the distribution of cholesterol. However, further analysis accounting for triglyceride levels demonstrated that in patients with elevated VLDL, total CoQ10 distribution was approximately equally divided between the VLDL and LDL fractions. Furthermore, in contrast to control subjects, CoQ10 concentrations did not correlate to triglyceride levels.

The ability of CoQ10 to prevent LDL-C oxidation has been previously established and reports have suggested higher LDL-C/CoQ10 ratios are associated with increased atherosclerosis risk (Tomasetti *et al.*, 1999). Moreover, higher ratios have also been described in patients with coronary heart disease with respect to controls (Hanaki *et al.*, 1993). This suggests LDL poor in CoQ10 has increased vulnerability to oxidative attack and could represent a marker of clinical risk. In the data shown here an association was observed when the content of all potentially atherogenic lipoproteins was considered, suggesting the use of non-HDL-C/CoQ10 is a more reliable indicator particularly in patients with elevated triglyceride levels.

The absolute concentration of CoQ10 carried by LDL was lower in hypertriglyceridaemic subjects compared to other dyslipidaemic classes, potentially signifying an increased susceptibility of LDL to oxidation. However, relative concentrations normalised to the concentration of cholesterol within the LDL fraction, an approximation for the actual number of LDL particles present, indicated an increased ratio of antioxidant to substrate. This infers there should be reduced oxidation stress in LDL particles in this group of patients. Conversely, the opposite was seen in the VLDL fraction, although this did not reach statistical significance

for normalisation to lipoprotein cholesterol. This would imply that elevated levels of cholesterol in lipoprotein fractions are not balanced by corresponding levels of CoQ10.

High density lipoproteins, responsible for efflux of cholesterol from tissue, may also be prone to oxidative damage that can prevent their proper functioning to confer protection against cardiovascular disease. Oxidised HDL lose the ability to reverse cholesterol transport thus lipid levels are allowed to accumulate. Several other protective functions are also ascribed to HDL including defence against endothelial dysfunction by enhancing synthesis of the vasodilator, nitric oxide (Rosenson *et al.*, 2016). Lower levels of HDL were associated with the hypertriglyceridaemic group but, as with LDL, the ratio of oxidisable substrate to CoQ10 present within the fraction was significantly higher. Thus any adverse effects that may be the results of diminished HDL in subjects with high triglycerides, cannot be attributed to reduced antioxidant protection from CoQ10. However, it should be noted that this data does not rule out oxidation as the influencing factor as other antioxidant compounds, chiefly α-tocopherol, are also present. Perugini and co-workers demonstrated HDL content of α-tocopherol, which is embedded in the phospholipid layer, almost exclusively comes from the transfer from LDL. This antioxidant was strongly associated with HDL cholesterol concentrations suggesting low HDL concentrations may increase the propensity for atherosclerosis through oxidation (Perugini *et al.*, 2000).

Contrary to expectation, total CoQ10 availability was approximately equally distributed between LDL and VLDL in the patients with hypertriglyceridaemia. Further analysis suggests, at least in these patients, the cholesterol in LDL had potentially enhanced oxidative protection whereas cholesterol in VLDL was more vulnerable to reactive oxygen species. Both these lipoprotein fractions are considered atherogenic.

From an alternative view point, the relatively low absolute concentrations of CoQ10 in LDL in type IV and V lipidaemia could support a more direct cytotoxic role for oxidation in the pathogenesis of atherosclerosis. The fact that only about 45 % of the total CoQ10 in type IV and V, compared to 71 % in type II hyperlipidaemias, is carried in LDL limits its absolute availability for transfer into target peripheral tissue, and possibly results in more CoQ10 being recycled back to the liver in VLDL. Endothelial cells lining the inner surface of blood vessels are involved in multiple aspects of vascular biology and an early indication of atherosclerosis,

and indeed cardiovascular disease, is signs of endothelial dysfunction. Notably this can be detected by evidence of diminished nitric oxide levels, either due to decreased synthesis or enhanced oxidation. The former method may be attributed to low levels of HDL whilst the latter can be associated with an imbalance of the cellular redox state (Bae *et al.*, 2001). The results presented here suggest a possible mechanism for the increased prevalence of conditions associated with endothelial dysfunction, such as diabetes and pancreatitis, in hypertriglyceridaemic patients mediated through the distribution of CoQ10 in lipoprotein fractions.

4.5 CONCLUSION

The data presented here suggest that contrary to the preferential sequestion of CoQ10 in LDL, in hypertriglyceraemic patients CoQ10 is roughly equal distributed between LDL and VLDL. As a result antioxidant protection by CoQ10 may be impaired, leaving VLDL highly susceptible to attack and limited CoQ10 available for transfer into tissue via LDL. In patients with moderate to severe hypertriglyceridaemia, this altered distribution of CoQ10 may be an important predisposing factor to a number of conditions including endothelial dysfunction, diabetes mellitus and pancreatitis mediated by reactive oxygen species, and may play an important role in atherogenesis (Bae *et al.*, 2001; Tiano *et al.*, 2007; Hegele *et al.*, 2014).

Chapter Five

Chapter 5. Method Development for the Measurement of Sterol Intermediates

5.1 INTRODUCTION

5.1.1 Gas chromatography mass spectrometry (GCMS)

One of the most widely applied analytical techniques in modern chemistry has its foundation in the early 1950s with the introduction of the gas chromatograph (GC) followed by the ability to directly introduce the GC effluent into a mass spectrometer (MS) in 1959 (Gohlke and McLafferty, 1993). Modern day gas chromatographs achieve separation of complex mixtures by partitioning between a stationary liquid or polymer phase packed into a narrow bore capillary column and a mobile gas phase, usually helium or hydrogen. The compounds for analysis are vaporised and their various physical and chemical properties promote an altered affinity for the stationary phase. Some molecules are retained by their interaction with the stationary phase and this enables separation of compounds as they are swept through the length of the column by the mobile gas. Elution from the column is achieved by increasing the temperature in a controlled manner, allowing the molecules to elute at characteristic retention times. When they emerge from the column, the downstream mass spectrometer ionises and detects the components using their mass-to-charge (m/z) ratio. A schematic of a basic GCMS system is shown in figure 5.1.

In contrast to electrospray ionisation commonly used in tandem mass spectrometry, GCMS commonly employs electron ionisation (EI) to introduce the molecules into the mass analyser. This is classed as a hard ionisation technique and results in almost complete destruction of the molecular mass unit and an abundance of low mass-to-charge fragments. The resulting mass spectrum can be compared to a library database and together with the retention time can be used to enable identification of the individual compounds (Bartle and Myers, 2002). The mass spectrometer can be utilized in either full scan mode, whereby all fragments are detected and used to identify unknown compounds, or in selective ion monitoring (SIM) mode. In this latter function only one or two ions that are unique to a particular compound are monitored. This removes interferences from co-elute compounds and enables maximum sensitivity and specificity to be achieved for the analysis of compounds present at low levels.

Column selection is a vital component to a successful GCMS application and improvements in manufacture and phase technology means there is now a wide portfolio of columns available. Typically available as 30 or 60 metre lengths with an internal diameter of 0.25 mm (although other sizes are obtainable), efficient, inert columns will produce tall, narrow peaks with greatest signal-to-noise ratios and increased analytical sensitivity. Low bleed columns commonly have a bonded stationary phase (or film) which reduces baseline noise and improves mass spectral clarity, and increased polymer stability enables higher operating temperatures to be used which provides shorter run times and longer column lifetimes. Solute retention is highly influenced by both the phase used and the film thickness.

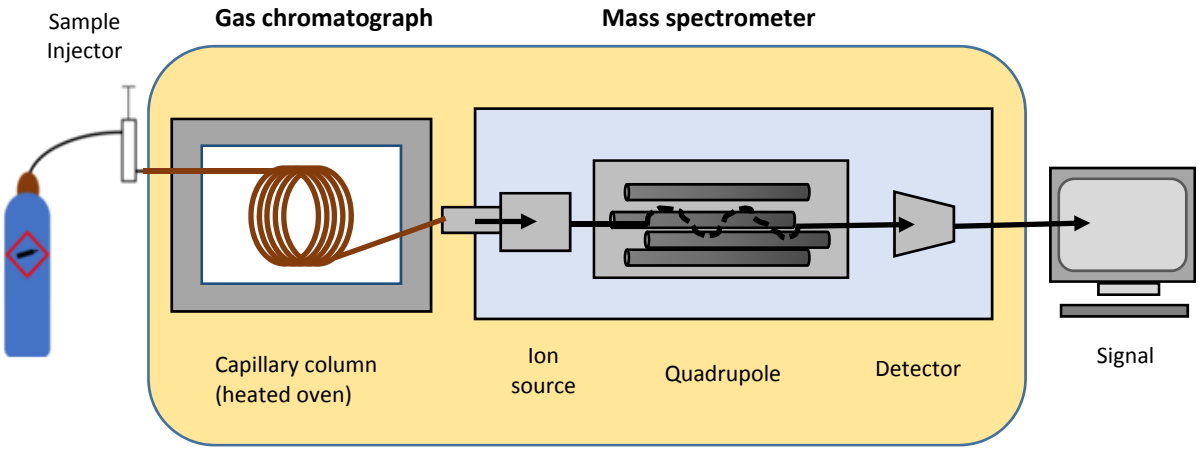


Figure 5.1 Schematic diagram of a basic gas chromatography mass spectrometer.
The column separates a mixture into its components which enter the ion source of the mass spectrometer and are ionised and fragmented. The fragments are scanned in the quadrupole and the mass spectral patterns recorded by the detector.

GCMS is an ideal analytical tool to analyse hundreds of relatively low weight, volatile and thermally stable compounds. It is commonly used as the tool of choice to diagnosis and monitor a range of inborn errors of metabolism in which trace-level detection of metabolites is required.

5.1.2 Analysis of cholesterol and sterol intermediates

Cholesterol is a well-established marker of cardiovascular disease risk and intervention therapies aimed at lowering LDL-cholesterol have proved a marked reduction in cardiovascular events and patient mortality (Jialal and Devaraj, 1996). The non-cholesterol sterols that serve as a measure of cholesterol homeostasis, indicating the balance between intestinal absorption from the diet and cholesterol biosynthesis, can prove to be a very useful tool in investigations in clinical practice. For instance, Kempen and co-workers proposed that the measurement of the lathosterol/cholesterol ratio was the preferable indicator of whole-body cholesterol synthesis which provides useful guidance in the monitoring of FH patients *(Kempen et al., 1988).*

Additionally, measurement of other sterol intermediates can serve as markers for several clinical diseases, including inborn errors of cholesterol metabolism. An example is sterol profiling in amniotic fluid which can assist in early identification of possible metabolic defects of sterol biosynthesis, such as SLO, in conjunction with abnormal foetal morphology examination by ultrasound (Chevy *et al.*, 2005).

Early analytical approaches to separate and measure non-cholesterol sterols used thin-layer chromatography separation followed by gas-liquid chromatography quantification (Kempen *et al.*, 1988; Kuksis, 2001). Both normal (Greenspan *et al.*, 1988) and reverse-phase (Hansbury and Scallen, 1978) HPLC coupled to diode array detectors and refractive index detectors have also been used. Phillips *et al* (Phillips *et al.*, 1999) used capillary gas-liquid chromatography with flame ionisation to quantify various cholesterol metabolites and phytosterols in human serum. The classic approach however for the detection of sterols is based on extraction using organic solvents and gas chromatographic separation prior to mass spectral analysis, which is much more specific than flame ionisation.

Some of the complications encountered during detection are the presence of other lipid categories (Harkewicz and Dennis, 2011) and relatively low concentrations of most analytes relative to cholesterol (Table 5.1). Variability in quantitation between labs does exist and may be due to the lack of a suitable commercial internal standard. Some researchers have synthesized deuterated standards prior to analysis (Acimovic *et al.*, 2009; Matysik *et al.*, 2012; McDonald *et al.*, 2012) or used a single labelled compound, commonly cholesterol, with

external calibration (Lembcke *et al.*, 2005). Others have relied on calibration using structurally related compounds, such as cholestane (Ahmida *et al.*, 2006), coprosterol (Honda *et al.*, 2008), epicholesterol (Phillips *et al.*, 1999) and 19-hydroxycholesterol (Yamaga, 2002).

Extraction procedures require sponification to cleave the ester bonds, using ethanolic potassium or sodium hydroxide, heated to between 60°C and 70°C. Plasma or serum is generally the sample of choice but blood spotted onto filter paper has been evaluated for the detection of SLOS (Starck and Lövgren, 2000; Johnson *et al.*, 2001). After cooling, the lipids are extracted into organic solvents, typically hexane or chloroform, then following solvent evaporation the dried extracts are derivatised to trimethylsilyl esters prior to analysis.

Sterol	Concentration umol/L		
	min	max	mean
Cholesterol	2890	8250	**4770**
Cholestanol	0.41	15.95	**6.75**
7-dehydrocholesterol	1.10	13.76	**6.00**
Desmosterol	0.10	4.68	**2.16**
Lathosterol	0.43	37.43	**8.42**
Lanosterol	0	3.47	**0.40**
Sitosterol	2.30	14.64	**6.66**
Zymosterol	0	2.58	**0.85**
Campesterol	1.72	40.93	**8.56**
Dihydrolanosterol	0.33	0.65	**0.49**

Table 5.1 Concentrations of sterols in normal human plasma and serum.
Data show the potential range taken from multiple literature sources and a calculated average based on those reported (Teunissen *et al.*, 2003; Lembcke *et al.*, 2005; Ahmida *et al.*, 2006; Honda *et al.*, 2008; McDonald *et al.*, 2012).

GCMS has proved an ideal tool enabling great sensitivity and selectivity for sterol quantitation (Ahmida *et al.*, 2006; Acimovic *et al.*, 2009), whilst newer instrumentation such as GC-MS/MS has expanded the scope for simultaneous determination of steroids and oxysterols in relatively short acquisition times using multiple reaction monitoring (MRM) mode (Matysik *et al.*, 2012). The use of picolinic acid to produce more polar picolinyl ester derivatives has enabled analysis to be conducted using liquid chromatography methods and both

atmospheric pressure photoionisation (APPI) (Lembcke *et al.*, 2005) and ESI (Honda *et al.*, 2008) platforms coupled to tandem mass spectrometry have been evaluated for rapid profiling of sterols and phytosterols in human serum. Honda and colleagues were able to comprehensively demonstrate the use of LC-MS/MS for sterol profiling in biological samples whilst the group lead by McDonald used a combination of LC-MS/MS and GCMS for the analysis of over 60 sterols and oxysterols in human plasma (Honda *et al.*, 2010; McDonald *et al.*, 2012) . LC-MS/MS methods benefit from small sample requirements and rapid throughput and whilst picolinyl esters enhance performance and reduce detection limits, the procedure required may by more laborious than most GCMS methods.

The advantage of sterol profiling is not only in the detection of clinical disorders but may also enable identification of a subgroup of people with abnormal absorption or synthesis rates of cholesterol. This may be advantageous if statin treatment is required and would aid in providing combination therapy to improve beneficial outcomes in some patients.

5.1.3 Aim

To develop and optimise an analytical procedure for the measurement of the intermediates of the Bloch and Kandutsch-Russell pathways of cholesterol biosynthesis in biological specimens using gas chromatography mass spectrometry.

5.2 MATERIALS AND METHODS

5.2.1 Reagents

All solvents and reagents were analytical grade or above. Methanol, pyridine and the derivatising reagent, N,O-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA + 1 % TMCS), were purchased from Sigma-Aldrich (Dorset, UK). Hexane was obtained from Fisher Scientific (Loughborough, UK), potassium hydroxide (KOH) from VWR International (Leicestershire, UK) and ethanol was purchased from Rathburn Chemicals (Walkerburn, UK).

Authentic standard compounds (cholesterol, 7-dehydrocholesterol [7DHC], cholestanol, desmosterol, lathosterol, sitosterol, campesterol, lanosterol and epicoprostanol [EPIC, 5β-cholestan-3α-ol]) were all available from Sigma-Aldrich. Zymosterol, 24,25-dihydrolanosterol (lanostenol) and cholesta-8(9)-enol (zymostenol) were purchased from Avanti Polar Lipids Inc. (distributed by Instruchemis B.V, The Netherlands). All stocks were prepared to a concentration of 1 mmol/L using high purity methanol and stored in light protected amber vials at -40°C.

5.2.1.1 Control material

Control serum for all analytes is not currently commercially available. ERNDIM (European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism, The Netherlands) offers a 'Special Assays in Serum Control' with target values available for cholestanol and 7DHC only. This was reconstituted according to manufacturer guidelines using HPLC grade water, aliquots prepared and stored at -40°C. This gave analyte reference concentrations as shown in table 5.2. Additional analytes were quantitated in this control and used for assay precision. A randomised pooled plasma specimen was also prepared and analysed for precision studies at physiological values.

Analyte	Unit	Mean conc.	Range
Cholestanol	µmol/L	56.8	41 – 73
7DHC	µmol/L	130	74 – 186

Table 5.2 Analyte reference concentrations in ERNDIM control material.
The assigned values represent the mean consensus concentrations obtained fron the ERNDIM External Quality Assessment Scheme and the range is based on the interlab variation of the 90% best performing laboratories.

5.2.2 Equipment

Quantitative analysis of sterols was performed using an Agilent 6890 gas chromatograph coupled to a 5975 MSD quadrupole mass spectrometer with electron impact ionisation (Agilent Technologies, Stockport, UK). One microlitre of sample was injected via a 7683B series autosampler and injector in splitless mode with a purge to split vent at 0.4 min. The inlet temperature was maintained at 265°C. The ionisation source was set to 230°C, the quadrupoles to 150°C and the transfer line to 300°C.

Helium was used as the carrier gas and separation was performed using a J&W® DB-5MS 25 m capillary column (0.25 µm x 0.32 mm id) purchased from Agilent. This column has an operational range of 60°C – 325°C with a maximum temperature tolerance of 350°C. Based on previous knowledge the GC conditions consisted of an initial column temperature at 150°C held for 2 minutes then a programmed rate increase as follows:

Rate (°C/min)	Final temp (°C)	Final time (min)
15.0	260	0
8.0	290	15

Data analysis was performed using Agilent Chemstation software.

5.2.3 Optimisation of the chromatography method

Optimisation for cholesterol, cholestanol, 7DHC, lathosterol, desmosterol, sitosterol and campesterol has previously been undertaken and parameters were available from the Blood Sciences department, Royal Victoria Infirmary, Newcastle-upon-Tyne. Further method evaluation relates to the additional analytes of lanosterol, zymosterol, lanostenol and zymostenol.

Retention times and mass spectra for additional sterol compounds were identified by analysis of trimethylsilylether (TMS) derivatives of authentic standards (100 nmols) in full scan mode (*m/z* 50 - 700) using the column temperature conditions as above. The eluent from the column was introduced to the ion source of the mass spectrometer where molecules were ionised and fragmented by collision with high energy electrons. The fragment ions were recorded by the mass detector to generate the mass spectrum. As all sterols share similar structures it is likely many fragments will be common to all compounds. Therefore it was preferable to choose unique ion fragments regardless of whether they are the most abundant. To improve specificity as some analytes may co-elute, multiple ions were tested for monitoring of each compound by selective ion monitoring (SIM).

5.2.4 Sample extraction

Samples were extracted using a modified version of the Folch method which employs liquid-liquid separation of aqueous sample and an organic solvent (Folch *et al.*, 1957). Plasma was mixed with an equal volume of internal standard (50 µl 1 mmol/L EPIC) and saponified by ethanolic potassium hydroxide (4 % w/v) at 64°C for 60 mins. After cooling, water was added then the sterols partitioned into hexane (top layer) by vigorous mixing using a multi tube vortexer (VWR, Leicestershire, UK). The ratio of aqueous to organic content was approximately 3:1. The organic layer was transferred to a clean tube using a glass pipette, dried under nitrogen at 40°C and silylated using pyridine and BSTFA + 1 % TMCS heated at 64°C for 60 min. Production of the TMS derivatives is necessary to produce compounds that are volatile and thermally stable thus amenable to GC analysis. Sample tubes were maintained in light protected conditions over night prior to transfer to GC autosampler vials for analysis.

5.2.5 Validation of the method

Linearity of each analyte was determined by sequential dilution of the stock standards using methanol and analysed in triplicate over the range of 0 - 8000 µmol/L for cholesterol and 0 - 80 µmol/L for the intermediates. Calibration curves were constructed by calculation of the peak area to the internal standard ratio and linear regression analysis.

Analyte recovery was determined by preparing a series of dilutions of the stock standards (10, 25, 50, 100 and 200 µmol/L) then adding 20 µl standard to 180 µl pooled plasma to maintain a consistent methanol:sample matrix ratio. This give final concentrations in the spiked samples of 1, 2.5, 5, 10 and 20 µmol/L. Each sample was analysed in duplicate and the mean concentration determined. The variation between replicates was expected to be less than 5 %.

A series of standards with a range 0.01 – 0.25 µmol/L were analysed 5 times. The detection limits (LOD) were determined as the lowest concentration at which a peak was distinguishable from background noise. Limits of quantitation (LOQ) were defined as the lowest concentration at which the analyte could be reliably detected with an impression of less than 20 %.

Imprecision of the method was derived by calculating the RSDs using two pooled plasma samples extracted and analysed on different days (n=13).

5.2.6 Co-elution

To evaluate interference from co-eluting analytes, plasma samples were analysed in duplicate after the addition of standards to give final concentrations between 1 – 20 µmol/L. In one experiment the sample was spiked with lathosterol and sitosterol and in a parallel experiment, lanosterol and zymosterol were added. The effect of the spike compounds on the closely related analytes was monitored i.e. sitosterol and lanosterol or lathosterol and zymosterol. In addition, 7-dehydrocholesterol and desmosterol were also monitored as independent variables as retention times and molecular ions for these sterols are distinct from the sterols under investigation.

Zymostenol and lanostenol did not co-elute with any other sterols in the panel therefore were not investigated further.

5.2.7 Evaluation of extended scan parameters.

To assess the impact the acquisition of the additional ions for lanosterol, zymosterol, lanostenol and zymostenol may have on the other sterols in the profile, samples received at Blood Sciences, Royal Victoria Infirmary, Newcastle for routine sterol profiling were immediately reanalysed using the newly created extended scan function. Cholesterol, as the major species present, and lathosterol and sitosterol, owing to their proximity to the new compounds were compared using linear regression. The sitosterol concentration in 2 patients with known sitosterolaemia was 2 orders of magnitude greater than in a control population and a subset of samples were from study participants in whom intervention elevated both cholesterol and lathosterol. Comparisons were therefore possible over a wide concentration range.

5.3 RESULTS

5.3.1 Chromatographic Method

In initial experiments authentic lanosterol produced 2 peaks when analysed in full scan mode. The majority of mass spectra fragments produced by the minor peak differed from the major peak by 2 mass units. This most likely reflected the purity of the standard (53 %), with dihydrolanosterol (lanostenol), formed by reduction across the C24-C25 double bond, being the major impurity. Subsequent analysis of 93 % purity lanosterol confirmed a significantly reduction of the concentration of this impurity so this stock was used for further analysis (Figure 5.2).

The mass spectra and retention times for the TMS derivatives of sterol standards are shown in figure 5.3. Mass spectral patterns for lanostenol only differed from those of sitosterol by 1 mass unit whilst zymostenol fragments were identical to those produced by lathosterol. This is not surprising given these two latter compounds differ only in the position of a double bond. However, the retention time for each compound was significantly different.

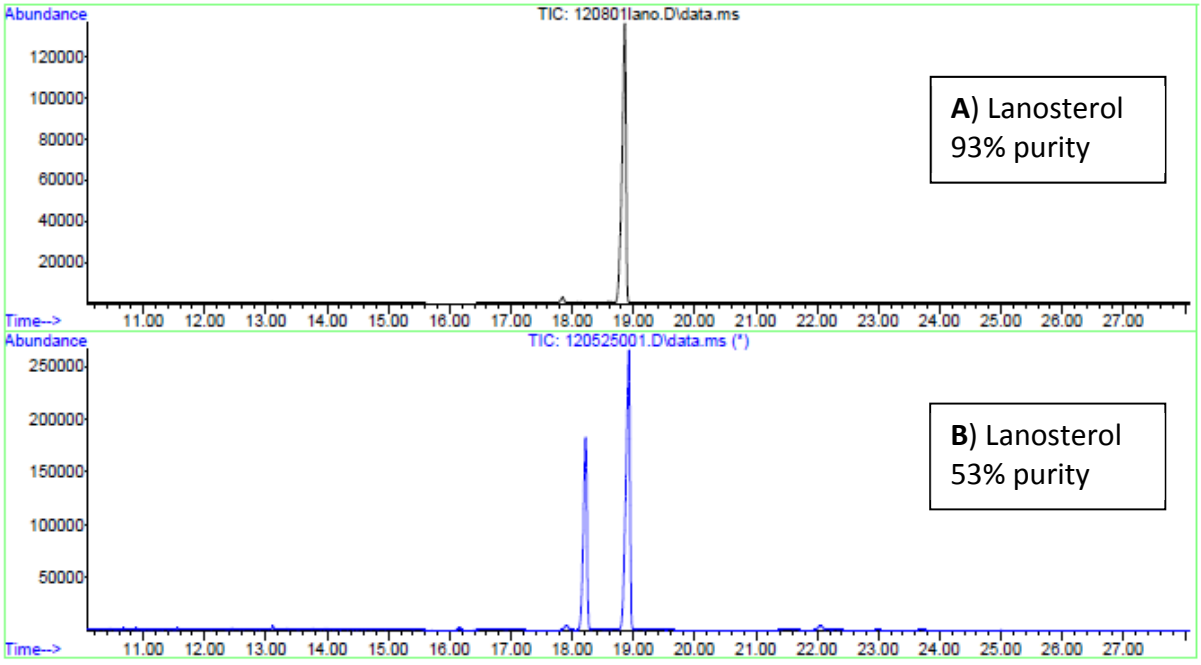


Figure 5.2 Retention time for authentic lanosterol, at two different purity levels.
A) 93% and **B**) 53% lanosterol, analyzed by GCMS using scanning acquisition. The minor peak (RT 18.2 mins) represents the impurity dihydrolanosterol.

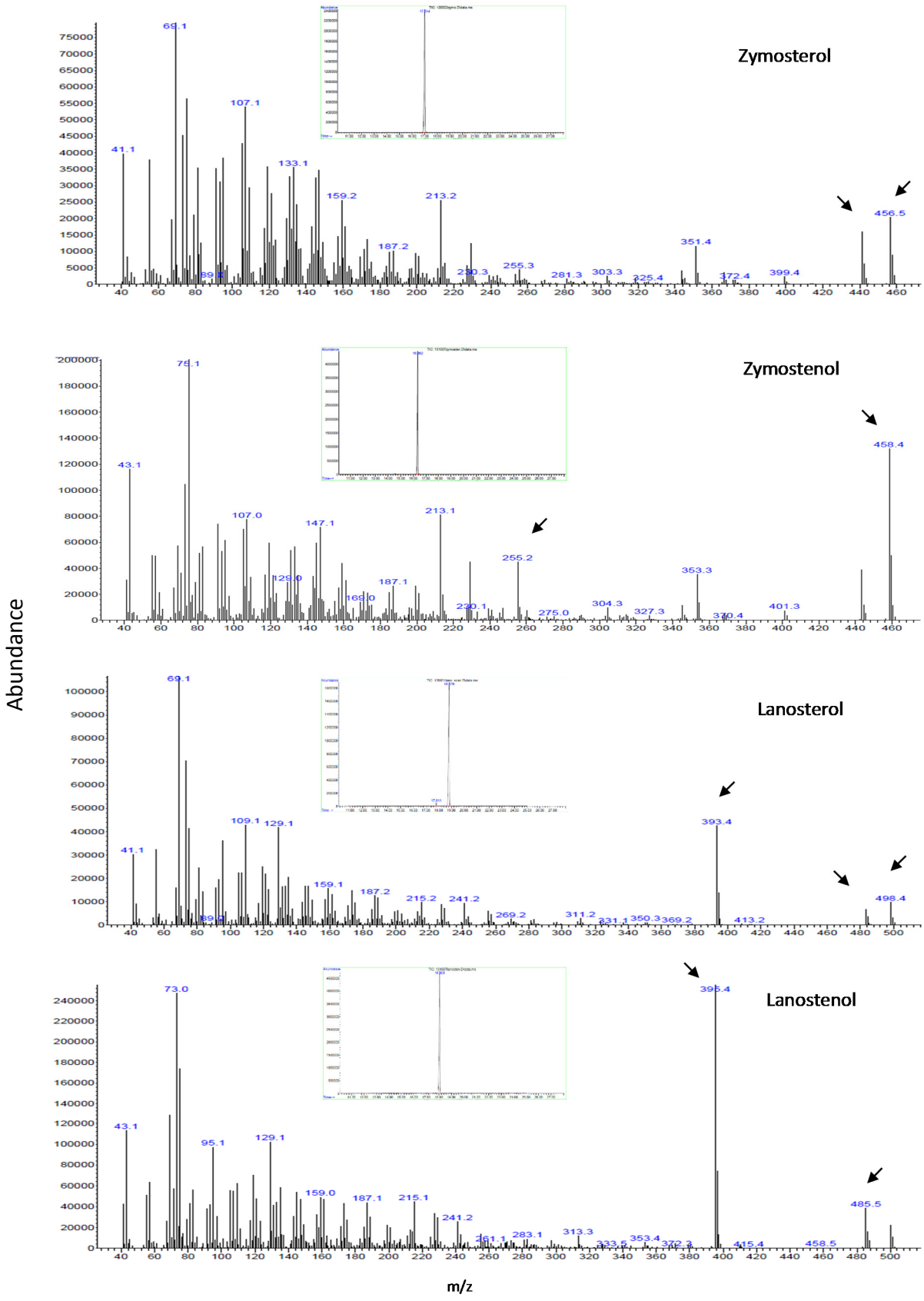


Figure 5.3 Product mass spectra of authentic sterols by GCMS.
100 nmols of each standard was derivatised using BSFTA to produce trimethylsilylether esters and analysed using scan mode (*m/z* 50-700). Ions used in SIM profile are indicated with an arrow. Inset: Chromatogram for each standard.

Lanosterol and zymosterol co-eluted with sitosterol and lathosterol respectively. The major fragments produced were also very similar between each pair, differing by only 2 – 3 mass units. Multiple ions were selected in order to improve selectivity for each compound. The optimal ions identified for each sterol intermediate in the full profile were used to create a SIM (selective ion monitoring) acquisition program (Table 5.3). The final elution profile for a mixture containing all 13 sterol compounds analysed using the conditions described above is shown in figure 5.4. Elution of all compounds was achieved in under 19 mins and the total run time, including column wash-out and pre conditioning was 27 min per sample.

Compound	*m/z* ions monitored	Approx. Retention time (mins)
Squalene	**69**, 341	13.03
EPIC (internal standard)	**355**, 445	15.30
Cholesterol	353, **368**	16.02
Cholestanol	403, **445**	16.20
Zymostenol	255, 458	16.30
Desmosterol	343, **372**	16.47
7-Dehydrocholesterol	325	16.52
Lathosterol	255, **458**	16.83
Zymosterol	**441,** 456	16.85
Campesterol	**382**, 472	17.35
Lanostenol	**395**, 485	17.96
Sitosterol	357, **396,** 486	18.62
Lanosterol	**393**, 483, 498	18.64

Table 5.3 SRM parameters for sterol analysis by GCMS. Quantitative ions are shown in bold.

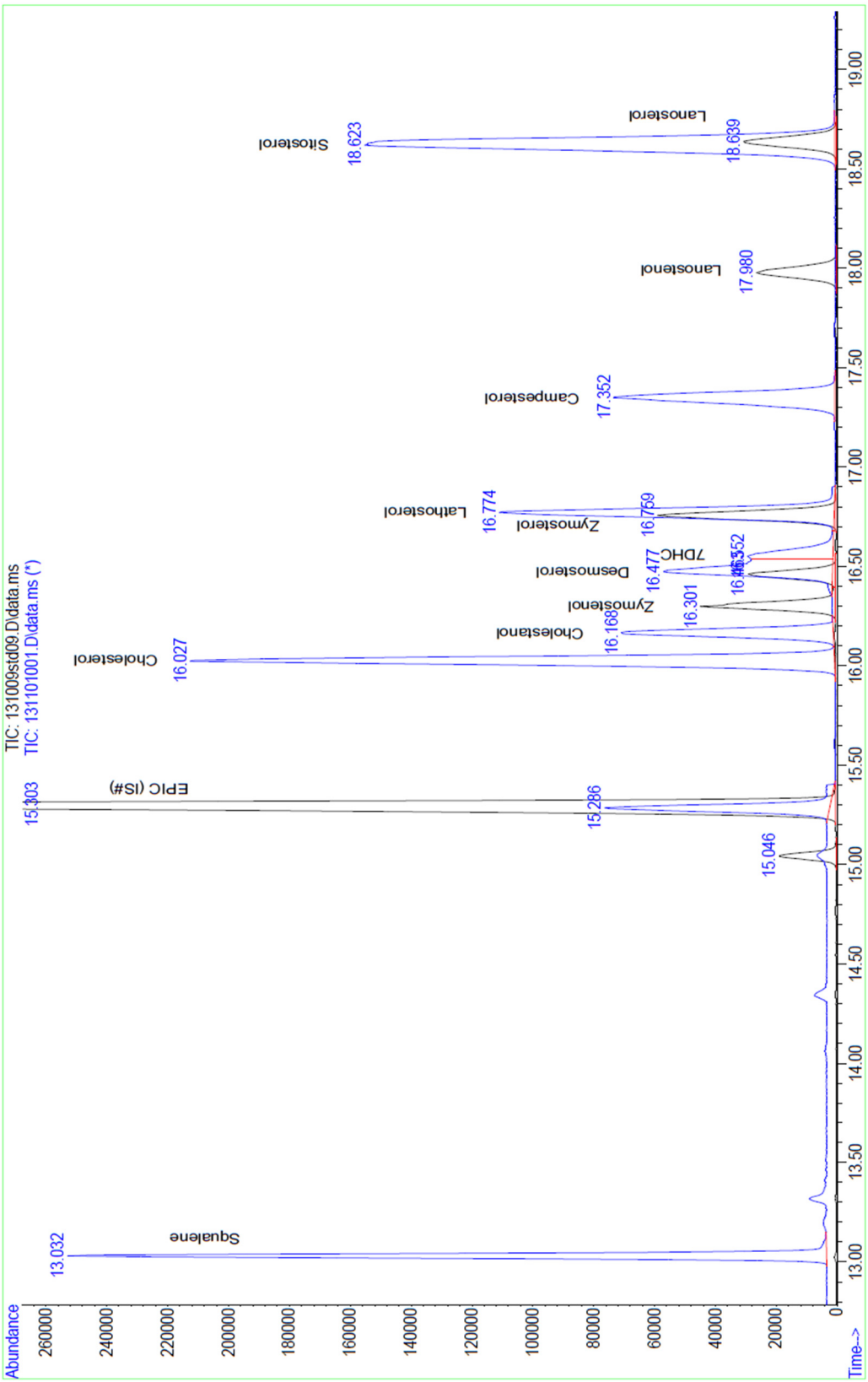


Figure 5.4 Total Ion Chromatogram for the trimethylsilyl derivatives of standard sterol compounds.
Profile acquired using SIM. Coeluting compounds zymosterol & lathosterol and sitosterol & lanosterol can be seen on these superimposed TICs.

5.3.2 Validation of the method

The calibration plots showed good linearity over the range 0 - 80 µmol/L for all ions monitored (Figure 5.5). For lanosterol, the intensities for ions 483 and 498 were significantly lower than ion 393 so these ions were retained as qualifier ions only.

Limits of detection were estimated to be 0.01 µmol/L for lanosterol and 0.05 µmol/L for lanostenol, zymosterol and zymostenol (n=5). The LOQ for all analytes was 0.1 µmol/L (n=5, CV < 20 %).

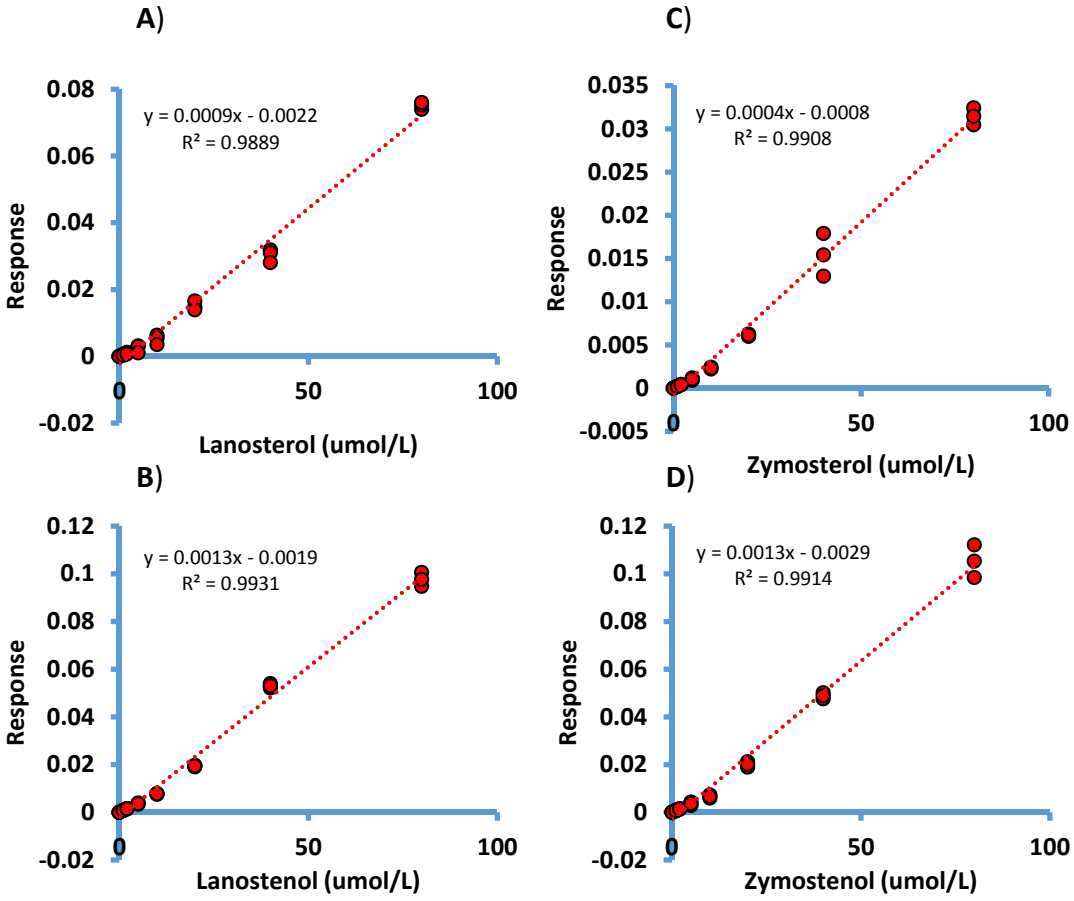


Figure 5.5 Linearity for A) lanosterol (m/z 393), B) lanostenol (m/z395), C) zymosterol (m/z441) and D) zymostenol (m/z 458).
Response is the peak area of the compound divided by the area of EPIC (IS# at 100 µmol/L). All points taken from 3 independent experiments are shown.

Recovery over the full spiked range for the major ions was as follows: lanosterol, 111 – 120 %; zymosterol 105 – 122 %; lanostenol, 93 – 100 % and zymostenol, 74 – 104 %. Low and high level recoveries are shown in table 5.4. Sitosterol and lathosterol were included due to their possible conflicting properties. A blank sample consisting of methanol and internal standard was extracted, derivatised and analysed within the same batch and no significant peaks for any of the sterols monitored were detected.

Between batch imprecision (RSD %) of the assay is shown in table 5.5. The relatively high margins of error are likely due to the very low levels of these compounds but recoveries and variation are comparable to other authors (Acimovic *et al.*, 2009).

Sterol	Ion (*m/z*)	endogenous (µmol/L)	Amount added (µmol/L)	Recovery (%)
Lathosterol	458	**2.51**	2.5	99.6
			10	98.3
Sitosterol	396	**2.00**	2.5	116.4
			10	121.0
Lanosterol	393	**0.26**	1	119.9
			5	115.5
Zymosterol	441	**0.24**	1	87.8
			5	121.0
Lanostenol	395	**0.06**	1	100.0
			5	95.8
Zymostenol	458	**0.84**	1	74.0
			5	93.2

Table 5.4 Recovery of sterols measured in plasma. All results are means of 2 extractions.

5.3.3 Co-elution

Elution of Lanosterol and zymosterol was very similar to sitosterol and lathosterol respectively. Standard mixtures containing these sterol 'pairs' were analysed and although baseline resolution was not possible the molecular ions monitored allowed them to be uniquely identified by mass spectrometry (Figure 5.6). Modifications to the temperature gradient were tested but failed to improve chromatographic resolution.

Sterol	Mean (µmol/L)	RSD %
Cholesterol	3841	9.8
	4110	9.2
7-DHC	0.75	37.6
	128.24	6.8
Cholestanol	7.0	11.0
	52.43	10.1
Desmosterol	2.32	20.8
	2.39	23.7
Lathosterol	5.13	12.6
	5.58	6.8
Sitosterol	6.01	9.0
	6.30	9.0
Campesterol	6.36	9.9
	9.09	9.4
Lanosterol	0.49	27.6
	1.76	15.6
Lanostenol	0.09	Below LOQ
	1.52	20.5
Zymosterol	1.58	23.9
	1.84	24.4
Zymostenol	1.12	5.3
	2.31	11.9

Table 5.5 Between batch imprecision of sterols in plasma at different concentrations of analytes (n=13)

Evaluation using spiked samples suggested there is a potential for interference from closely eluting compounds at high concentrations. Depending on the ion monitored and the concentration added, sitosterol increased the quantitation of lanosterol between 32 and 96 % (n=12), with the most significant impact occurring with *m/z* 483 and the least using *m/z* 393 (mean change 60.2 % vs 47.4 %). This equated to a maximal change from 0.26 µmol/L to 0.43 µmol/L in the presence of 20 µmol/L sitosterol. The mean increase in zymosterol by lathosterol was 128 % (n=12, range 0.24 - 1.42 µmol/l) with the most abundant ion, *m/z* 456 showing the greatest influence (5.4 % - 373 %). Conversely, quantitation of sitosterol and lathosterol in samples spiked with lanosterol and zymosterol were less significantly affected,

with an average increase of approximately 30 % ($p > 0.0744$ for both analytes). This is possibly a reflection of the higher endogenous levels for these analytes. It should be noted that normal ranges, provided by the Blood Sciences Department, Newcastle, for lathosterol and sitosterol in plasma are less than 7.6 and 11.3 µmol/L respectively, thus these experiments were spiked beyond physiological levels. Results also confirmed independent analytes (desmosterol and DHC) were not affected during either experiment (RSD < 6 %, n=22, $p > 0.9$) (Figure 5.7).

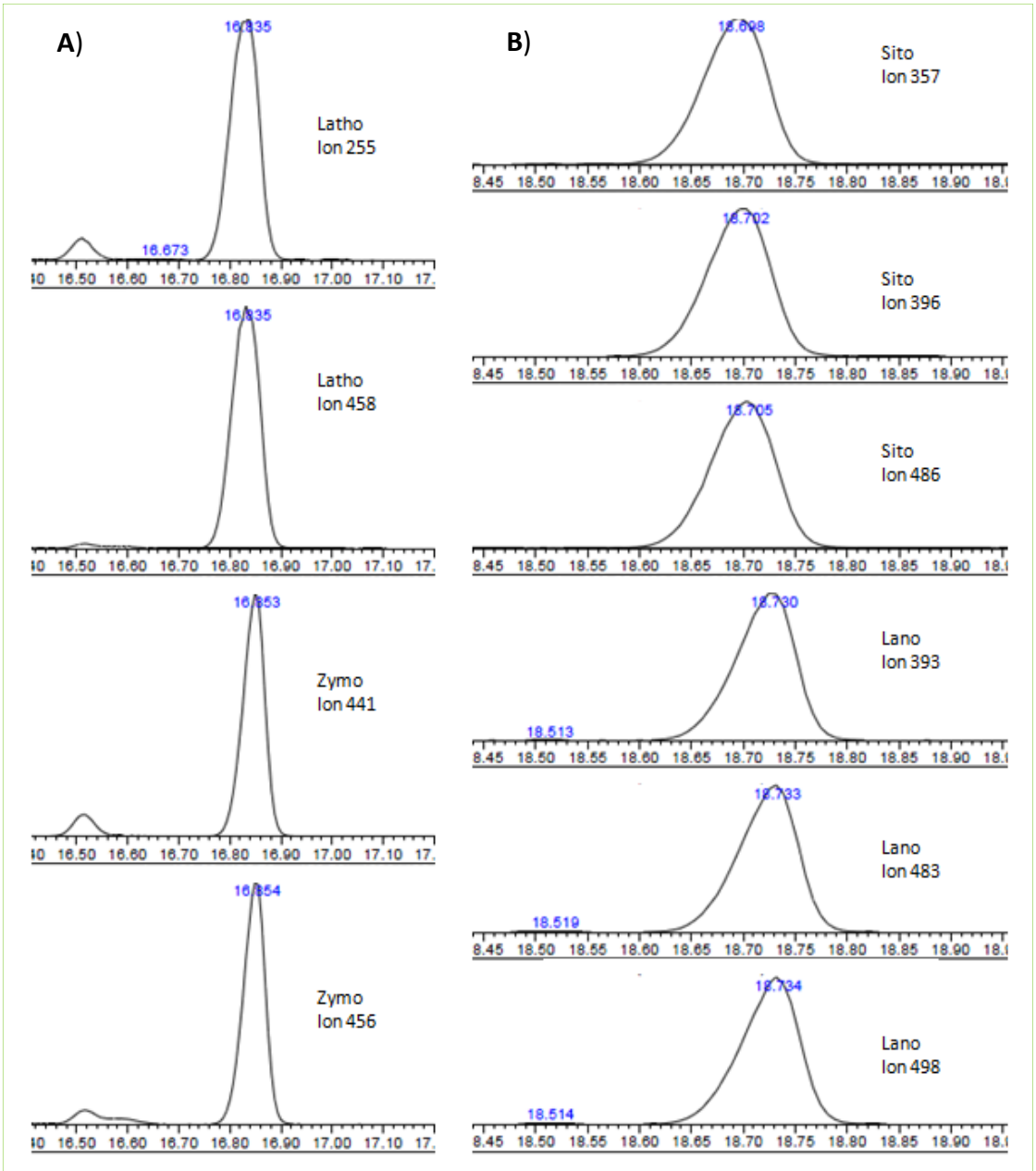


Figure 5.6 Extracted ion chromatograms showing co-elution of authentic lathosterol and zymosterol (panel A), and sitosterol and lanosterol (panel B).
Baseline resolution was not possible but monitoring multiple ions for each analyte allows the compounds to be separated based on mass and identifies a slight shift in the peak elution.

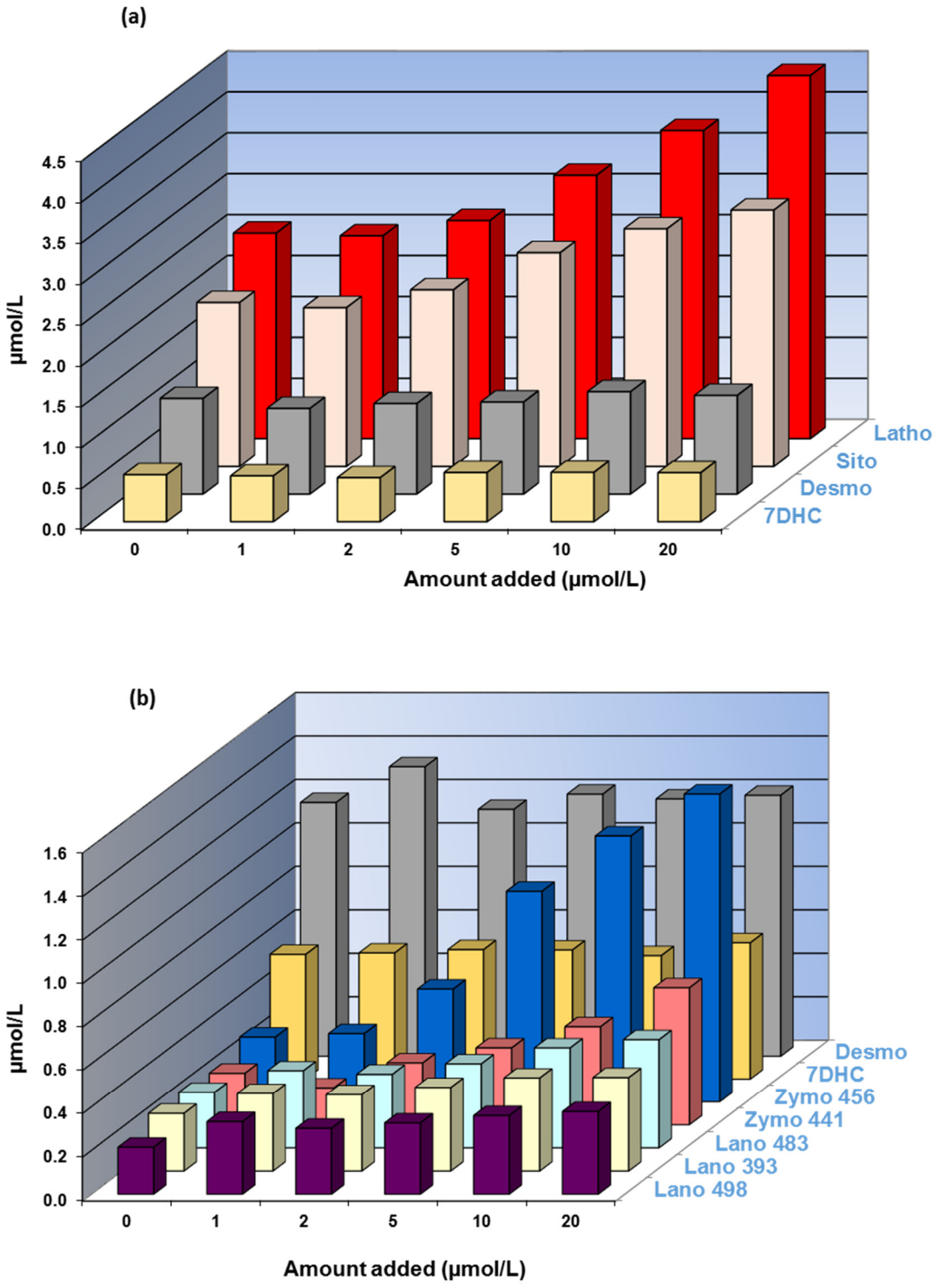


Figure 5.7 Effect of increasing sterol concentrations on closely related compounds.
(a) Plasma sample spiked with lanosterol (Lano) and zymosterol (Zymo) indicating an increase in the quantitation of sitosterol (sito) and lathosterol (Latho). **(b)** Plasma sample spiked with lathosterol and sitosterol, indicating a significant increase in the determination of zymosterol, particularly when quantitation is performed using the 456 ion. Desmosterol (Desmo) and 7DHC are included as independent analytes and showed no change in concentrations with increased spiked analytes. Numbers after the analytes name represent the ion monitored.

5.3.4 Evaluation of extended scan parameters

The acquisition parameters for methods with and without the additional analytes were compared by the analysis of 60 samples. Good correlation was found for all sterols tested demonstrating that the additional ions do not interfere with existing protocols. Example correlations are shown in figure 5.8.

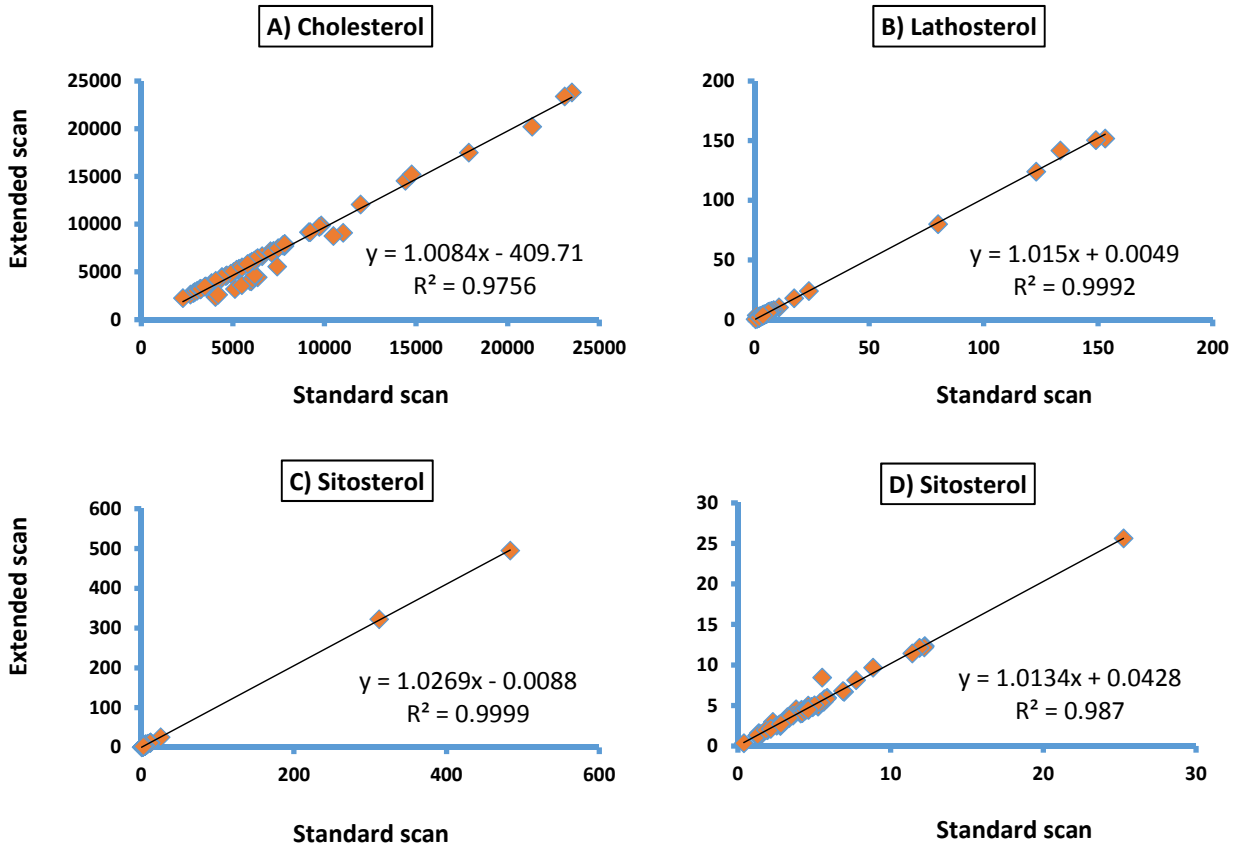


Figure 5.8 Correlations for sterols analysed using the current proticol and the extended profile containing additional ions (n=60).
The data set included normal and abnormal sterol profiles. Examples shown are for **A)** cholesterol, **B)** lathosterol, **C)** and **D)** represent the correlation for sitosterol shown over the maximal and physiological range respectively. All axis represent the concentrations expressed in µmol/L.

5.3.4.1 Quantitation of lanosterol and zymosterol in patient samples

From the data set mentioned above, 26/60 were quality controls and were thus not included in further correlations owing to their artificial nature. Of the remaining samples, 18 were from patients enrolled in a clinical trial and 16 were received for diagnostic sterol profiling, of which 3 were patients with known defects of sterol metabolism, and 2 gave abnormal sterol profiles,

both with cholesterol and lathosterol concentrations above the normal range as quoted by the Blood Sciences laboratory, Newcastle upon Tyne Hospitals NHS Foundation Trust.

Assessment of conflicting influences was undertaken by correlation of all values obtained for lanosterol and zymosterol with sitosterol and lathosterol (Figure 5.9). Sitosterol is a phytosterol therefore biologically has no expected correlation to endogenously synthesized sterols. These results also indicate no analytical complications would arise in the determination of lanosterol in plasma as excessive sitosterol (> 300 µmol/L) present in patient samples did not correlate to increased lanosterol measurement.

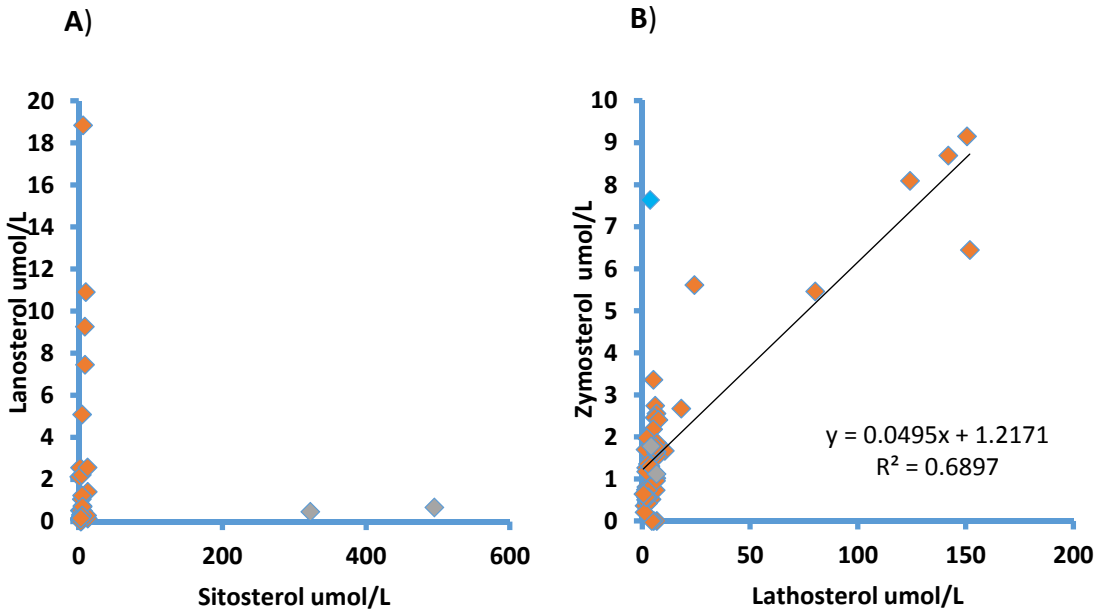


Figure 5.9 Correlation between endogenous co-eluting sterol levels in 34 plasma/serum samples.
A) Green points represent patients diagnosed with sitosterolaemia (sitosterol > 300µmol/L) and indicate excessive sitosterol does not interfere with the determination of lanosterol as no correlation is observed. **B**) A weak correlation between lathosterol and zymosterol suggests a biological origin rather than analytical interference. The blue point is from a patient under treatment for CTX (high cholestanol levels).

Lathosterol and zymosterol are both intermediates in cholesterol biosynthesis and can indicate flux through the pathway. Weak correlation between these two sterols was seen which did improve ($R^2 = 0.76$) when abnormal profiles were removed. Previous experiments revealed spiking plasma with 20 µmol/L lathosterol more than doubled the quantitation for zymosterol, but this equated to an actual increase of approximately 0.4 µmol/L. However,

analysis of authentic plasma samples indicates analogous increases in zymosterol with lathosterol; samples with endogenous lathosterol around 20 µmol/L have zymosterol concentrations of approximately 3 µmol/L. This suggests the association is most probably of true biological rather than analytical origin.

Diagnostic samples deemed to have normal sterol profiles as assessed by the existing sterol profile procedures, were used to determine physiological ranges for the additional compounds. These were, mean (95 % CI); lanosterol 0.41 (0.018 – 1.154) µmol/L, zymosterol 1.79 (0.30 – 5.58) µmol/L and zymostenol 1.14 (0.60 – 2.64) µmol/L. Lanostenol was undetectable in the majority of samples and had a maximum concentration of 0.11 µmol/L.

5.4 DISCUSSION

Non-cholesterol sterols are valuable surrogate markers for the efficiency of cholesterol absorption and biosynthesis. As shown in table 5.1, compared to cholesterol, these cholesterol precursors and plant sterols are present in plasma in only small amounts. They can be used to investigate patients with metabolic defects of cholesterol homeostasis but could also prove beneficial to patients with hypercholesterolaemia. Although statin intervention has great efficacy in reducing LDL-cholesterol, combination therapy with cholesterol absorption inhibitors, such as ezetimibe, could be recommended in patients identified as high cholesterol absorbers in order to maximise cholesterol reduction and improve clinical outcomes.

Two main routes for cholesterol biosynthesis downstream of farnesylpyrophosphate have been proposed; the Bloch and Kandutsch-Russell pathways. If reduction of the C24 double bond occurs early, cholesterol synthesis from lanosterol proceeds via lanostenol, zymostenol, lathosterol and 7-dehydrocholesterol. Alternatively, zymosterol, 7-dehydrodesmosterol and desmosterol are produced if the double bond reduction occurs at the final stage. DHCR24 is known to facilitate C24 bond reduction at multiple points throughout the pathway. Little evidence exists regarding evaluation of non-cholesterol sterols, in part due to the analytical restraints, therefore the experiments undertaken in this section were designed to help improve the current monitoring of cholesterol homeostasis.

5.4.1 Sample preparation and analysis

The data presented here indicate an improved analytical method has been established to measure an extended range of post-squalene sterol intermediates in plasma. Twelve structurally related cholesterol precursors and plant sterol can be analysed in a single analytical run using GCMS. These included the precursors lanosterol and zymosterol present at concentrations less than 1 μmol/L as well as their reduced counterparts. The GC conditions provided excellent resolving power and using the MS in SIM mode enhanced the specificity for each analyte as well as improving signal to noise ratio. The total run time for each analysis for 25 min.

Sample extraction was based on a modification of the Frolch method for lipid extraction. Lipids were saponified to cleave the ester bonds using 4 % KOH in ethanol. Hexane replaced the more toxic chloroform during the liquid-liquid extraction stage. This also simplified the removal of the partitioned organic phase, which in this case forms as the top layer. The TMS derivatives were directly injected onto the column, thus preventing possible analyte loss by omitting a second drying stage. The procedure required the use of only 100 µl of plasma or serum. For optimal quantitation, plasma should be separated and stored in the dark to prevent auto-oxidation of cholesterol and the intermediates, particularly 7DHC which converts to vitamin D_3 with ultraviolet light (Kelley, 1995).

Calibration curves for individual sterols were constructed with each batch of samples analysed and calculated using a single internal standard. This method was chosen firstly due to the lack of commercial availability of suitable stable isotopes. Secondly, the internal standard method can correct for any impact sample extraction may have on each analyte. It also enables the same internal standard to be used for a range of analyte concentrations which is useful for the simultaneous determination of cholesterol, which is generally at least 3 orders of magnitude greater than the intermediates.

5.4.2 Validation of the method

The limit of quantitation for the minor sterol intermediates was 0.1 µmol/L and the detection limit was 0.01 µmol/L. In normal control samples zymosterol and zymostenol were easily detected and quantifiable. Lanosterol and lanostenol, when detectable, were very close to the quantitation limit. However, in six samples analysed with evidence of hypercholesterolaemia (Chol > 10 mmol/L) both these compounds were easily detectable (range 9 – 18 µmol/L for lanosterol and 1-4 µmol/L for lanostenol). This suggests that any alterations or disturbances in the normal flux through the mevalonic pathway would be detected using this method and the currently available GCMS analyser.

Imprecision of the assay was evaluated and demonstrated variation up to 20 % for some analytes. This is not uncommon for assays of this type and complexity. Analytical variation should be considered in relation to the magnitude of the change expected in clinical cases which is often at least one order different. In this respect the analytical impression is small therefore useful inferences can still be made.

A normal range was established for the new analytes. However, it has been shown that ranges can vary within discrete populations and depend on the methods of extraction used (McDonald *et al.*, 2012). Therefore comparing reference ranges to alternative sources can provide variable results. That said, the ranges determined by this method do compare favourable to others in the literature (Honda *et al.*, 2008; McDonald *et al.*, 2012).

5.4.3 Co-elution

Influence of co-elution of analytes was considered for sitosterol with lanosterol, and lathosterol with zymosterol as it was not possible to achieve baseline resolution of these analytes. The most abundant ion detected for zymosterol was *m/z* 456. However, this also tended to be the most significantly affected by artificially high levels of lathosterol. For this reason, *m/z* 441 was chosen as the quantifier ion and 456 monitored as a qualifier ion. The four analytes all showed some degree of co-elution effects, although the impact on sitosterol and lathosterol did not reach statistical significance.

Analysis of authentic patient samples suggests quantitation of lanosterol is not significantly influenced by its co-eluting peak, as demonstrated in samples from patients diagnosed with sitosterolaemia, in whom sitosterol levels exceeded 300 µmol/L. Similarly, the analytical influences on zymosterol due to high lathosterol would appear to be inconsequential to the inherent biological relationship between these analytes.

5.4.4 Conclusion

A robust GCMS method has been established to measure an extended range of non-cholesterol intermediates in a single analysis in plasma. Expansion of the sterol profile enables more information to be gained regarding cholesterol homeostasis, including flux through the sterol biosynthetic pathway as well as insight into cholesterol absorption rates. The method can be applied to diagnostic samples for inherited metabolic diseases or used as potential markers or outcome measures for hypercholesterolemia.

Chapter Six

Chapter 6. Inhibition, biosynthesis and mitochondrial function of CoQ10

6.1 INTRODUCTION

6.1.1 Biosynthesis and inhibition

CoQ10 is present in virtually all tissues and cells and performs a variety of functions. It is essential to the mitochondrial respiratory chain, has independent antioxidant properties and the ability to recycles other antioxidants such as tocopherol (Mitchell, 1976), and has a role in cell signalling, gene expression, uncoupling proteins, apoptosis and anti-inflammatory processes (Crane, 2001; Bentinger *et al.*, 2010).

Its structure is a quinone ring attached to an all *trans* isoprenoid side chain (Teclebrhan *et al.*, 1993; Okada *et al.*, 1996). The enzyme, parahydroxybenzoate polyprenyltransferase, encoded by *Coq2,* catalyses the condensation of the tyrosine derived 4-hydroxybenzoate (4HB) ring to the mevalonate produced isoprenoid tail (Figure 6.1). The lack of substrate specificity of Coq2 enables the selective inhibition of CoQ10 biosynthesis using 4HB analogues. Forsman and colleagues demonstrated 4-nitrobenzoate (4NB) actively competes with 4HB in cell cultures and reduces CoQ10 concentration to 24 % of controls within 6 days post incubation *(Forsman et al., 2010)*. This analogue showed none of the toxicity or unknown chromatographic intermediates that had been observed previously with the use of 4-chlorobenzoate and 4-aminobenzoate respectively. These researchers also showed addition of exogenous 4HB tripled the CoQ9 concentration in murine cell lines, suggesting 4HB and not the isoprenoid chain was rate-limiting in this system. Co-treatment with both analogues effectively restored normal cell growth, counteracting the 11 % decrease in total protein and 50 % reduction in whole cell respiration seen in 4NB treated cells.

Studies using human fibroblasts with varying degrees of CoQ10 deficiency due to different molecular defects have revealed inconsistent consequences, such as ROS production, cell viability and mitochondrial function. For instance, cells with 30 - 45 % CoQ10 showed increased ROS production and lipid peroxidation whereas cells with <20 % or between 50 - 70 % of CoQ10 did not generate increased ROS (Quinzii *et al.*, 2012). However, as 4NB inhibits Coq2 in a dose-dependent manner, this can prove to be a valuable tool to assess the role of

CoQ10 *in vitro* in CoQ10 deficient fibroblasts without the influence of any other factors that may have contributed to the phenotype, thus allowing the degree of CoQ10 deficiency to be intrinsically linked to the extent of biochemical defects.

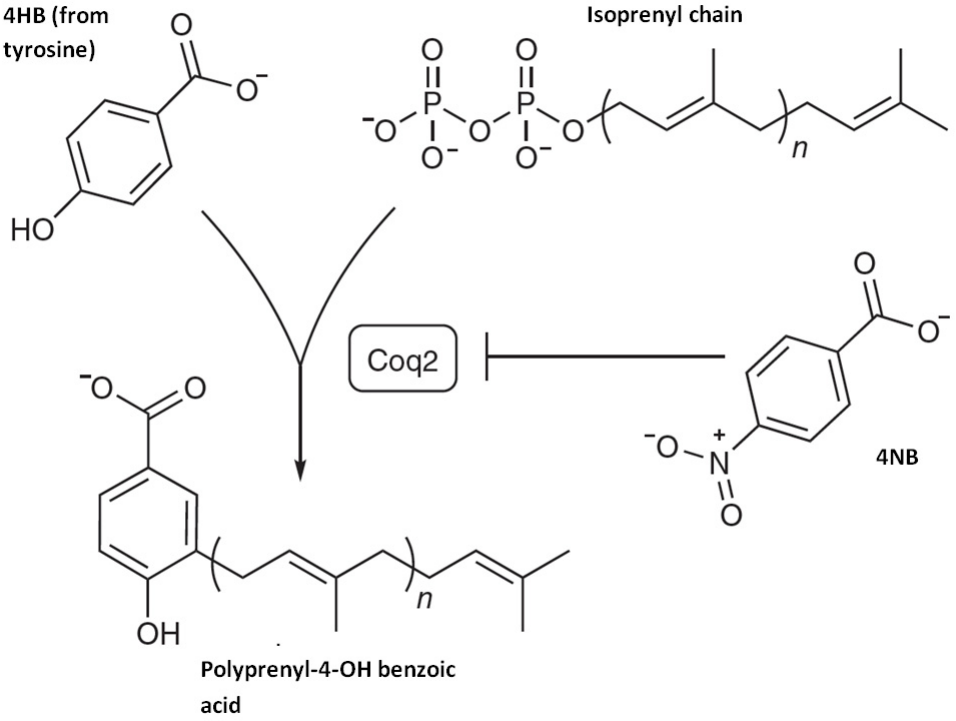


Figure 6.1 Condensation reaction of tyrosine and mevalonate metabolites catalysed by Coq2.
Inhibition of Coq2 using 4NB is indicated (adapted from (Forsman *et al.*, 2010))

As previous studies have indicated exogenous 4HB enhances the synthesis of CoQ10, the addition of carbon ring labelled isotopes of 4HB are useful in assessing the rate at which new CoQ10 is synthesized. Coq2 activity can be measured using $^{14}C_4$-HB (Forsman *et al.*, 2010) but to enable quantitation by mass spectrometry a carbon ring labelled stable isotope, $^{13}C_6$-HB, was evaluated.

6.1.2 Mitochondrial structure and superoxide production

An estimated 2 % of electrons from the mitochondrial electron transport chain are incompletely reduced resulting in the formation of mitochondrial superoxide, the predominant ROS species (Bentinger *et al.*, 2007). Whilst some degree of ROS production is

necessary for normal cellular functions including cell signalling and homeostasis, increased levels contribute to various disease pathologies and have been implicated in the cause of statin associated muscle symptoms (Lass *et al.*, 1997; Apostolopoulou *et al.*, 2015). CoQ10 is an integral redox carrier in oxidative phosphorylation, and as such is a plausible mediator of mitochondrial dysfunction and myopathy. It seems prudent therefore to investigate the impact of depleted CoQ10 levels, either by specific inhibition using 4NB or more broad based inhibition caused by statin treatment, on the generation of ROS.

Furthermore, the use of cell permeable fluorescent dyes suitable for live cell staining enables visualisation and quantitation of cellular changes as a consequence of *in vitro* treatments resulting in an altered level of CoQ10.

6.1.3 Statins

Statins inhibit the mevalonate pathway at the level of HMG-CoA reductase. Consequently all downstream metabolites are potentially affected. Conversely, the 4HB analog, 4NB, is specific for the inhibition of CoQ10 biosynthesis at the level of Coq2. Simvastatin is a hydrophobic 3-HMG-CoA reductase inhibitor which, although well tolerated in most patients, has been shown to decrease cell viability *in vitro* (Itagaki *et al.*, 2009). Recently it has been confirmed that two different subcellular pools of CoQ10 contribute differently to the protection of the cell (Mugoni *et al.*, 2013). The biosynthesis of the mitochondrial pool is controlled by the prenylation of 4HB by Coq2 whist the enzyme, Ubiad1, has been proposed to control the equivalent reaction in the cytosolic pool. It is possible that whilst statins have been shown to decrease the total CoQ10 content, mitochondrial reserves may be preserved in favour of the cytosolic component. This would result in normal mitochondrial function being maintained but increased ROS-mediated oxidative stress in cellular membranes. The use of, 4NB, however would ensure a reduction in the mitochondrial pool of CoQ10. Thus comparing the possible effects of CoQ10 reduction caused by statin treatment with inhibition by 4NB may provide some insight into a causal link between statin use and myopathy.

6.1.4 Aims

This chapter aims to examine the rate of CoQ10 biosynthesis *in vitro* within human fibroblasts, exploring the impact statin use may have on this process. Additionally, I will investigate the impact that CoQ10 reduction, either as a result of statin treatment or via specific inhibition of

the CoQ10 biosynthesis pathway, has on the production of mitochondrial superoxide. Furthermore, potential changes in mitochondrial membrane potential as a result of statin treatment and CoQ10 inhibition will be investigated.

6.2 MATERIALS AND METHODS

The reagents and methods described here are in addition to the general procedures described in Chapter 2. Ubiquinone analysis was performed as detailed in Chapter 3 using LC-MSMS.

6.2.1 Cell culture and treatment

All fibroblast control cells, obtained from the Newcastle biobank, were cultured in 25 cm^2 culture flasks in MEM supplemented with 10 % FBS, vitamins, amino acids, penicillin-streptomycin and glutamine until 70 % confluent.

Experiments were performed after incubation in medium additionally supplemented with one of the following; 4-hydroxybenzoic acid (4HB), 4-ntirobenzoic acid (4NB) and $^{13}C_6$ 4-hydroxybenzoic acid (labelled 4HB) which were purchased from Sigma-Aldrich (Dorset, UK) and prepared as 100 mmol/L stock solutions in PBS with the pH adjusted to 7.4 with saturated potassium hydrogen carbonate. Aliquots were stored at -40°C and added as indicated.

Simvastatin was supplied as the inactive lactone from Calbiochem (Leicestershire, UK). Stock solutions were prepared at 10 mmol/L either in ethanol or activated by heating at 50°C for 60 min with sodium hydroxide before neutralizing with HCl. Both formulations were tested with 96 h incubations following dilution with supplemented medium to give final concentrations of 1 µmol/L and 20 µmol/L. Aliquots were stored at -40°C and added as indicated.

Cells were seeded at the same density (1 x 10^5), harvested by trypsinisation and washed with PBS prior the analysis. Incubation times varied according to experiments and the split day was used as day 0.

6.2.2 Biosynthesis of CoQ10.

The tandem mass spectrometry transitions used to monitor CoQ10 requires the presence of the complete CoQ10 molecule with subsequent loss of the isoprenoid side chain. Therefore any newly synthesized CoQ10 that has incorporated the labelled 4HB should have a parent ion that is 6 mass units greater than its non-labelled counterpart. The SRM transitions were evaluated following a 96 h incubation of a control cell line with 5 µmol/L labelled 4HB. Duplicate experiments were performed using non-supplemented medium and cells incubated

in the presence of either 5 µmol/L labelled or unlabelled 4HB to assess any effects of the reagent preparations.

Dose response for uptake of labelled 4HB was evaluated by dilution with supplemented MEM to give final concentrations of 0.05, 0.1, 0.25, 0.5, 1, 5, 10 and 50 µmol/L. Cells were incubated for 72 h before harvesting by trypsinisation. The experiment was performed in duplicate using 2 different cell lines and the mean of the results reported.

The rate of incorporation of 4HB into CoQ10 was assessed by incubation of a control cell line, in duplicate, with 5 µmol/L $^{13}C_6$ 4HB and analysing the concentrations of labelled and unlabelled CoQ10 after 24 h, 48 h and 72 h. Additionally, the rate of incorporation of $^{13}C_6$ 4HB was compared using 7 independent cells lines incubated, in duplicate, for 72 hrs.

6.2.3 Inhibition of ubiquinone using 4-nitrobenzoate.

Following passage, control fibroblast cells were allowed to settle in standard supplemented medium for 24 hr before changing to treatment medium. Cells were grown in the presence of 1, 4 or 10 mmol/L 4NB and harvested after 2 or 7 days incubation, with a media change at day 4. Experiments were performed after 2 passages with each measurement carried out in duplicate. To demonstrate the reduction in measured CoQ10 is caused by reduced biosynthesis, and not CoQ10 breakdown, cell lines were additionally incubated for 7 days in the presence of excess 4HB (50 µmol/L) or 1 mmol/L 4NB + 10 µmol/L 4HB.

6.2.4 Effect of simvastatin on ubiquinone.

Dose effect of simvastatin on CoQ10 concentrations was investigated by dilution of stock simvastatin (prepared in ethanol) with supplemented medium to give final concentrations of 0.01, 0.1, 0.5, 1 and 5 µmol/L. A reagent control, consisting of a similar ratio of ethanol to medium as used in the treatments, was also tested. Co-treatment of control cells with simvastatin (1 µmol/L) and $^{13}C_6$ 4HB (10 µmol/L) was also examined. Following passage, cells were allowed to settle in standard supplemented medium for 24 hr before changing to treatment medium and harvested after 72 h.

6.2.5 Oxidative stress analysis

To estimate the production of ROS, cells were treated with either 0.5, 1 or 2 µmol/L simvastatin, or 1 or 2 mmol/L 4NB for 72 h before being harvested using trypsin and resuspended to a concentration of 1 x 10^6 cells/ml. A proportion of the cells was exposed to MitoSOX Red as described in Chapter 2 and analysis of ROS performed using flow cytometry. The gated population was determined based on the detected response of untreated cells. The remaining cells were extracted for quantitation of CoQ10 as described previously. The change in fluorescence signal comparative to untreated cells was determined and correlated with the associated change in CoQ10. Each experiment was performed in duplicate using 2 passage numbers.

6.2.6 Live cell imaging

Four fibroblast cell lines were incubated in either control medium or treatment medium containing either 1 mmol/L 4NB or 1 µmol/L simvastatin. For the live cell staining of fibroblasts using TMRM and CellTracker Green stains, incubations were performed in the same multiwall plate, using 9 wells each per cell line for control and simvastatin treatment and 6 wells per cell line for treatment with 4NB. Confocal microscopy was then used to investigate mitochondrial membrane potential and mitochondrial mass as described in Chapter 2. Results were calculated as the mean of all results per treatment. Standard 25 cm^2 culture flasks were used for the corresponding measurement of CoQ10, performed in duplicate. All treatments were left for 72 h before analysis.

6.2.7 Statistical analysis

All statistical analyses were performed using MedCalc for Windows, version 11.2.1.0 (MedCalc Software, Ostend, Belgium).

Data was assessed for normality using the D'Agostino-Pearson omnibus test and different treatments were compared using an independent t-test. A *p*-value of <0.05 was taken as significant in all cases.

6.3 RESULTS

6.3.1 Biosynthesis of CoQ10

The chromatograms obtained following CoQ10 analysis of cells cultured in the presence or absence of $^{13}C_6$-4HB are shown in figure 6.2. The peak obtained using the transition *m/z* 901 > 197 elutes at the same retention time as unlabelled CoQ10 and demonstrates that the labelled 4HB ring compound was incorporated into the cells. No peaks were detected using this transition when cells were incubated in control medium.

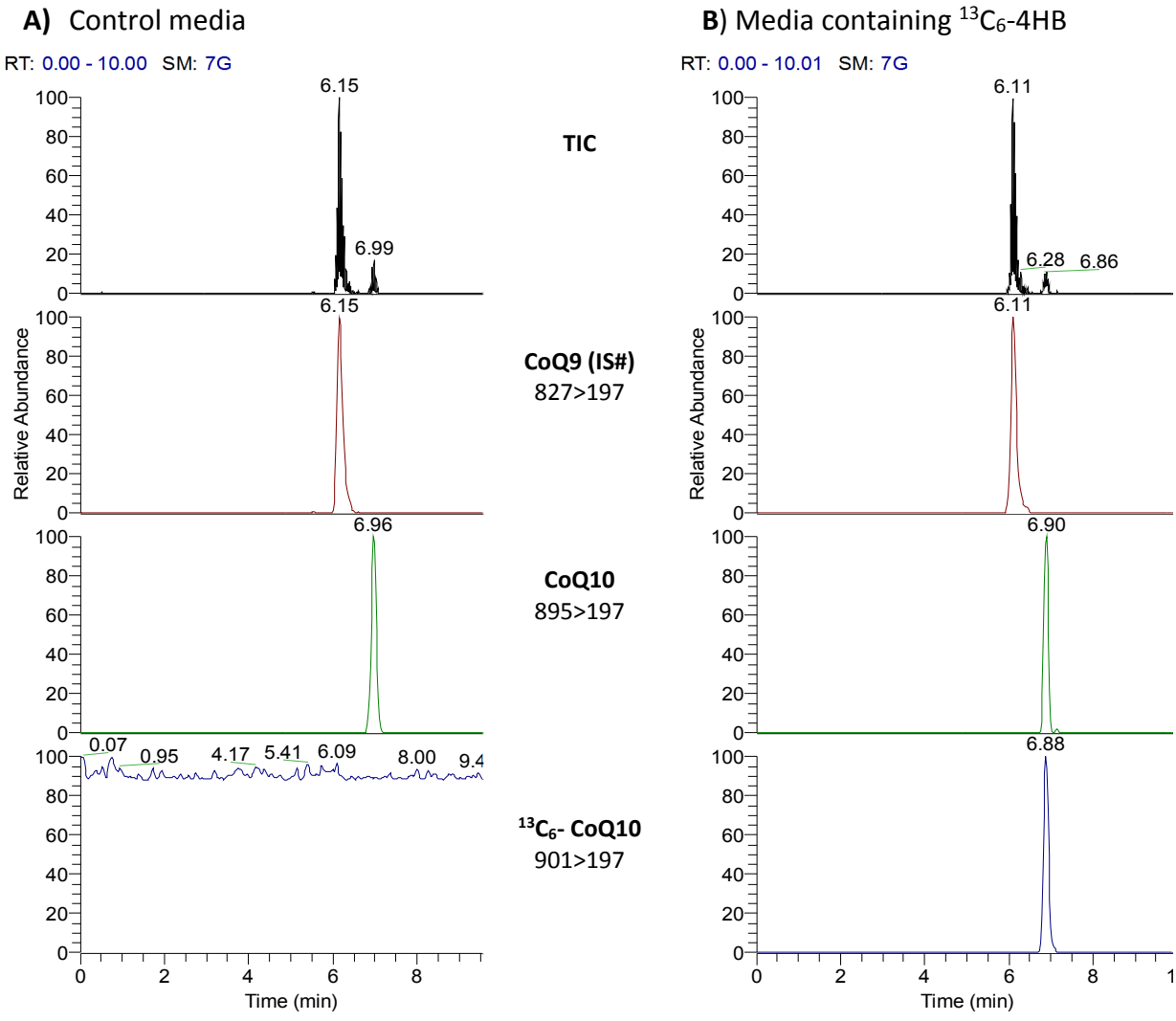


Figure 6.2 Positive electrospray mass spectrometry chromatograms of the 3 quinone compounds isolated from fibroblast cells.
The graphs show the TIC and the extracted ion chromatograms obtained when cells are incubated for 96 h in **A)** control media and **B)** supplemented media containing 5µmol/L $^{13}C_6$-4HB. A peak for transition 901 > 197 indicates incorporation of the labelled 4HB ring into the final CoQ10 molecule.

No statistical difference was observed in the total CoQ10 concentrations in cells following incubation in control medium or medium supplemented with either 5 μmol/L unlabelled or labelled 4HB. CoQ10 values were 198.3, 181.6 and 208.2 nmol/g protein respectively (n=2). Significance testing using independent t-test were p = 0.7669 for unlabelled 4HB and p = 0.7516 for labelled 4HB versus control medium.

Uptake of $^{13}C_6$-4HB showed a dose response for concentrations between 0.05 - 0.5 μmol/L. After 72 h incubation the maximal incorporation of $^{13}C_6$-4HB accounted for approximately 80 % of the total CoQ10. Concentrations of $^{13}C_6$-4HB greater than 0.5 μmol/L did not increase the percentage of CoQ10 containing the labelled isotope suggesting a finite rate of uptake was achieved (Figure 6.3). No difference in the total CoQ10 measured was observed (124 - 145 nmol/g protein).

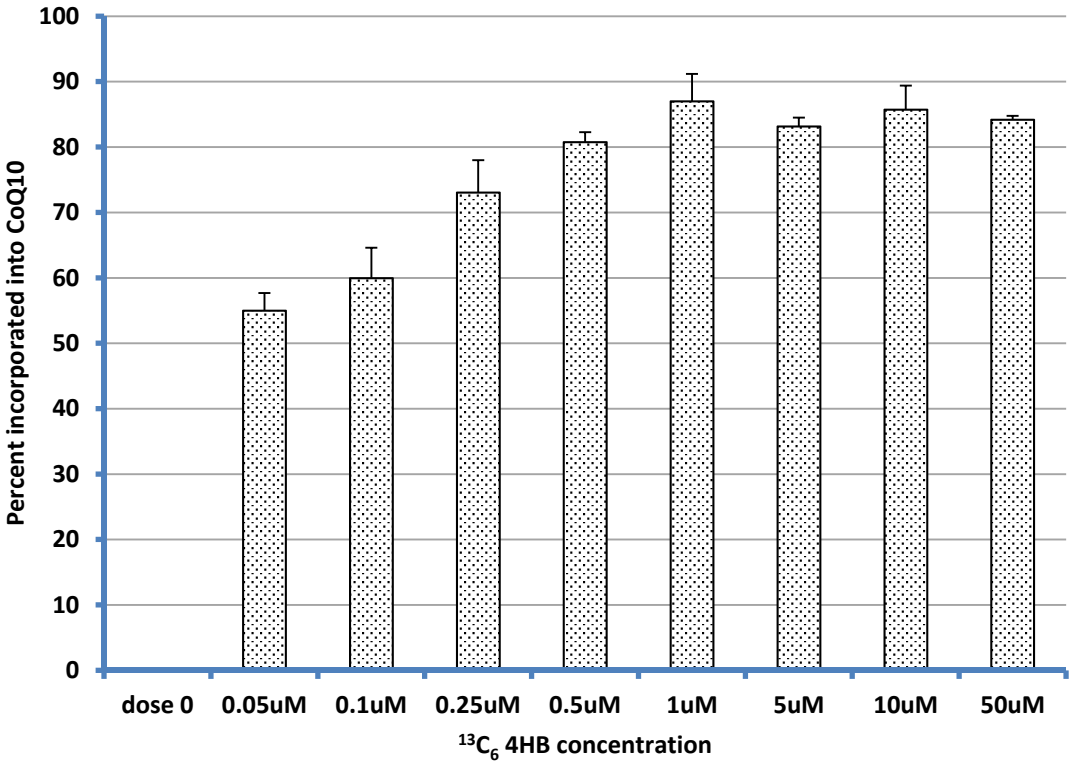


Figure 6.3 Dose response for uptake of $^{13}C_6$-4HB into CoQ10 in fibroblast cells following 72 h incubation. Maximum uptake of 80 % was achieved using 0.5 μmol/L labelled 4HB. Results are the mean and standard deviation of 2 cell lines analysed in duplicate in separate experiments (n=4).

Cells harvested at 24, 48 and 72 h post incubation with labelled 4HB indicated a steady increase in CoQ10 synthesis over time. Conversely, the concentration of unlabelled CoQ10 decreased by 70 % after 72 h compared to 24 h (Figure 6.4). The total concentration of CoQ10 detected increased by 66.7 % between 24 h and 72 h.

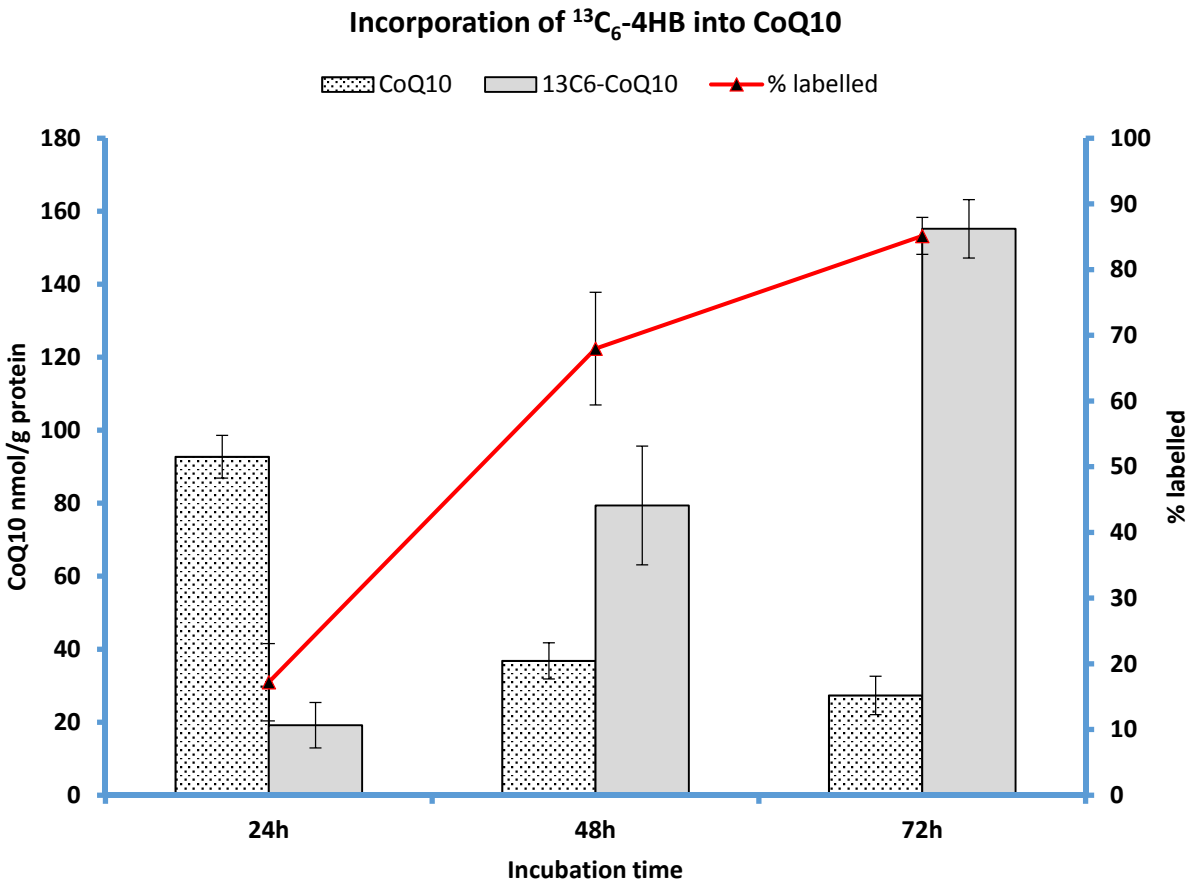


Figure 6.4 Incorporation of $^{13}C_6$-4HB (5 µmol/L) into CoQ10 in fibroblast cells.
Results are the mean of 2 incubations. Error bars represent the range of the calculated results

Assessment of the incorporation of labelled 4HB in 7 different control cell lines showed the variation in the rate of newly synthesised CoQ10 over a period of 72 h was between 76.8 % and 87.9 % (Figure 6.5).

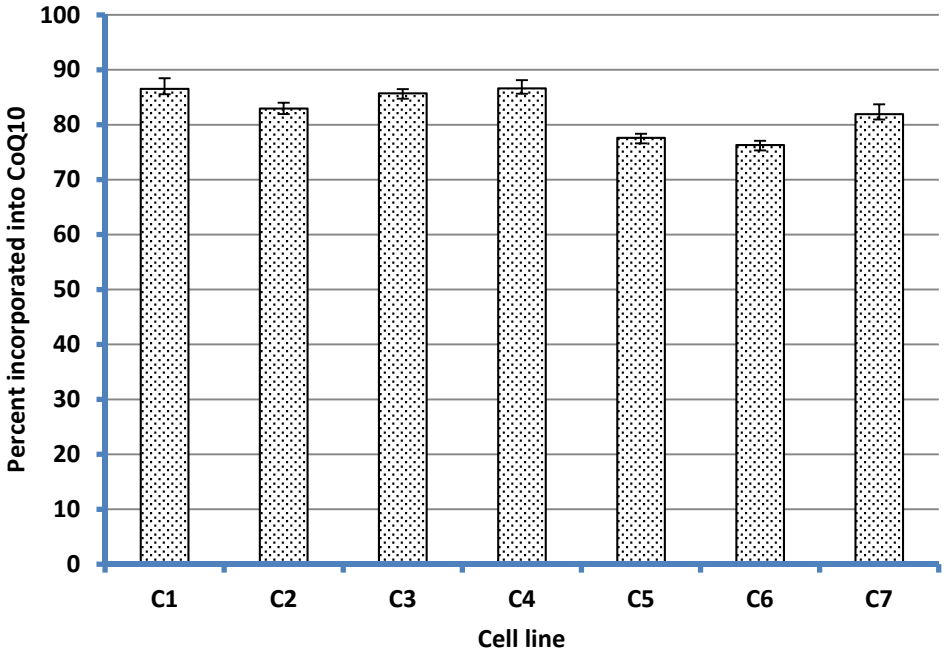


Figure 6.5 Rate of CoQ10 biosynthesis over 72 h in control cell lines as determined by the incorporation of $^{13}C_6$-4HB (5 µmol/L).
Each cell line (designated C1 –C 7) was incubated in duplicate in independent experiments and the mean and the ranges are shown.

6.3.2 Inhibition of CoQ10 with 4-nitrobenzoic acid

Treatment of fibroblast cells with 1 mmol/L 4NB for 7 days, which was the maximum time permitted to maintain a monolayer of cells in each flask, decreased the concentration of CoQ10 to 62 % (p = 0.0237) of the control level. A dose-dependent decrease was observed with CoQ10 concentrations of 41 % (p = 0.0036) and 15 % (p = 0.0005) of the control value for 4 mmol/L and 10 mmol/L 4NB respectively (Figure 6.6). Concentration of 4NB less than 1 mmol/L were not evaluated as this level was based on previous reported values.

After 2 days incubation, total protein concentration, which was measured as an indicator of cell number, was similar in all treatments and only decreased by 4 % using 1 mmol/L 4NB for 7 days, indicating no apparent toxic effect on the cells. However, 4 mmol/L and 10 mmol/L 4NB significantly decreased the protein concentration by 42 % and 73.5 % respectively (Figure 6.7). This toxic effect could also be observed using an inverted phase light microscope which showed morphological changes, such as rounded cells instead of the characteristic elongated spindle structure and an increased number of detached cells (approximately 50 % confluency compared with 80 % in control conditions) with 10 mmol/L 4-NB treatment.

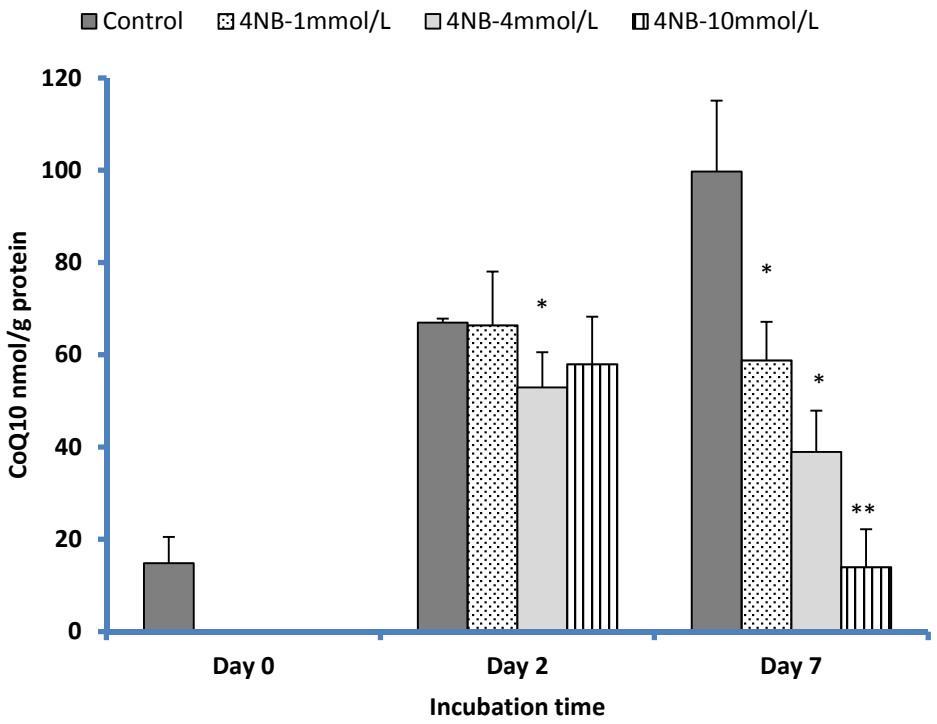


Figure 6.6 Dose response of CoQ10 concentration to 4NB treatment harvested at 2 and 7 days post incubation. Experiments were performed in a single fibroblast cell line, analysed in duplicate at two passage numbers Bars represents the mean of the 4 results, error bars are the standard deviations. *p < 0.05, **p < 0.001 versus same day control.

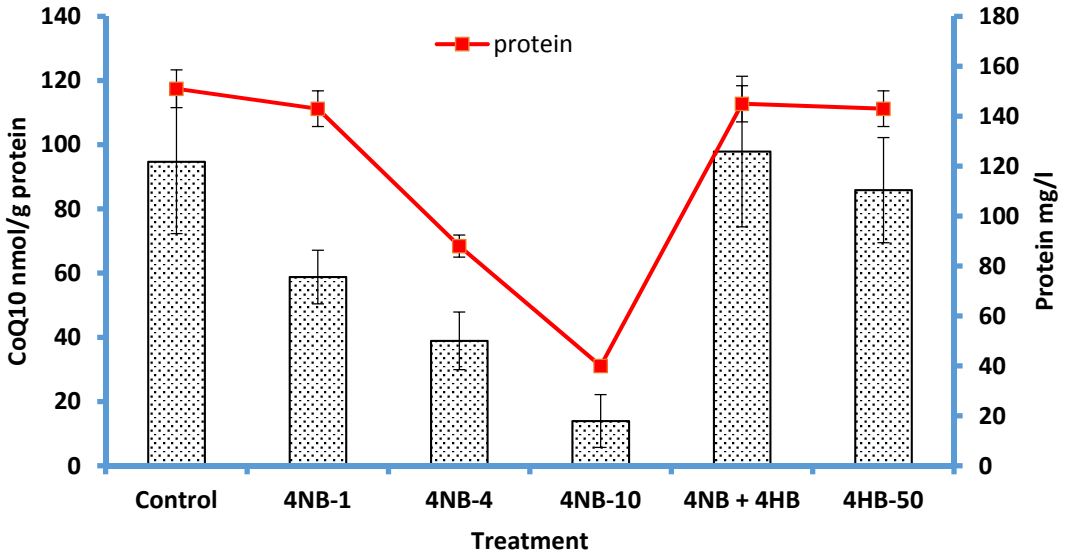


Figure 6.7 CoQ10 concentrations in the presence of 4NB and/or 4HB.
CoQ10 was measured in fibroblasts after 7 days treatment with 4NB at 1, 4 and 10 mmol/L. 4HB was added at 50 µmol/L. Co-treatment used 1 mmol/L 4NB + 10 µmol/L 4HB. Protein concentrations are also shown Results are the mean and standard deviation of 2 incubations at two passage numbers (n=4).

Co-treatment of the cells with 4NB + 4HB completely restored CoQ10 biosynthesis. The mean concentrations were 94.7 nmol/g for untreated controls and 97.8 nmol/g for co-treated cells (p = 0.85). Incubation medium containing 50 µmol/L 4HB did not significantly affect the concentration of CoQ10 after 7 days (mean 85.9 nmol/g, *p* = 0.55) (Figure 6.7).

6.3.3 Inhibition of CoQ10 by statins

With both formulations, 20 µmol/L simvastatin caused visible cell death after 96 hr incubations. This time frame was used as under ideal conditions, fibroblast cultures will generally reach full confluency of the monolayer when cultured in standard tissue culture flasks (25 cm^2). The absolute concentrations of CoQ10 and protein in fibroblasts were significantly reduced compared to controls which resulted in aberrant CoQ10 concentrations when corrected for the protein. These results are likely to represent the original levels within the cells rather than suggestive of a reduced rate of cellular CoQ10 inhibition. However, 1 µmol/L simvastatin, activated with either ethanol or an acid/alkali reaction, resulted in a significant decrease (58 %) in CoQ10 concentration without any apparent toxic effects (Figure 6.8).

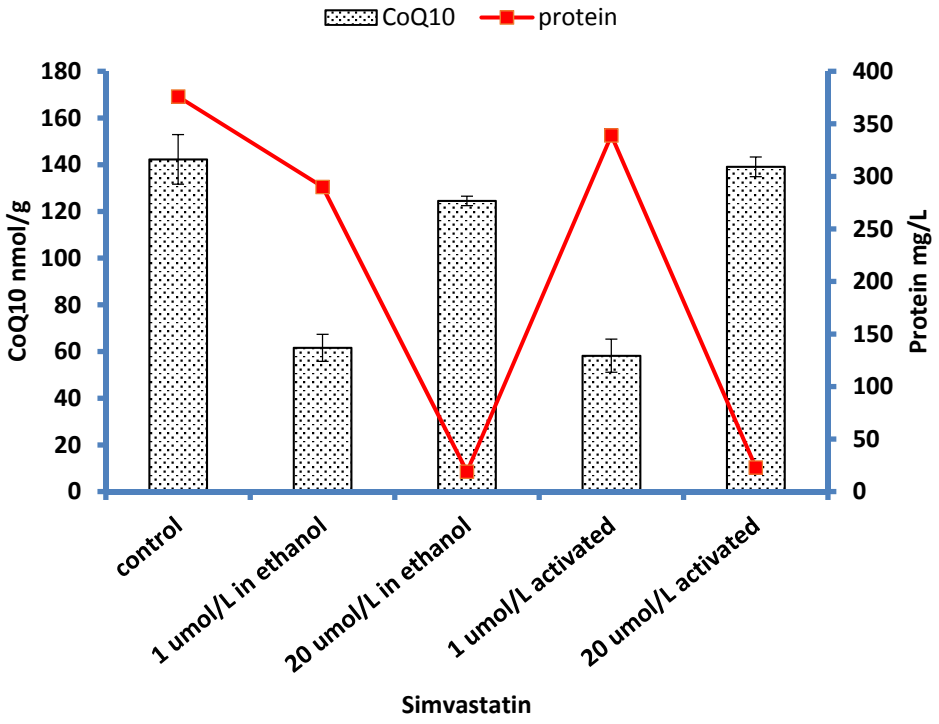


Figure 6.8 Effect of two formulations of simvastatin on total CoQ10 concentration in fibroblasts.
Cells were incubated for 96 h. The decrease in protein reflects significant cell death. Results are the mean of 2 incubations performed in a single experiment. Error bars represent the range of the individual results.

Simvastatin treatment consistently resulted in reduction of CoQ10 when tested at concentrations between 0.01 – 1 µmol/L without any significant change in the protein content. A trend towards a dose response was seen, with values declining to 62.6 %, 60.5 %, 59.2 % and 56.5 % of the control level with increased simvastatin concentration. Significant toxicity was evident at concentration above 1 µmol/L (Figure 6.9).

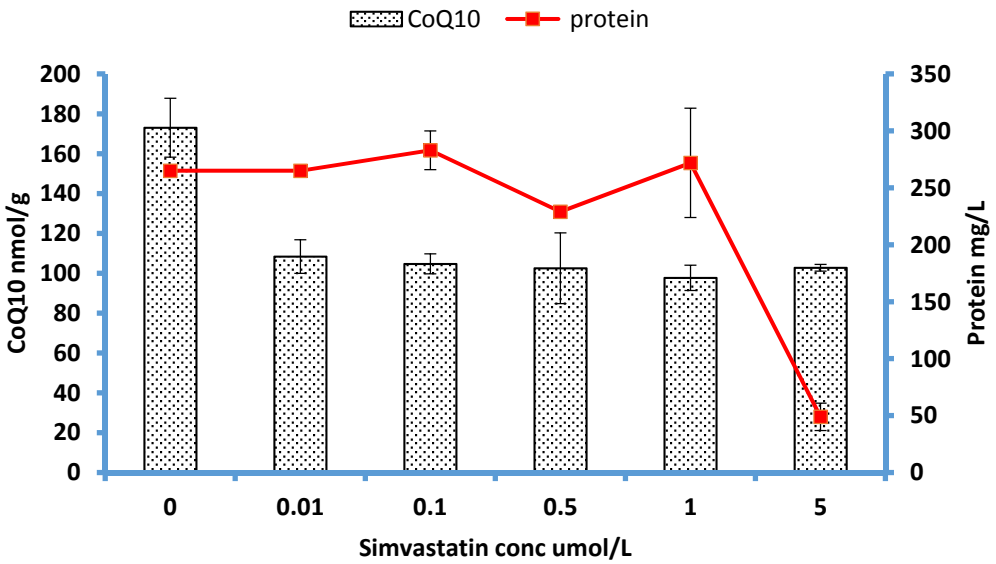


Figure 6.9 CoQ10 and protein concentration in fibroblasts after 72 h treatment with increased concentration of simvastatin.
Concentrations above 1 µmol/L caused significant cell death. Results represent the mean of duplicate incubations and the error bars show the range. Less than 10 % variation was observed for all duplicate results.

The concentration of CoQ10 in cells incubated with the ethanol spiked medium did not show any significant difference from control levels. The previous experiments determined the maximum dose of simvastatin required to effectively reduce CoQ10 concentrations was 1 µmol/L and that concentrations of 4HB greater than 1 µmol/L were well tolerated by cells. However, in contrast to inhibition due to 4NB treatment, decreased levels of CoQ10 as a result of simvastatin treatment (1 µmol/L) were not rescued by co-treatment with an excess of 4HB (10 µmol/L) (Figure 6.10).

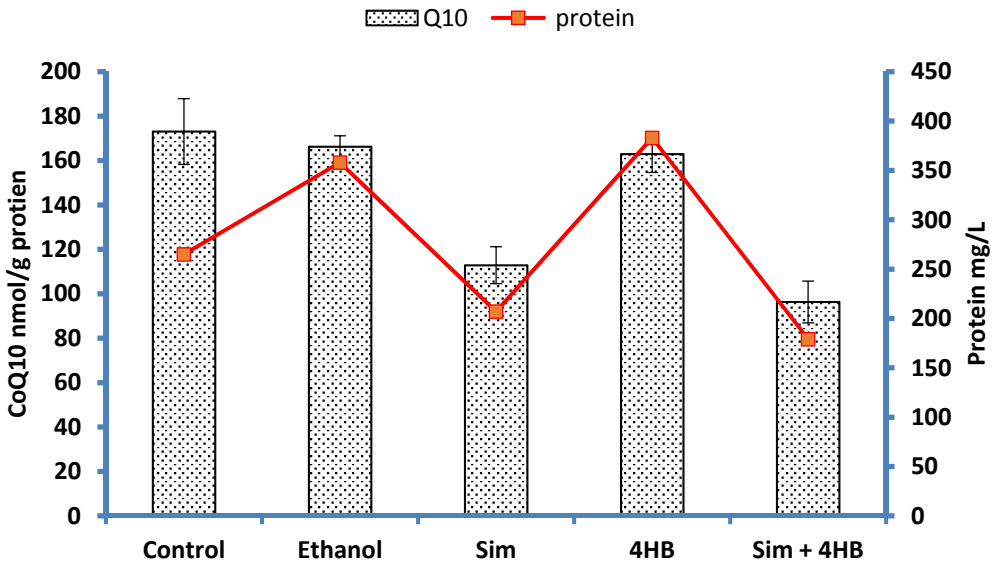


Figure 6.10 CoQ10 concentrations after 72 h under different treatment conditions.
Simvastatin (Sim) was added at 1 µmol/L and 4HB was at 10 µmol/L. Simvastatin caused a decrease in total CoQ10 concentrations which was not rescued by co-treatment of the fibroblasts with the artificial substrate 4HB. Bars represent the mean and error bars the range of duplicate incubations.

In addition, the cells incubated with $^{13}C_6$-4HB alone showed 71 % label incorporation into CoQ10 whereas in the presence of the statin only 41.5 % of the total CoQ10 detected contained the labelled substrate (p <0.001).

6.3.4 Oxidative stress

Flow cytometry analysis was used to assess the production of ROS in fibroblasts by detection of mitochondrial superoxide using the fluorogenic dye, MitoSox Red. A gated window (P1), excluding points with low forward and side scatter which generally represents cell debris, was manually established using unstained cells as shown in figure 6.11. All further investigation used cells that fell within this gated area to ensure any treatment conditions did not alter the physical characteristics, such as size, of the cells.

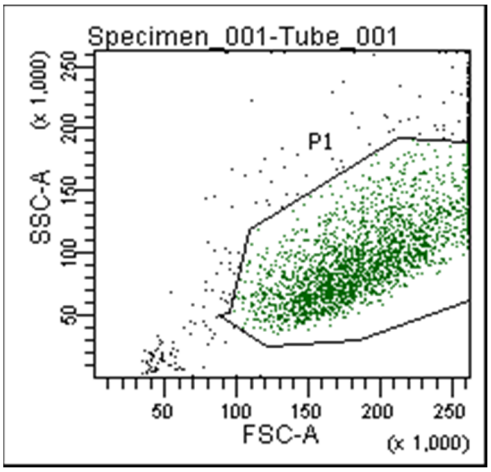


Figure 6.11 Gated cell population (unstained control fibroblasts) used for flow cytometry investigations.
The physical characteristics of approximately 6000 cells (events) were captured and a gated window (P1) established to encompass the majority of the cells. Cells debris, represented by low forward (FSC-A) and side (SSC-A) scatter, was excluded from the gated population.

In two independent experiments using control cells not treated with MitoSOX dye, 76 % of the population fell within the P1 gated area. Control cells plus dye and those incubated with either 1 mmol/L 4NB or 0.5 μmol/L simvastatin did not show significant alteration in the morphology of the cells. In contrast, cells incubated with 1 μmol/L and 2 μmol/L simvastatin, and 2 mmol/L 4NB showed a shift towards smaller, more compact or granulated cells. The decrease in P1 population correlated with increased concentration of inhibitor and ranged from 52.8 – 64.6 %, $p < 0.05$, $n=4$.

As expected, the addition of MitoSOX Red to control cells produced a significant increase in fluorescence compared to untreated cells. Quantitative measurements of the mean fluorescence intensities of samples treated with 0.5 μmol/L simvastatin resulted in a further 17.8 % increase compared to dye treated controls and a 45 % increase when high concentrations of 2 μmol/L were added to the medium. Both concentrations of 4NB tested (1 mmol/L and 2 mmol/L) demonstrated a comparable increase in MitoSOX fluorescence intensity (28.6 % and 29.6 % respectively), which was similar to that seen with 1 μmol/L simvastatin (27.7 %). All the changes in fluorescence following treatments reached statistical significance using the independent t-test and a *p*-value of < 0.05. Results are summarised in Table 6.1 and representative histograms of the FACS analysis for each treatment are shown in figure 6.12.

	Cell population within P1 (%)		Fluorescent intensity		Change in Fluorescence from control (%)		CoQ10 nmol/g		Change in CoQ10 from control (%)	
Control - no dye	**76.8**	(0.1)	**248**	(30.5)						
control	**73.3**	(3.5)	**2814**	(348.1)	**0**		**119.6**	(13.9)		
Sim 0.5 μmol/L	**71.7**	(4.0)	**3314**	(224.1)	**17.8**	(7.7)	**67.1**	(12.6)	**56.1**	(12.1)
Sim 1 μmol/L	**64.6**	(5.3)	**3592**	(352.5)	**27.7**	(12.5)	**44.4**	(3.9)	**37.1**	(3.2)
Sim 2 μmol/L	**52.8**	(3.4)	**4086**	(350.3)	**45.2**	(12.4)	**40.8**	(11.5)	**34.1**	(9.6)
4NB 1 mmol/L	**70.7**	(3.1)	**3649**	(403.6)	**29.6**	(14.3)	**35.9**	(9.9)	**30.0**	(8.2)
4NB 2 mmol/L	**61.6**	(3.8)	**3618**	(249.1)	**28.6**	(8.8)	**25.2**	(1.6)	**21.1**	(1.3)

Table 6.1 Results of oxidative stress analysis in fibroblasts.
Fluorescent intensity, measured using MitoSOX dye, was recorded for the cells detected within the gated P1 area using flow cytometry. CoQ10 concentrations were calculated using LC-MSMS. Percent change is determined as the difference from the control cells treated with MitoSOX dye. Data represent the mean ± standard deviation, n=4. Sim = simvastatin.

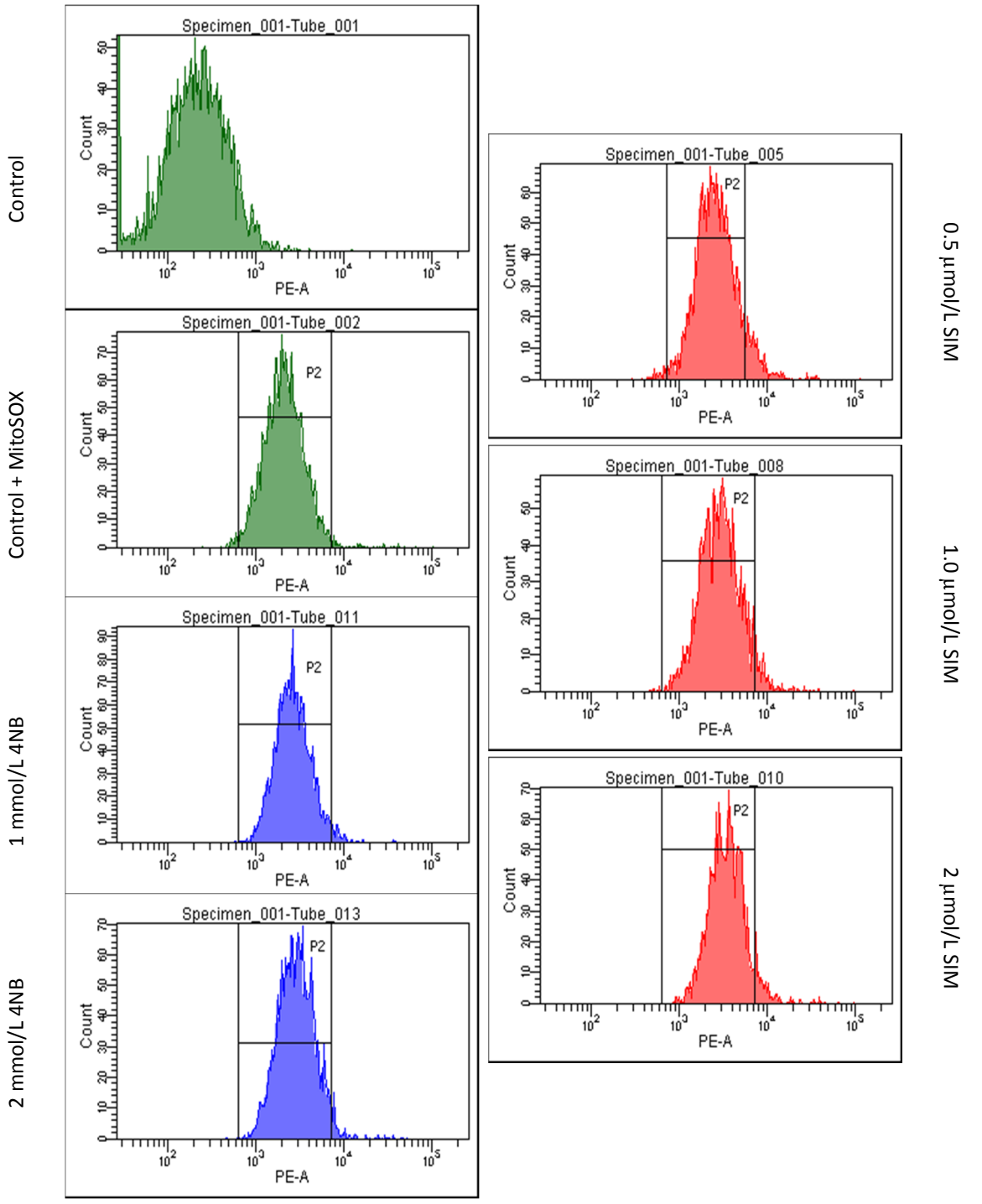


Figure 6.12 Representative histograms of flow cytometry experiments demonstrating increase in mean fluorescent intensity of oxidised MitoSOX following treatment as indicated.
The peak window (P2) was set based on the analysis of control fibroblasts following treatment with MitoSOX dye and used as a guide to visualise shifts in fluorescence as a result of treatment with either simvastatin (red) or 4NB (blue). All analysis were performed within 1 hour of incubation with MitoSox and approximately 6000 events were recorded for each experiment. Each treatment was repeated in 4 experiments.

Quantitative measurement of CoQ10 in the corresponding treatments demonstrated an inverse relationship with mean fluorescence (Figure 6.13). Compared to controls, total CoQ10 was reduced by 62 % with 1 μmol/L simvastatin and 69 % when synthesis was inhibited with 1 mmol/L 4NB ($p < 0.05$ in both cases). Higher concentrations of these inhibitors decreased CoQ10 by 66 % and 78 % respectively.

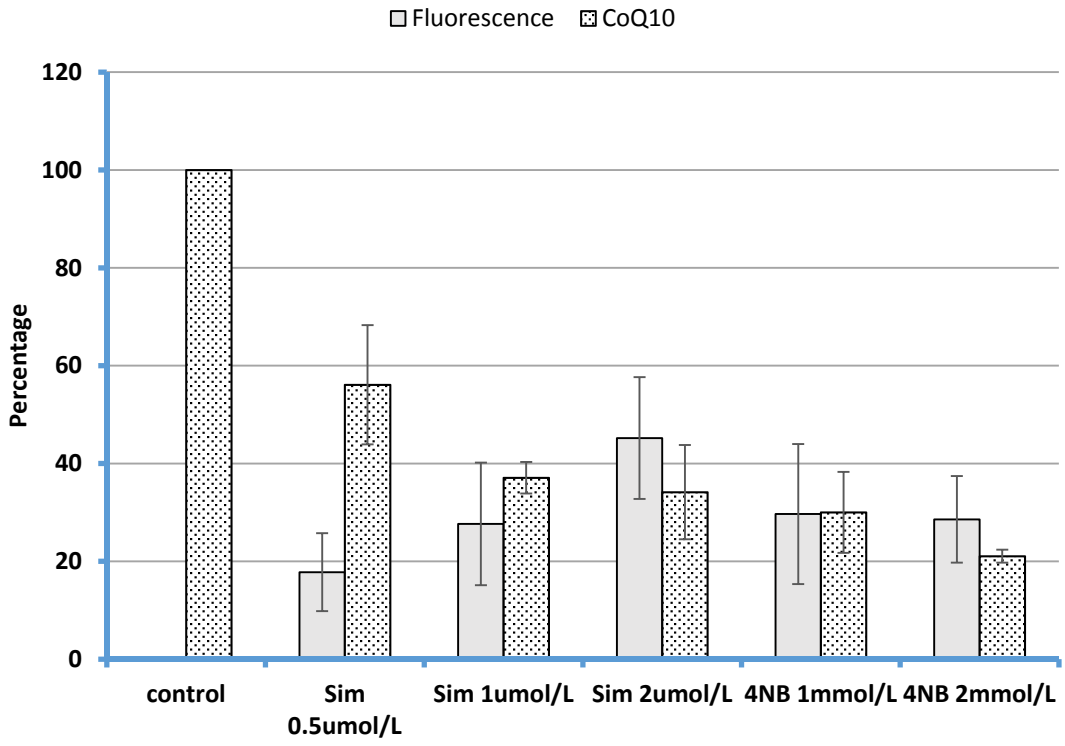


Figure 6.13 Quantitative data expressing changes in CoQ10 and ROS following various treatments in fibroblast cells for 72 h.
ROS is represented by the change in fluorescence intensity of MitoSOX (expressed as percentage of the controls after treatment with MitoSOX only) measured by flow cytometry. CoQ10 represents the calculated concentration as a percent of the control value. Data bars represent the mean and errors bars the standard deviation from 4 experiments. All results were significant ($p < 0.05$) compared to controls.

6.3.5 Mitochondrial imaging

Using the ImageJ software, the total cell area coverage in each well was determined by the degree of green fluorescence. Red fluorescence, caused as a result of the membrane potential driven accumulation of TMRM within healthy functioning mitochondria was used to determine the area of the cells comprising mitochondria. Figure 6.14 shows sample images of the cells from one well after 72 h in treatment conditions and stained with CellTracker Green and TMRM.

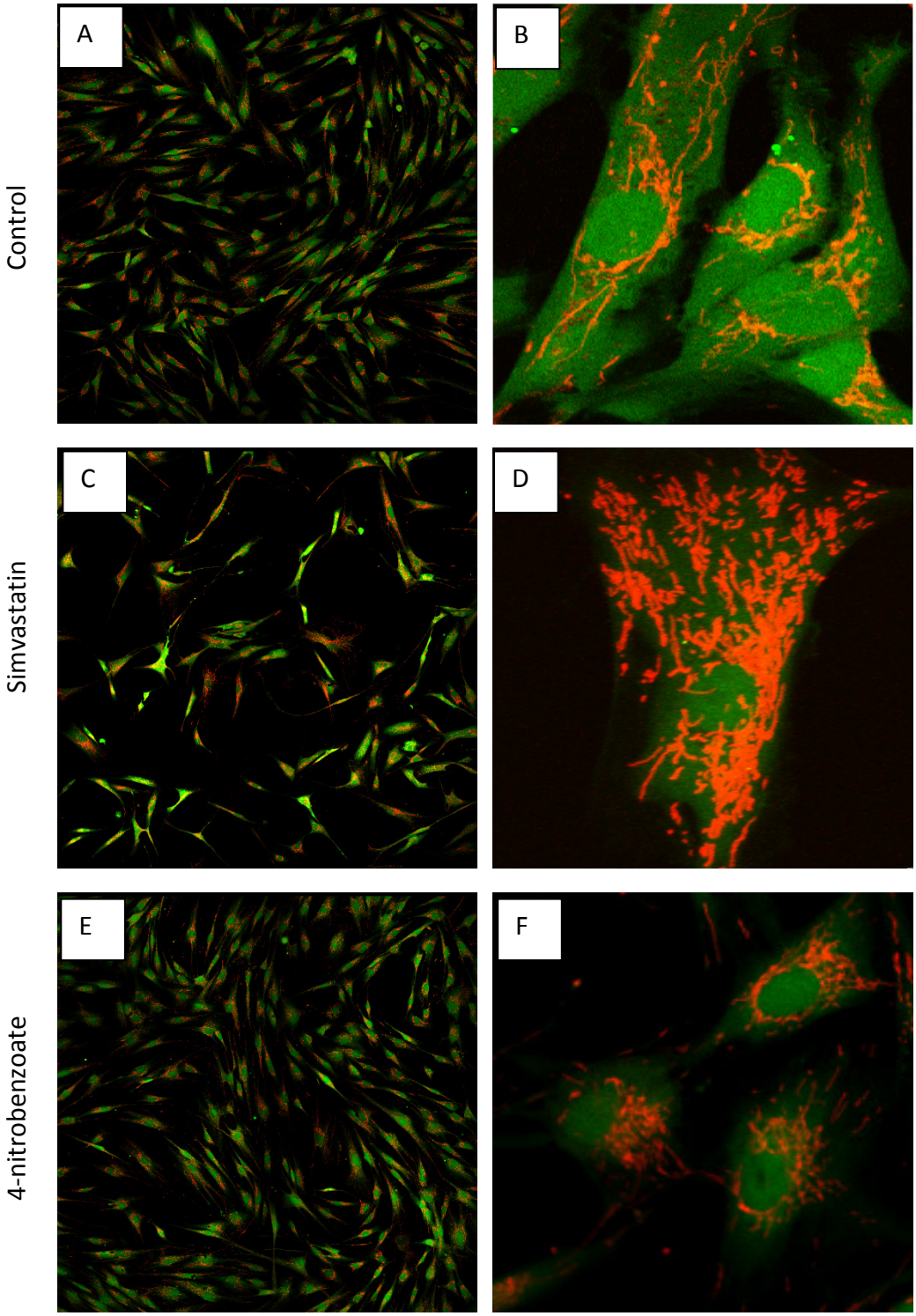


Figure 6.14 Confocal images of fibroblast cells showing changes in cell number and mitochondrial mass. Control cells are shown in panels **A-B, C-D** were treated with 1 µmol/L simvastatin, and **E-F** were incubated with 1 mmol/L 4-nitrobenzoate. Images represent the contents of one well **(A, C, E)** and a separately imaged enhanced image of individual cells **(B, D, F).** Analysis was performed using a 96-well plate. In total, 36 wells were used for each of the control and simvastatin treatments and 4-nitrobenzoate was included in the remaining 24 wells. Cells are stained with CellTracker (green) and TMRM (red).

There was a significant decrease in the cell number ($p < 0.0001$) when cells were incubated in the presence of simvastatin compared to control medium. 4-nitrobenzoate also caused a reduction in cell number but this was not as pronounced ($p < 0.05$) (Figure 6.15A).

Cells treated with simvastatin had a decreased total mitochondrial mass but an increased area of the cell comprising mitochondria (mean 28.6 %, 95 % CI 25.7 – 30.5 %) compared to both controls (25.6 %, 23.1 – 28.6 %) and 4HB treatment (25.7 %, 23.3 – 29.1 %) ($p < 0.0001$). Although the total mitochondrial mass in 4NB treated cells was lower than controls, this was not significant when the mitochondrial content per cell was examined (Figure 6.15B).

The concentration of CoQ10 measured under the same treatment conditions was significantly lower in both inhibitor treated cell groups. Compared to the controls, CoQ10 levels decreased by 53 % with simvastatin and 41 % with 4NB ($p < 0.01$), which is similar to levels seen in previous experiments (Figure 6.16).

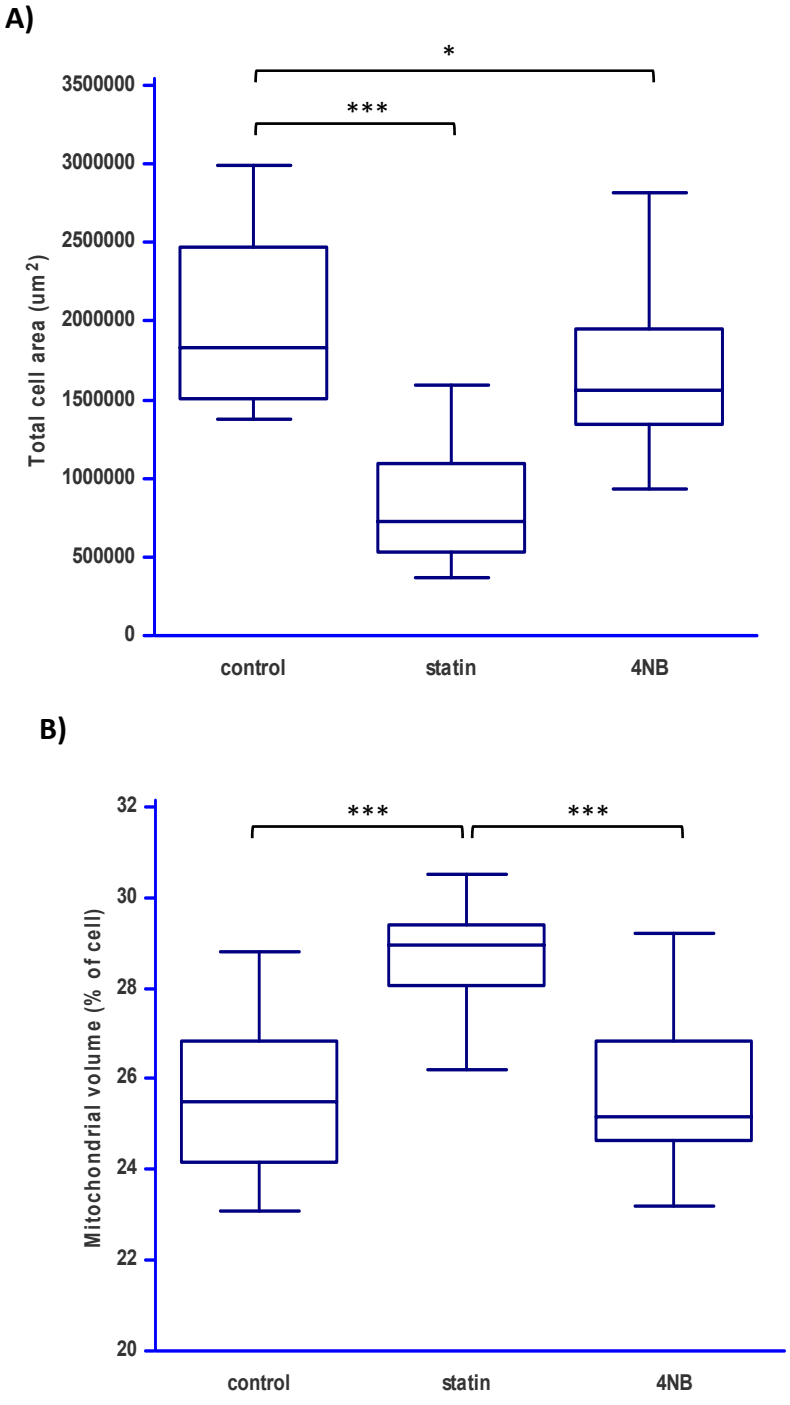


Figure 6.15 Variation in A) cell number and B) mitochondrial mass per cell, in fibroblasts incubated for 72 h with 1 µmol/L simvastatin (statin) or 1 mmol/L 4-nitrobenzoate (4NB).
Cell area was determined by the degree of staining using CellTracker Green. Mitochondrial volume per cell was calculated as the ratio of red (TMRM) to green (CellTracker) staining per well. N = 36 for control and statin treatment and n=24 for 4NB treatment. A two-tailed t-test for significance was performed, *** $p < 0.0001$, * $p < 0.05$

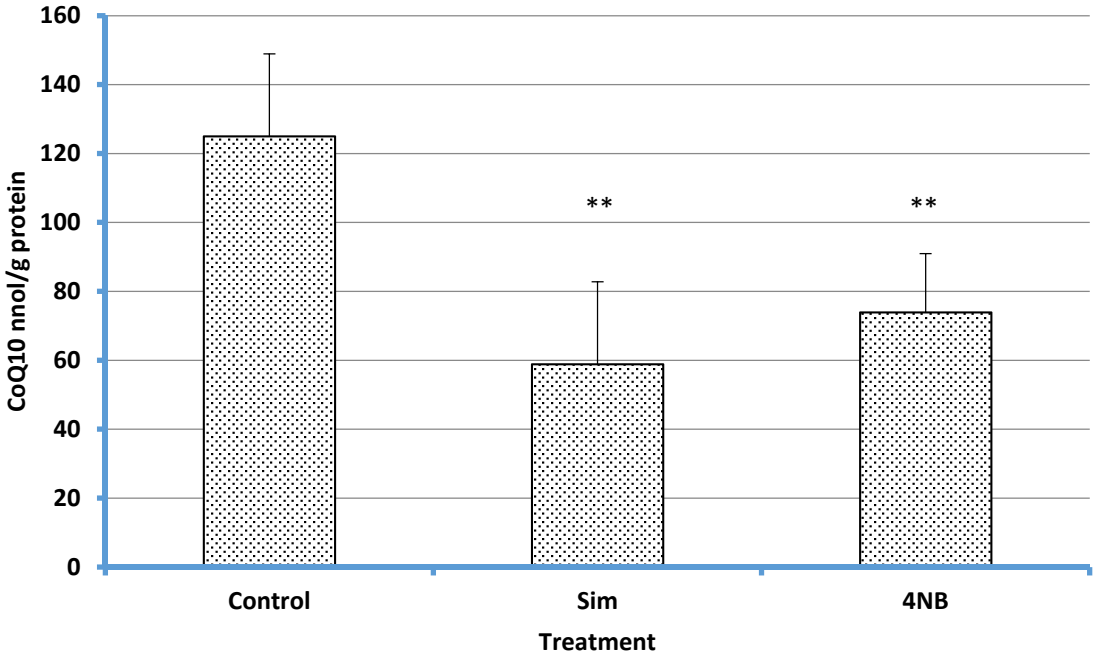


Figure 6.16 Concentration of CoQ10 in fibroblasts analysed in parallel with experiments for confocal mitochondrial imaging.
Results are the mean (plus standard deviation) of 8 duplicate extractions from 4 different cell lines.
**$p < 0.001$ compared to controls using Mann-Whitney U test for significance

6.4 DISCUSSION

6.4.1 CoQ10 biosynthesis

The data presented in this chapter confirmed the incorporation of 4-hydroxybenzoate as the ring structure in the synthesis of CoQ10. The LC-MSMS method described in Chapter 3 was modified to enable differentiation between concentrations of unlabelled CoQ10, containing 4HB derived from amino acid metabolism, and newly synthesised CoQ10 containing an exogenously supplied carbon ring labelled stable isotope, $^{13}C_6$-4HB. The maximal degree of inclusion after 3 days incubation was approximately 80 %, achieved using 0.5 μmol/L $^{13}C_6$-4HB. This was consistent in all 7 control cell lines evaluated (range 76.8 – 87.9 %). Concentrations in excess of this level did not increase the percent of labelled CoQ10 formed over the time period investigated. Moreover, incubation of fibroblast cells in high concentrations of 4HB, up to 50 μmol/L, did not increase the total concentration of CoQ10 measured compared to control cells. This is in contrast to in vitro studies using mouse 3T3-Swiss cells (Forsman *et al.*, 2010) which suggest relatively low concentrations (5 μmol/L) of 4HB more than tripled the synthesis of CoQ9, a clear indication that 4HB was rate limiting in the process. Conversely, and in keeping with the data presented here, a previous study using control skin fibroblasts did not detect increased total CoQ10 after 6 days incubation with 2 mmol/L 4HB (Quinzii *et al.*, 2012). This suggests that, in these cells at least, 4HB is not rate-limiting in the biosynthesis of CoQ10 and that the availability of mevalonate derived isoprenoid chain may be the limiting factor.

The increase in the proportion of CoQ10 containing the labelled substrate and the corresponding decrease in unlabelled CoQ10 over different time points during one cell passage suggests there is a high turnover rate of endogenous synthesis. It further implies the condensation reaction catalysed by parahydroxybenzoate polyprenyltransferase (COQ2) preferentially used the exogenous 4HB supplied in the media over amino acid derived 4HB. This is likely due to the incubation medium in these experiments containing an excess of $^{13}C_6$-4HB. Further time course experiments using less concentrated artificial substrate could be performed to investigate this.

6.4.2 CoQ10 inhibition by 4-nitrobenzoate

Experiments demonstrated pharmacological inhibition of COQ2 by 1 mmol/L 4NB decreased CoQ10 synthesis by 38 – 69 % without apparent toxic effect. Co-treatment with 4HB was able to restore CoQ10 concentrations to those of untreated cells. As the concentration of 4HB needed to displace the inhibitor was significantly less (100 fold) this suggests the enzyme has a greater affinity for 4HB than for 4NB.

Further reduction in residual CoQ10 levels were seen using higher concentrations of 4NB. However, this was accompanied with significant cellular toxicity with decreased protein concentrations and increase visible cell death. This is consistent with previous evidence that poor cell growth was not solely related to inhibition of CoQ by 2 mmol/L 4NB as growth could not be rescued even when CoQ levels were restored to normal (Forsman *et al.*, 2010). In contrast to this report however, 4NB treatment resulting in 30 % residual COQ10 did indicate a slight increase in mitochondrial superoxide production thus possible evidence of oxidative stress occurring in these cells. Experiments using co-treatment with either 4HB or a short-chain CoQ analogue to restore CoQ10 levels were not performed before assessment of ROS which could have confirmed whether the increased ROS observed was directly related to the decreased CoQ10 and not some other effect of the 4NB.

6.4.3 CoQ10 inhibition by statins

Simvastatin, a commonly prescribed statin, was supplied as the inactive lactone which requires conversion to an active acid form to elicit inhibitory effects on the mevalonate pathway. Stock preparations were tested using a method for prior activation using an acid/alkali reaction (Indolfi *et al.*, 2000; Dong *et al.*, 2009), or using an ethanol solubilised stock diluted in culture medium which has previously been shown to fully convert to the active form within 24 h (Masters *et al.*, 1995). Similar results were noted following incubations using either preparation, therefore, for simplicity, further experiments were carried out using stock simvastatin prepared in ethanol.

Similar reductions in CoQ10 concentrations to those produced using the COQ2 inhibitor were observed following incubations with simvastatin. Residual levels of 37 – 56 % occurred in fibroblast cells treated with 1 μmol/L simvastatin. Even using a 100 fold dilution of simvastatin still resulted in an approximate 60 % reduction of CoQ10. At these levels, protein

concentrations did not change compared to control cells indicating no apparent toxic effects. However, concentrations of statin greater than 5 μmol/L caused significant toxicity and cell death.

In contrast to inhibition by 4NB, a similar percent reduction in CoQ10 concentration by simvastatin was not rescued by co-treatment with 4HB. This would suggest that synthesis was limited by the availability of the mevalonate derived isoprenoid chain and confirms the previous findings that 4HB was not the rate-limiting component in skin fibroblast cells under the conditions described here. Moreover, there was a significant reduction in the amount of CoQ10 containing the labelled substrate in statin treated cells compared to controls (41.5 % vs 71 % respectively), indicating a reduced rate of synthesis.

6.4.4 Mitochondrial superoxide production

MItoSOX red is highly selective for the detection of superoxide in the mitochondria of live cells. Using flow cytometry, a detectable change in the generation of mitochondrial superoxide was shown to occur in simvastatin treated fibroblast cells which correlated with increased statin concentration. A similar change was seen using 4NB. A concomitant decrease in CoQ10 concentrations was also observed in both treatments. This provides some evidence in support of the theory that a reduction in CoQ10 during statin therapy could be directly responsible for increased reactive oxygen species and oxidative stress within the mitochondria leading to respiratory chain defects and activation of cell-death related pathways.

It is acknowledged that cells treated with greater than 1 μmol/L simvastatin or 1 mmol/L 4NB showed some evidence of morphological changes and possible cell death. The percentage of cells within the gated region fell by approximately 11 % following statin treatment which suggests the concentrations used had a mild cytotoxicity which may account for increased detection of ROS. A marginal decrease in protein content was also noted which is likely due to reduced cell division rather than decreased content per cell, although this cannot be confirmed as a count of cell numbers was not performed. However, even using concentrations of simvastatin that did not significantly affect the cell morphology, a shift towards increased production of superoxide was noted.

6.4.5 Mitochondrial imaging

The cellular effects of simvastatin treatment and isolated inhibition of CoQ10 synthesis using 4NB were briefly examined using a molecular dye that is readily sequestered by active mitochondria. Exploitation of the mitochondrial membrane potential was used as this can be easily assessed by live cell imaging using TMRM mitochondrial dye, which requires the mitochondrion to be functioning and generating a membrane potential in order to retain the dye and produce a measurable level of orange-red fluorescence. In apoptotic or metabolically stressed cells, the mitochondrial membrane potential collapses and the dye disperses throughout the cytosol, resulting in minimal fluorescence detection. It should be noted that these experiments were performed to primarily aid visualisation of the cells, no actual measure of the membrane potential was determined. Four different cell lines was analysed simultaneously to account for possible variation between different lines. Overall, the experiments performed suggest reduction of CoQ10 synthesis by 40 - 50%, either by inhibition of HMG-CoA reductase or COQ2, results in decreased cell division and growth. This was more severe in the statin treated cells. Both the compounds tested had also demonstrated an increase in ROS production at the same concentrations.

There was a significant increase in the mitochondrial mass per cell in statin treated cells, as indicated by the increased ratio of TMRM staining to CellTracker Green stain. This could explain a possible mechanism, via increased ROS production, for the muscle symptoms experienced by some patients taking regular statins. However this hyperpolarisation of the mitochondrial membrane potential was not observed in isolated CoQ10 reduction. It is possible that this consequence may be due to the concentrations of each inhibitor used as previous studies have reported that membrane potential increased proportionally to ROS formation and inversely to CoQ10 concentrations using 4NB at 4 mmol/L (Quinzii *et al.*, 2006; Quinzii *et al.*, 2012). Also, the degree of reduction in total cell area seen in simvastatin treated cell in this particular experiment would suggest a higher level of cellular toxicity than had been demonstrated using the same concentration in other experiments performed during this project. It should be noted that an attempt was made to repeat this experiment but technical issues with the confocal microscope prevented clarification of these earlier findings and time restraints limited any further investigations to date. Ideally, repeat experiments using a range of inhibitor doses would help to clarify if the increased mitochondrial was linked to CoQ10 content or to an additional, as of yet, unknown side effect of statins. Furthermore, I did

attempt to assess functional metabolic status, such as glycolsis and mitochondrial respiration, during statin treatment of fibroblast cells, but on two occasions the Seahorse XF Analyser, which measures oxygen consumption rates, failed mid-run. Time restraints prevented further evaluation of the cells by Seahorse microscale oxygraphy.

6.4.6 Conclusion

This chapter has, very briefly, focused on *in vitro* investigations of the basic influences of statin treatment on CoQ10 levels. Results have shown statins do inhibit CoQ10 synthesis with a concomitant increase in production of mitochondrial superoxide. The changes observed were similar to these caused by isolated inhibition of CoQ10 biosynthesis, using an analogue of 4HB which competes for the activity of parahydroxybenzoate polyprenyltransferase (COQ2), and leads to possible evidence relating adverse effects of statins directly to reduced synthesis of CoQ10. Live cell imaging demonstrated that statins appear to have an influence on mitochondrial volume although this could not be directly linked to cellular changes in CoQ10. Further work in this area would certainly be beneficial in providing further insight into the link between statin therapy, CoQ10 depletion and adverse cellular effects.

Chapter Seven

Chapter 7. Biochemical Parameters in Statin Related Myopathy

7.1 INTRODUCTION

Statins are among the most commonly prescribed drugs worldwide. Around 1 in 3 people over the age of 45 are on statins, with an estimated seven million people in the UK regularly taking a prescribed statin (Trusler, 2011). These cholesterol-lowering agents reduce de novo cholesterol biosynthesis by inhibition of HMG-CoA reductase, an essential enzyme in the mevalonate pathway. Statin therapy is effective for treatment and prevention of cardiovascular disease and in general statins are considered safe and well tolerated. However, in the Prediction of Muscular Risk in Observational Conditions (PRIMO) study, adverse effects were reported by approximately 10 % of the 7.924 patients included and statin associated muscle symptoms (SAMS) are one of the principle reasons for non-adherence or discontinuation of statin therapy (Bruckert *et al.*, 2005). Nonetheless, it is generally accepted that the beneficial outcomes for reduced cardiovascular morbidity and mortality surpass the associated risk of statin use.

While several hypotheses have been suggested, the underlying mechanism for reduced statin tolerance in some patients is still not clear (Stroes *et al.*, 2015). The dose and type of statin, drug-drug interactions, as well as patient related factors such as co-morbidities, age and gender have all been associated with an increased risk of adverse effects (Alfirevic *et al.*, 2014; Schirris *et al.*, 2015). Genetic polymorphisms have also been investigated based on single nucleotide polymorphisms in the *CoQ2* gene, and have led to putative mechanisms to explain statin associated muscle symptoms with the CoQ10 pathway (Oh *et al.*, 2007).

The aetiology for skeletal muscle symptoms, the most prevalent side effect of cholesterol lowering statin therapy, remains poorly understood (Vaklavas *et al.*, 2009). Mitochondrial impairment, increased reactive oxygen species and decreased antioxidant capacity may aggravate adverse reactions which can range from muscle pain or aching to myopathy and severe rhabdomyolysis (Bouitbir *et al.*, 2011; Larsen *et al.*, 2013). Cerivastatin was withdrawn from the market due to unacceptably high rates of fatal rhabdomyolysis (Seehusen *et al.*, 2006). As the bioavailability and ability to inhibit HMG-CoA reductase varies significantly between statins, patients exhibiting symptoms with one statin can often be successfully re-

challenged with a lower dose or different statin to achieve the desired lipid levels (Stroes *et al.*, 2015).

One theory to explain statin related myopathy (SRM) implicates CoQ10, due to its biosynthesis being integrated, via the mevalonate pathway, to the ultimate target of HMG-CoA reductase inhibitors, namely the production of cholesterol. A relationship between statin intake and plasma CoQ10 concentrations is debatable and the correlation between CoQ10 levels and myopathy is even more questionable (Marcoff and Thompson, 2007). It has been proposed that the affinity of the branch point enzymes for the available substrate distal to the mevalonate pathway could be sufficiently diverse to allow for a reduction in cholesterol synthesis with the use of statins whilst maintaining an adequate level of CoQ10 biosynthesis (Hargreaves *et al.*, 2005). Moreover, the benefits of CoQ10 supplementation to alleviate statin associated adverse effects are also disputed (Caso *et al.*, 2007; Young *et al.*, 2007b). However there is evidence of improvement in some patients and as no noted side effects of CoQ10 are known, concomitant treatment with statins is often recommended to improve statin tolerability (Mortensen *et al.*, 2014).

Reduced cholesterol content may itself be a trigger for myopathy, causing alterations in the muscle membrane affecting fluidity and ion channel function (Mas and Mori, 2010). This theory however does not explain the lack of myopathic effects associated with inherited disorders of cholesterol synthesis or non-statin lipid lowering agents such as fibrates (Joy and Hegele, 2009; Littlefield *et al.*, 2013).

Cholesterol homeostasis is a balancing act between intestinal absorption of dietary cholesterol and endogenous biosynthesis to prevent cholesterol accumulation in the circulation. The measurement of non-cholesterol sterols can be used as indicators of cholesterol homeostasis (Gylling *et al.*, 2014). Precursor intermediates act as surrogate markers for cholesterol biosynthesis, whilst phytosterols and cholestanol may be used as cholesterol absorption markers.

The ultimate precursor for cholesterol can be either desmosterol or 7-dehydrocholesterol and two major routes have been proposed depending on when reduction of the double bond at the C24 (Δ^{24}) position occurs. In the Bloch pathway, which encompasses desmosterol, the

double bond is retained in all intermediate compounds, whereas early reduction of the double bond results in 7DHC. This latter pathway consisting of Δ^{24} saturated intermediates is defined as the Kandutsch-Russell (KR) pathway. Reduction occurs via the action of 24-dehydrocholesterol reductase (DHCR24) and can potentially occur at any point downstream of lanosterol, thus intertwining the two routes (Zerenturk *et al.*, 2013). A simplified schematic of the intermediates in the two cholesterol synthesis pathways is shown in figure 7.1.

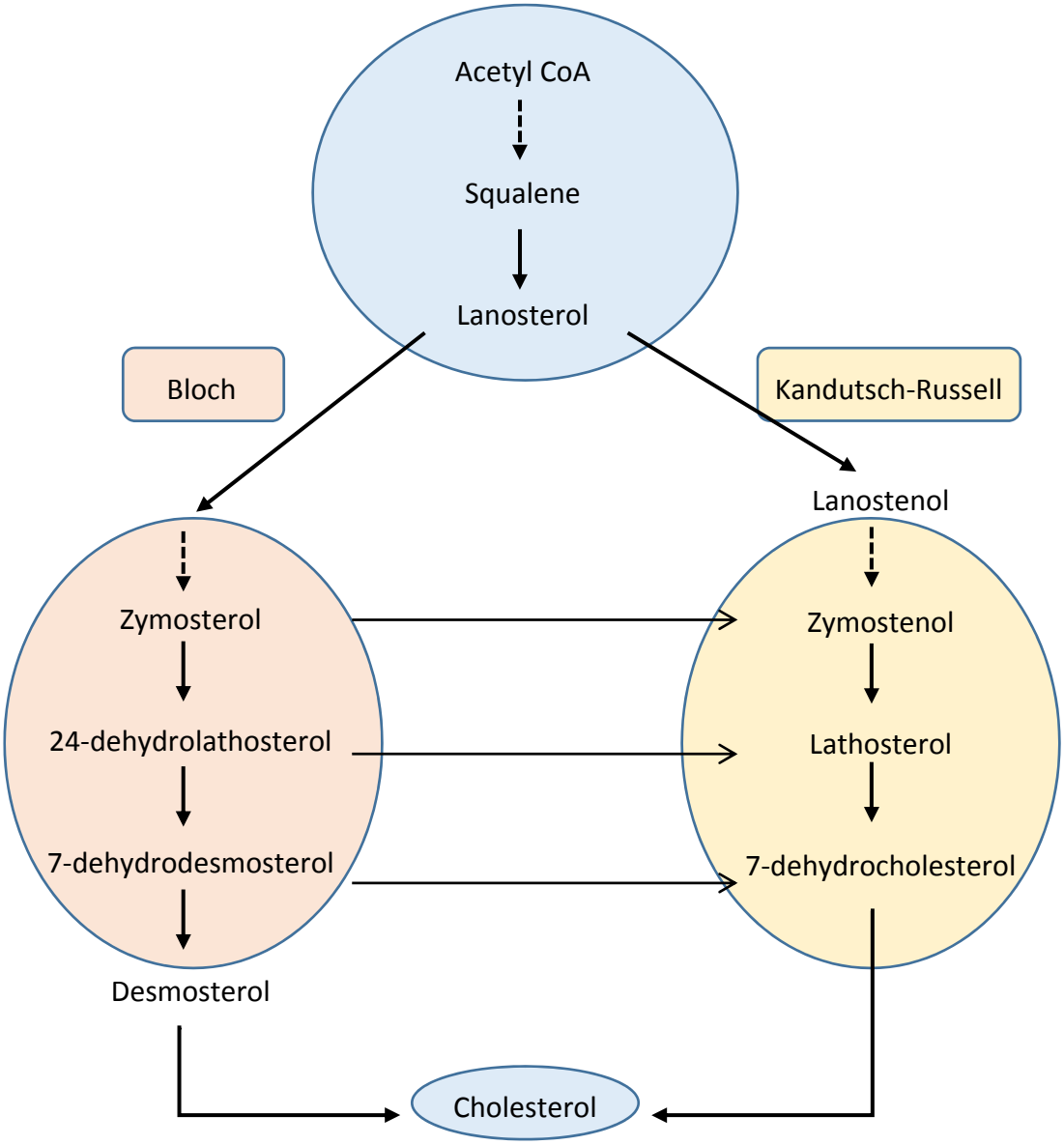


Figure 7.1 Simplified schematic of the two alternative pathways of cholesterol biosynthesis.
Unsaturated intermediates form the Bloch pathway whilst the Kandutsch-Russell Pathway consists of the saturated intermediates following reduction of the double bond at C24 by DHCR24

Alterations in the cholesterol homeostasis profile have been assessed in relation to risk of cardiovascular disease (CVD). Data from relatively small scale studies as well as the Framingham Offspring Study, consisting of a cohort of 155 normolipidaemic subjects with documented CVD and/or carotid stenosis who were not on lipid lowering medications, have consistently reported increased risk to be associated with higher absorption markers compared to controls. The relationship to levels of synthesis markers however is contradictory (Matthan *et al.*, 2009). Similar profiles have also been reported in diabetic patients with evidence of heart disease (Hallikainen *et al.*, 2008). Furthermore, baseline levels of non-cholesterol markers have been assessed to potentially predict the efficacy of statin treatment in reducing LDL-C concentrations (Wu *et al.*, 2013).

Although some data is available on the influence of statins on the major non-cholesterol sterols (Uusitupa *et al.*, 1992), most are small scale studies and limited information exists concerning the relationship and impact of statin therapy on a comprehensive array of non-cholesterol sterols, particularly those to enable further discrimination between the alternative pathways of cholesterol synthesis. Additionally, association of non-cholesterol sterols and adverse muscle symptoms associated with statin treatment has virtually been overlooked.

7.1.1 Aims

This chapter focuses on a large cohort of patients with or without evidence of statin intolerance. Using the analytical methods developed in previous chapters I measured the concentrations of CoQ10 as well as the cholesterol and non-cholesterol sterols in plasma samples. I then investigated the relationship between the measured biochemical parameters to determine whether altered biochemistry of mevalonate derived compounds is related to statin associated muscle symptoms.

7.2 MATERIALS AND METHODS

7.2.1 Study subjects

Ethical approval for the study was provided by NRES Committee North East – County Durham & Tees Valley (number 10/H0908/53). Participants were recruited to the study from patients attending the Lipid and Cardiology Clinics at the Royal Victoria Infirmary, Newcastle upon Tyne between May 2011 and June 2013. All patients required statin therapy for primary or secondary prevention of cardiovascular disease.

The statin control group (statin controls) were defined as patients who had received a daily dose of statin for at least 6 months without new onset of symptoms. The statin intolerant group (cases) consisted of patients in whom statin therapy had been discontinued because of new muscle symptoms (pain or weakness, with or without elevated CK) which had developed within 6 months of commencement of therapy and which abated on withdrawal but recurred with re-challenge at the same or reduced statin dose. Based on the classification proposed by Alfirevic (Alfirevic *et al.*, 2014), which grades SAMS using a scale from 0 - 6 based on elevations of CK, severity of symptoms and end organ damage, these cases were classified as grade 2.

The normal population (controls) were derived from data from samples sent to the Blood Sciences department, Royal Victoria Infirmary, for analysis of plasma sterols. Patients with known metabolic disorders and hypercholesterolaemia were excluded.

Venous blood was collected into tubes containing either EDTA or Lithium heparin as anticoagulant, centrifuged and the plasma aliquoted and stored at -80°C until analysis.

7.2.2 Measurement of lipid profiles and creatinine kinase

Total cholesterol, HDL-cholesterol, triglycerides and CK were measured in plasma as a single determination using the Cobas Modular analyser and methods described in Chapter 2.

Non-HDL-cholesterol was calculated as the total cholesterol minus HDL-cholesterol.

7.2.3 Analysis of sterol intermediates.

Sterol analysis was performed, as a single determination, using the method described in Chapter 5. In brief, 50 µl of plasma was mixed with internal standard (1 mmol/L EPIC) and hydrolysed using 4 % (w/v) ethanolic potassium hydroxide heated to 64°C for 1 hour. A double liquid-liquid extraction step using hexane was performed to partition the sterols into the upper layer which was then removed and evaporated to dryness using nitrogen. The extract was derivatised using BSTFA to produce trimethylsilyl compounds that are amenable to analysis by GCMS.

Data were acquired using SIM mode and quantitation of each sterol was achieved by reference of the peak area for the specific ion to that of the internal standard. Concentrations were determine using calibration curves which were extracted and analysed with every batch. Total analysis time for each sample was 27 mins. Agilent Chemstation software was used for data analyses.

7.2.4 Analysis of CoQ10

CoQ10 was analysed using the LC-MSMS method developed and described in Chapter 3. Each sample was analysed in duplicate and the mean result determined. In brief, 100 µl each of sample and internal standard (CoQ9 0.5 µg/ml) were vortex mixed in a microtube. The CoQ was double extracted using hexane, the organic phase evaporated to dryness using nitrogen then reconstituted in 100 µl methanol and transferred to 96-well microplate for analysis by LC-MSMS.

Data were acquired using SRM mode and quantitation was performed using the ratio of the peak area relative to the area of the internal standard peak and reference to a calibration curve. Total run time was 10 min per sample. Data was analyses were performed using Thermo Xcalibur software.

7.2.5 Statistical analysis

All statistical analyses were performed using MedCalc for Windows, version 11.2.1.0 (MedCalc Software, Ostend, Belgium).

Data were assessed for normality using the D'Agostino-Pearson omnibus test. Almost all variables failed the test for normality therefore comparisons were performed using the Mann-Whitney U test for non-parametric data. Where necessary, the data were $\log_{10}$ transformed to achieve normality and the geometric mean calculated. The correlation coefficient (r) between variables were calculated using Spearman rank correlations. Unless stated, data are presented as median and 95 % confidence intervals (CI). A *p*-value of < 0.05 was taken as significant in all cases.

7.3 RESULTS

7.3.1 Study groups

In total 414 samples were investigated in this study. The demographics are summarised in table 7.1. A greater proportion of females were present in the cases group (p = 0.004). The mean age at enrolment was similar between the cases and statin control groups but significantly younger in the controls (p < 0.001). Simvastatin, at 40 mg/day, was the most commonly prescribed statin in both groups followed by atorvastatin at 80 mg/day. The total duration of statin treatment was markedly different between the statin groups with cases undergoing significantly shorter total statin regimes (p <0.001). The length of time from cessation of statin in the case subjects was not available but was generally within two weeks of the sample being taken (personal correspondence with Dr Neely).

Variables	Controls (n = 69)	Cases (n = 108)	Statin controls (n = 237)
Sex			
Male, N (%)	40 (58 %)	43 (39.8 %)	115 (48.5 %)
Female, N (%)	29 (42 %)	65 (60.2 %)	122 (51.5 %)
Age at enrolment (mean years, 95 % CI)	49.2 (19.2 – 72.8)	59.2 (43 – 76.8)	56.2 (27.7 – 79.2)
Duration on statin (months), median (IQR)		8 (3 - 18)	24 (12 - 65)

Table 7.1 Demographics of study cohorts.

7.3.2 Lipid profiles

Cases had higher concentrations of cholesterol (6.25 [4.22 – 10.45] mmol/L) compared to both the controls (5.19 [2.56 – 7.22] mmol/L) and the statin control subjects (4.98 [2.89 – 7.60] mmol/L) (p <0.0001) at the time of recruitment (Figure 7.2).

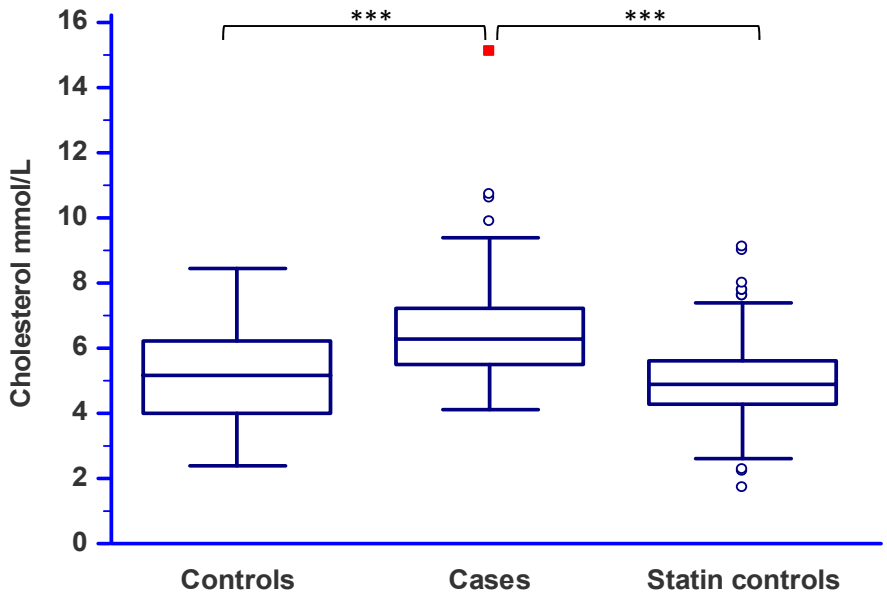


Figure 7.2 Comparison of total plasma cholesterol concentrations between study groups.
The horizontal bar in the box and whisker plots represents the median and the box the interquartile range (IQR). Circles and red squares represent results which exceed plus/minus 1.5 and 3 times the IQR respectively. ***p < 0.0001 calculated using a Mann-Whitney U test for significance.

The cholesterol content in high density lipoproteins was similar between the cases (1.40 [0.90 – 2.28] mmol/L) and statin control subjects (1.45 [0.70 – 2.66] mmol/L) but non-HDL cholesterol was significantly higher in the cases (4.70 [3.10 – 8.76] vs 3.40 [1.64 – 6.10] mmol/L respectively) (p <0.0001) (Figure 7.3).

Triglyceride concentrations were also slightly higher in the cases (1.95 [0.70 – 5.29] mmol/L) compared to the statin controls (1.60 [0.50 – 5.91] mmol/L) (p = 0.0012). No difference was observed in CK concentrations between the groups receiving statin therapy (Figure 7.3).

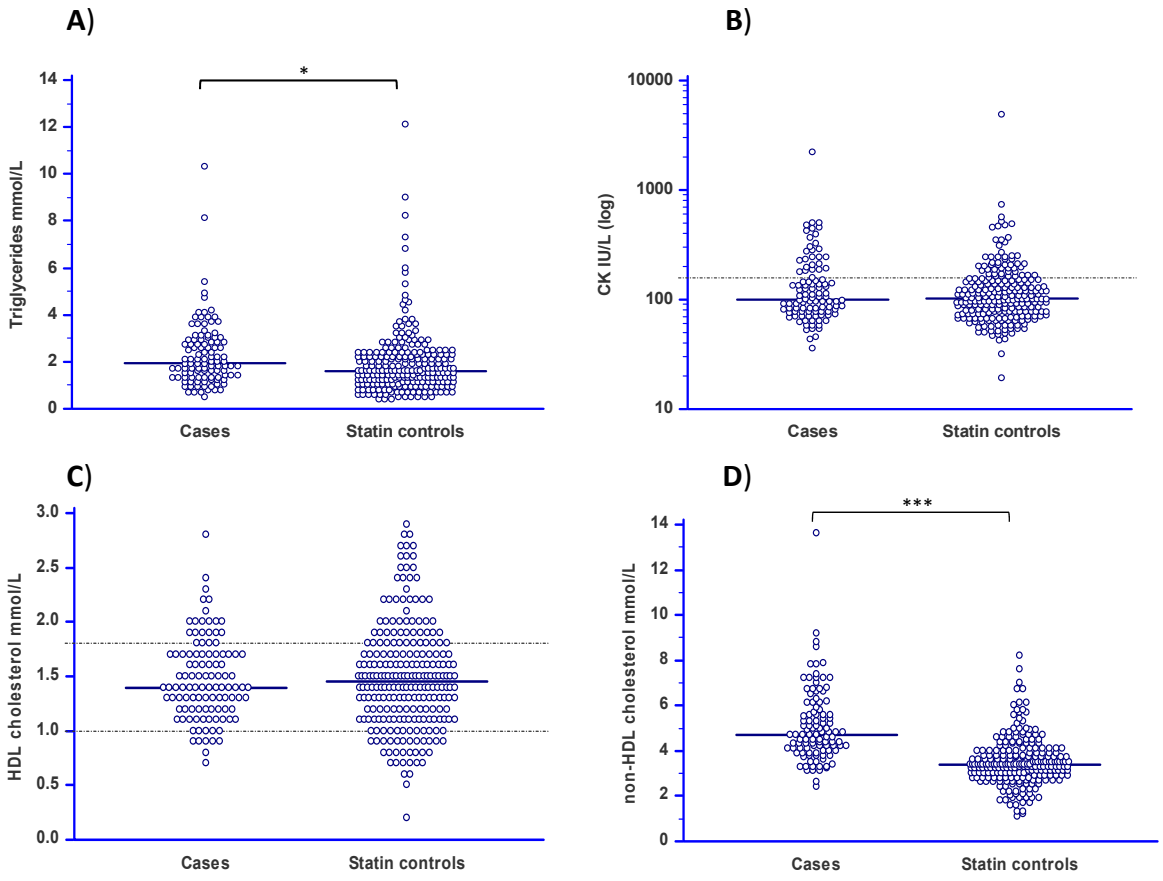


Figure 7.3 Variation in lipid profiles and CK levels between subjects taking statins with myopathy (cases n=108) or without myopathy (statin controls n=237).
All analytes were measured in plasma. A**)** triglycerides, **B)** CK concentrations**, C)** HDL cholesterol and **D)** non-HDL cholesterol. CK levels are shown using a log scale. Dotted line represents the ULN (CK) and the normal range (HDL-C). P-value calculated using a Mann-Whitney U test *$p < 0.05$, ***$p < 0.0001$.

7.3.3 CoQ10 concentrations

The absolute molar concentration of plasma CoQ10 was significantly lower in statin controls compared to both the cases (0.70 [0.21 – 1.75] vs 0.97 [0.31 – 2.77] µmol/L respectively, $p < 0.0001$) and the controls (0.81 [0.36 – 2.54] µmol/L, $p < 0.001$) (Figure 7.4). Comparisons of the concentration of CoQ10 between the cases and the statin naive controls did not reveal any significant difference ($p = 0.098$).

However, when the CoQ10 concentration was normalised for cholesterol, significant difference remained for the statin tolerant controls (0.14 [0.050 – 0.346] µmol/mmol) vs controls (0.16 [0.062 – 0.711] µmol/mmol) ($p = 0.0012$), but was resolved between the two statin treated groups (cases, 0.14 [0.052 – 0.501] µmol/mmol) ($p = 0.13$).

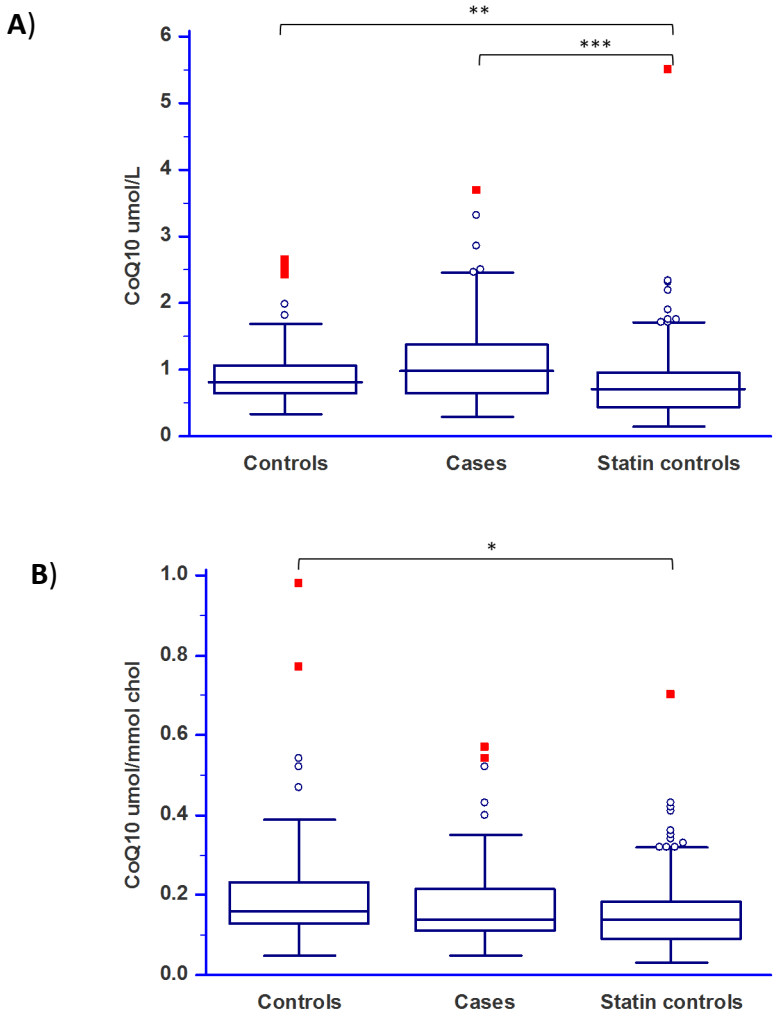


Figure 7.4 Comparison of plasmaCoQ10 concentrations between study groups.
Data are shown as **A)** absolute and **B)** cholesterol normalised concentrations. n= 69, 108 and 237 for controls, cases and statin controls repectively. P-values calculated using mann-Whitney U Test *p <0.05. **p <0.001, ***p < 0.0001. Circles and red squares represent results which exceed plus/minus 1.5 and 3 times the IQR respectively.

7.3.4 Sterol Intermediates

The sterol intermediates were assessed based on their absolute concentrations in plasma and on the concentration normalized to cholesterol.

Analytes produced downstream of squalene during the synthesis of cholesterol were grouped as synthesis markers. These were further divided after the branch compound lanosterol based on their position in either the Bloch pathway (zymosterol and desmosterol) or the Kandutsch-Russell pathway (lanostenol, zymostenol, lathosterol and 7DHC).

Across all groups lathosterol was the most prominent cholesterol precursor, followed by desmosterol then zymosterol. Lanostenol showed the lowest incidence and was undetectable in more than 70 % of all samples analysed. Of the absorption markers, cholestanol was present in the highest concentration followed by campesterol then sitosterol (Table 7.2).

Sterol		Controls	Cases	Statin controls
Cholesterol		**5.19**	**6.25**$^{*\,\#}$	**4.9**
	mmol/L	(2.55 - 7.223)	(4.220 - 10.456)	(2.885 - 7.60)
Lanosterol		**0.37**	**0.565**$^{\#}$	**0.2***
	µmol/L	(0.018 - 1.154)	(0 - 1.128)	(0 - 0.646)
Bloch Intermediates				
Zymosterol		**1.45**	**1.835**$^{*\,\#}$	**1.17***
	µmol/L	(0.30 - 5.580)	(1.054 - 3.990)	(0 - 2.407)
Desmosterol		**1.5**	**1.945**$^{*\,\#}$	**1.41**
	µmol/L	(0.544 - 4.158)	(0.930 - 4.126)	(0.242 - 2.766)
Kandutsch-Russell Intermediates				
Lanostenol		**0**	**0**$^{*\,\#}$	**0**
	µmol/L	(0 - 0.103)	(0 - 0.476)	(0 - 0.310)
Zymostenol		**1.04**	**1.195**$^{\#}$	**0.65***
	µmol/L	(0.60 - 2.643)	(0.562 - 2.674)	(0 - 1.747)
Lathosterol		**4.1**	**4.65**$^{\#}$	**2.08***
	µmol/L	(0.894 - 12.292)	(1.444 - 13.606)	(1.077 - 6.606)
7DHC		**0.5**	**0.935**$^{*\,\#}$	**0.58**
	µmol/L	(0.10 - 2.433)	(0 - 2.426)	(0 - 1.240)
Absorption Markers				
Sitosterol		**3.73**	**4.605***	**5.34***
	µmol/L	(0.889 - 8.689)	(1.736 - 14.290)	(1.814 - 16.459)
Cholestanol		**7.7**	**6.085***	**6.37***
	µmol/L	(4.923 - 12.478)	(2.985 - 12.740)	(2.815 - 12.740)
Campesterol		**4.97**	**4.985**	**6.28***
	µmol/L	(1.453 - 13.697)	(1.529 - 17.885)	(1.958 - 18.947)

Table 7.2 Concentrations of sterol intermediates in statin treated and control subjects. Mean (95 % CI). Significance testing was calculated using Mann-Whitney U test * $p < 0.01$ cases and statin controls vs controls, # $p < 0.01$ cases vs statin controls

7.3.4.1 Absorption markers

Assessed individually, absolute sitosterol concentrations were lower and cholestanol concentrations were higher in controls compared to subjects receiving statins (Table 7.2). Collectively, no significant differences were observed in the concentration of absorption markers between the three groups (Figure 7.5).

Conversely, when corrected for the cholesterol concentration, a marked decrease in absorption markers was found in the case subjects (2.52 [0.93 – 7.33] µmol/mmol). This was most significant when compared to the statin control group (3.75 [1.68 – 8.24] µmol/mol, $p < 0.0001$) but was also significantly lower than in the control subjects (3.40 [1.66 – 6.59] µmol/mmol, $p < 0.001$) (Figure 7.5).

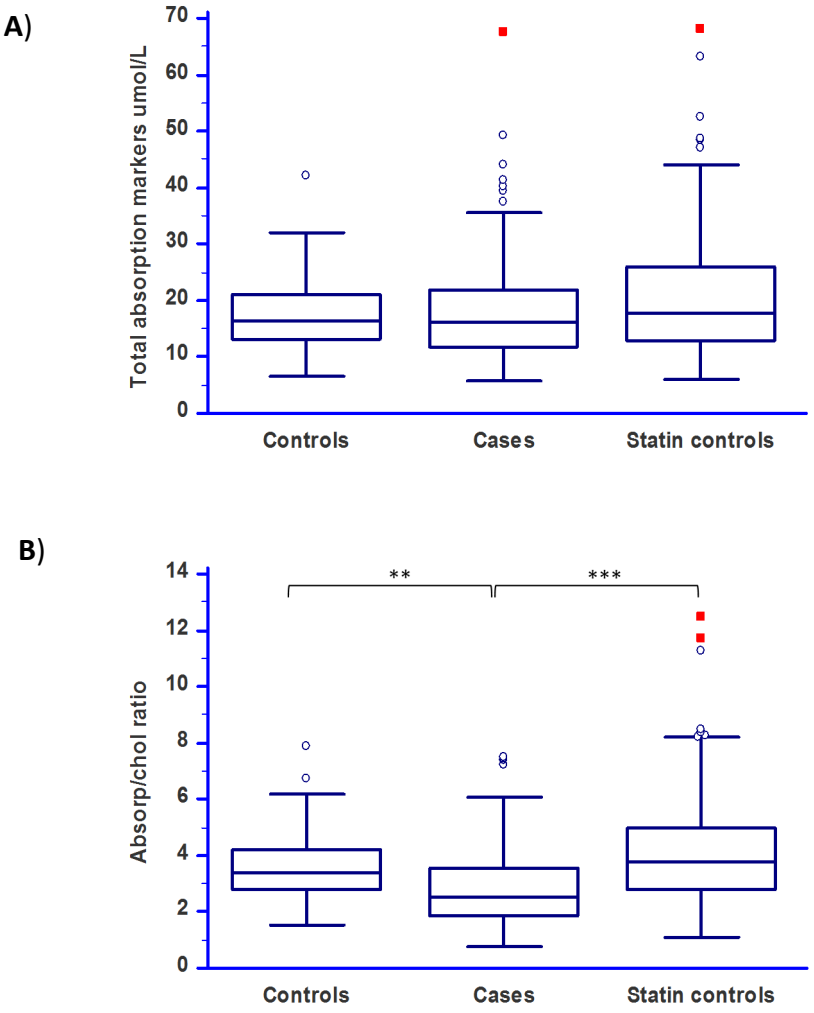


Figure 7.5 Concentrations of plasma total cholesterol absorption markers.
A) Absolute concentrations and **B)** cholesterol normalised data for the combined concentrations of sitosterol, cholestanol and campesterol. n = 69 controls, 108 cases, and 237 statin controls. Circles and red squares represent results which exceed plus/minus 1.5 and 3 times the IQR respectively. $^{**}p < 0.001$, $^{***}p < 0.0001$ calculated using the Mann-Whitney U test for significance.

7.3.4.2 Cholesterol biosynthesis markers

The ratio of lanosterol to cholesterol, a common intermediate preceding both branches of cholesterol biosynthesis, was significantly lower ($p < 0.0001$) in the statin control group (0.04 [0 – 0.13] µmol/mmol) compared to both controls (0.07 [0.005 – 0.27] µmol/mmol) and cases (0.08 [0 – 0.17] µmol/mmol).

Similarly, using the ratio of lathosterol to cholesterol as an indication of whole body cholesterol synthesis clearly shows that the subjects taking statins without evidence of myopathic symptoms have significantly reduced flux through the biosynthetic pathway for cholesterol (0.44 [0.23 – 1.23] µmol/mmol) ($p < 0.0001$) (Figure 7.6).

Subjects with myopathy taking the maximally tolerated dose of statin showed no significant difference in the rate of cholesterol synthesis, assessed using the lathosterol to cholesterol ratio, compared to subjects in whom no statin therapy had been prescribed (0.72 [0.25 – 2.13] vs 0.73 [0.25 – 2.65] µmol/mmol/L respectively) (Figure 7.6).

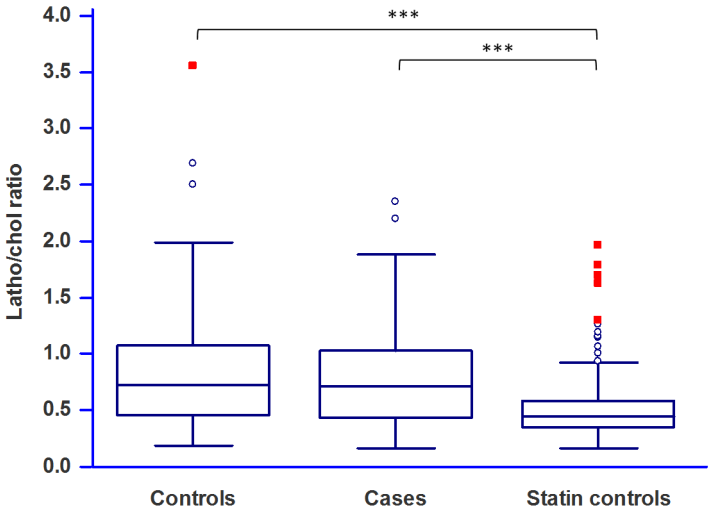


Figure 7.6 Lathosterol to cholesterol ratio as a measure of total cholesterol synthesis.
Subjects with good adherence to statin regimes (n=237) show significantly reduced cholesterol biosynthesis when compared to both control (n=69) and statin intolerant subject (n-108). P-value calculated using a Mann-Whitney U test . ***$p < 0.0001$

Furthermore, significantly lower concentrations of total biosynthesis markers were observed in the statin controls (6.05 [3.33 – 14.26] µmol/L) compared to the cases (11.79 [4.95 – 25.09] µmol/L), which was maintained when concentrations were normalised to cholesterol ($p < 0.0001$) (Figure 7.7).

There was a slight reduction in synthesis indicators in cases (1.82 [0.84 – 4.00] µmol/mmol) compared to controls (1.98 [1.03 – 4.19] µmol/mmol) (p =0.032) when corrected for the cholesterol concentration but absolute concentrations were similar.

The statin control group showed consistently lower rates of cholesterol biosynthesis compared to both the cases and control groups. The median concentration of all synthesis markers, corrected for cholesterol, in the statin control group was 1.26 µmol/mmol (95 % CI, 0.74 – 2.60 µmol/mmol) (Figure 7.7).

Interestingly, cholesterol normalisation of desmosterol was the only intermediate that showed consistency across the groups. The median values were 0.31, 0.31, and 0.29 µmol/mmol for controls, cases and statin controls respectively.

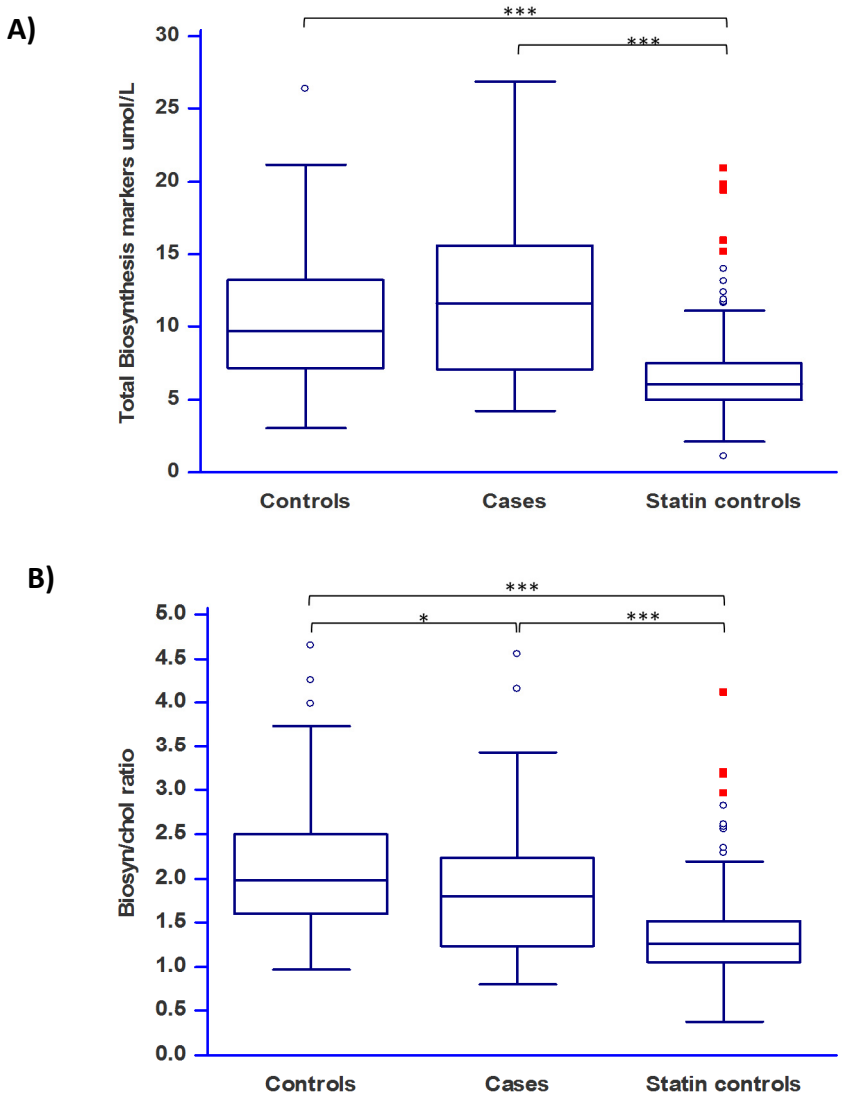


Figure 7.7 Concentrations of plasma total cholesterol biosynthesis markers.
Combined concentrations of lanosterol, zymosterol, desmosterol, lanostenol, zymostenol, lathosterol and 7DHC represented as A) absolute concentration and B) normalised to cholesterol. n = 69 controls, 108 cases, and 237 statin controls. Circles and red squares represent results which exceed plus/minus 1.5 and 3 times the IQR respectively. A Mann-Whitney U test was used to test for significance *p <0.05. **p <0.001, ***p < 0.0001.

7.3.4.3 Bloch pathway intermediates

Of the two compounds measured that retained the double bond at position C24 only zymosterol exhibited any variation between the study groups when normalised to cholesterol, which resulted in marked differences in total intermediates from the Bloch pathway between the groups.

Statin control subjects had the lowest concentrations both in terms of absolute levels (2.59 [0.66 – 4.74] µmol/L) and ratios to cholesterol (0.52 [0.16 – 0.91] µmol/mmol). Cases had the highest absolute values (3.82 [2.09 – 7.06] µmol/L) but were significantly less than controls when corrected for cholesterol (0.61 [0.37 – 1.21] µmol/mmol) (p = 0.0129). Absolute and cholesterol normalised concentrations in the control group were 3.39 (1.18 – 7.25) µmol/L and 0.70 (0.24 – 1.54) µmol/mmol respectively.

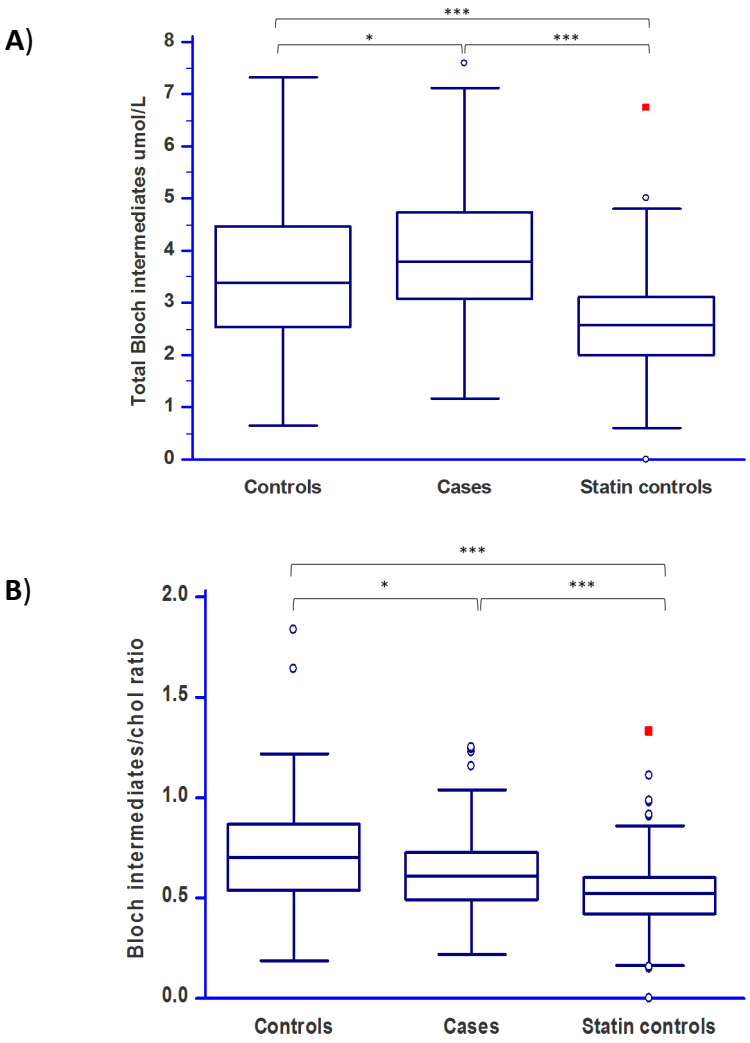


Figure 7.8 Comparison of the total Bloch intermediates (zymosterol and desmosterol)
A) Absolute concentrations and **B)** cholesterol normalised data in plasma. n = 69 controls, 108 cases, and 237 statin controls. All significance testing was calculated using the Mann-Whitney U test *p <0.05, ***p < 0.0001.

7.3.4.4 Kandutsch-Russell intermediates

Concurrent with the data for the Bloch intermediates, the total concentration for all the KR intermediates was significantly lower ($p < 0.0001$) in the statin control group (3.36 [1.39 – 9.47] µmol/L). Absolute concentrations in the controls were 5.57 (1.98 – 15.41) umol/L and in the cases it was 7.23 (2.55 – 18.25) umol/L. This relationship did not change when the ratio to cholesterol was examined (Figure 7.9).

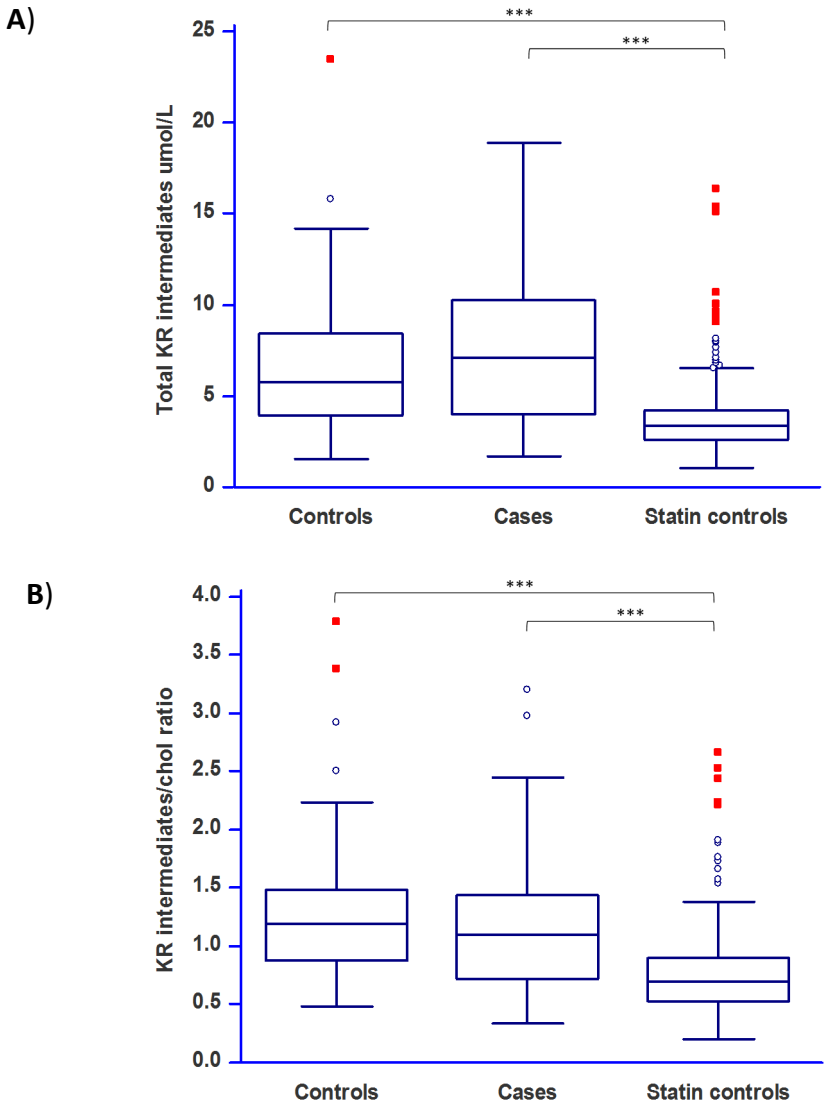


Figure 7.9 Comparison of the total Kandutsch-Russell (KR) intermediates (lanostenol, zymostenol, lathosterol and 7DHC).
A) Absolute concentrations and **B)** cholesterol normalised data in plasma. n = 69 controls, 108 cases, and 237 statin controls. All significance testing was calculated using the Mann-Whitney U test ***$p < 0.0001$.

7.3.5 Correlation between sterol intermediates

In all groups, positive correlations were observed between plasma cholesterol and intermediate compounds relative to both alternative pathways in the post-lanosterol biosynthesis of cholesterol (Figure 7.10).

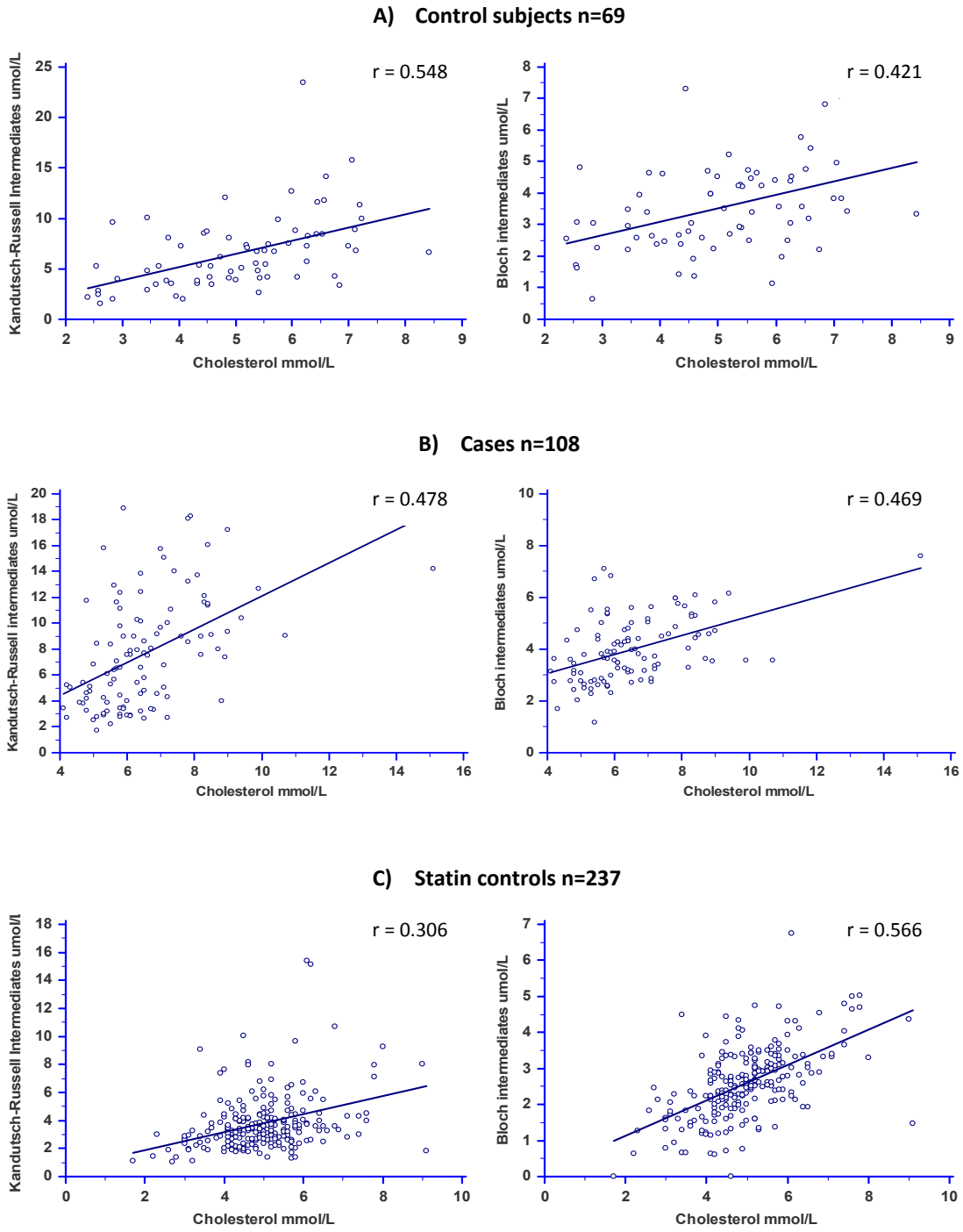


Figure 7.10 Correlation between cholesterol concentration and sterol biosynthesis markers.
Graphs represent the combined concentrations of intermediates specific to either the Kandutsch-Russell or Bloch pathways. In control subjects **(panel A)** the degree of correlation was stronger with the KR pathway whilst subjects tolerant to statins had a preponderance towards unsaturated intermediates **(panel C).** Both pathways were equally represented in the cases subjects **(panel B),** Spearman correlation coefficient (r), p <0.001.

The relationship between the two alternative pathways showed positive correlation in all subject groups. However, this was noticeably stronger (r = 0.735) for the cases compared with the two control groups (Figure 7.11).

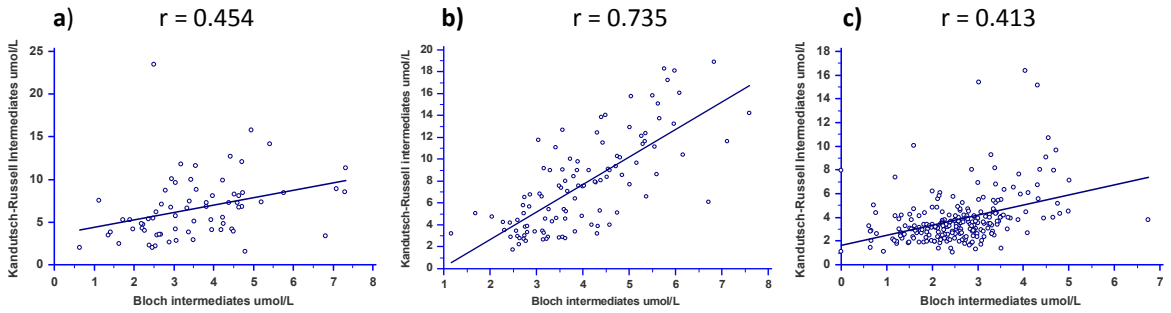


Figure 7.11 Correlation between the two alternative pathways of cholesterol biosynthesis.
a) Control subjects n=69, **b)** Case subjects n=108 and **c)** Statin control subject n=237. Spearman correlation coefficient (r), *P* <0.001

An increase in cholesterol also positively correlated to the total concentration of absorption markers in all study groups (Figure 7.12). Sitosterol consistently showed the weakest correlation (r < 0.3, *p* <0.01) whilst cholestanol and campesterol demonstrated an equal association with plasma cholesterol (*p* <0.001).

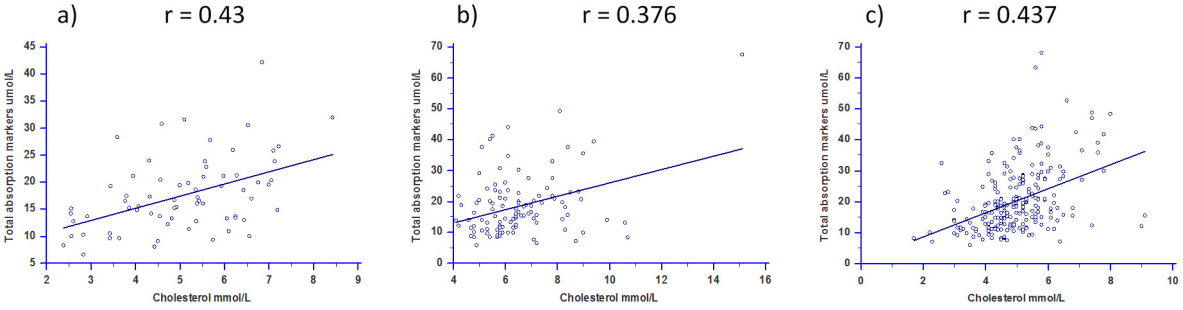


Figure 7.12 Correlation between cholesterol concentration and total absorption markers (cholestanol, sitosterol and campesterol).
a) Control subjects n=69, **b)** Case subjects n=108 and **c)** Statin control subject n=237. Spearman correlation coefficient (r), *P* <0.001

7.3.6 Contribution of sterol intermediates to biosynthesis

The contribution of each individual cholesterol precursor was calculated as a percent of the total pool of biosynthesis markers. Lathosterol accounted for approximately 40 % of the total concentration with lanostenol representing less than 1 % (Table 7.3 and Figure 7.13).

	Percent of total biosynthesis intermediates		
	Controls n = 69	**Cases n = 108**	**Statin controls n = 237**
Lanosterol	4.2	3.58	3.4
Lanostenol	0.161	0.25	0.78
Zymosterol	18.39	18.03	16.92
Zymostenol	13.53	11.18	9.92
Lathosterol	40.41	41.19	37.08
Desmosterol	17.03	17.9	23.34
7DHC	6.26	7.87	8.54

Table 7.3 Percent contribution of sterol intermediates to the total cholesterol biosynthesis pool.
Data are calculated based on the mean concentration for each analyte within each group. Lathosterol was the most significant analyte detectable.

A discernible difference was observed in the mean percent contribution of lathosterol which was significantly lower in statin control subjects compared to the cases ($p < 0.0001$), and marginally lower compared to the control group ($p = 0.024$).

Zymostenol was also lower in statin controls compared to both the controls ($p = 0.004$) and cases ($p = 0.008$).

Conversely desmosterol was significantly higher ($p < 0.0001$) in statin controls in comparison to both of the other study groups.

In comparison to the controls, case subjects had a slightly higher percent contribution from 7DHC ($p = 0.0057$) and lanostenol ($p = 0.0019$). Both these were lower than seen in the statin

control group, and although 7DHC did not approach statistically significance, the lanostenol contribution did reach a level of significance ($p = 0.0013$).

No statistical differences were observed in the percent contribution of lanosterol or zymosterol between any of the three study groups (Figure 7.13).

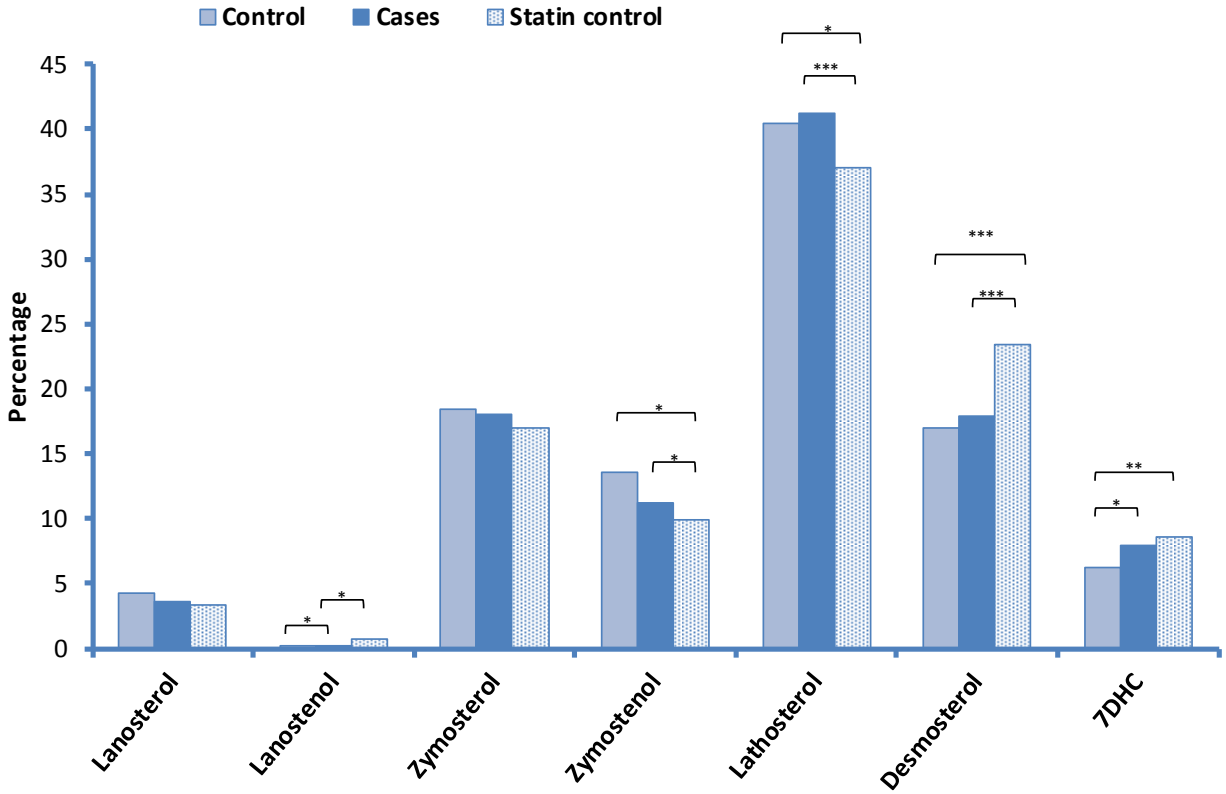


Figure 7.13 Percent contribution of the cholesterol precursors to the total pool of biosynthesis markers. Data represent the mean percent in controls and subjects taking statins with or without signs of statin associated muscle symptoms. P-values were calculated using a Mann-Whitney U test for significance. *$p < 0.05$. **$p < 0.001$. ***$p < 0.0001$.

7.3.7 CoQ10 and cholesterol intermediates

The relationship between plasma CoQ10 and total cholesterol varied between the three groups. There was a relatively high degree of association in the subjects taking statins without myopathic symptoms (r = 0.39, p <0.001) but only a weak correlation was seen in the case subjects (r = 0.235, p = 0.015). No correlation between CoQ10 and cholesterol was observed in the control population (r = 0.027, p = 0.828) (Figure 7.14).

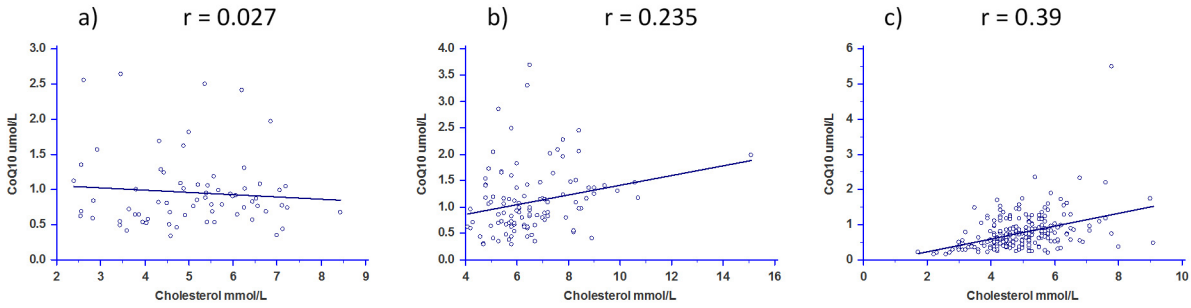


Figure 7.14 Correlation between plasma CoQ10 and cholesterol in study populations.
a) Control subjects (n=69), **b)** Case subjects (n=108) and **c)** Statin control subject (n=237). Spearman correlation coefficient (r), controls p < 0.001

Using the ratio of CoQ10 and lathosterol to cholesterol to assess the relative rates of biosynthesis of these mevalonate derived compounds did not indicate any associations, suggesting the synthesis of the two compounds are independent from each other (Figure 7.15). Similarly, no correlations were found between CoQ10 and the cholesterol absorption markers.

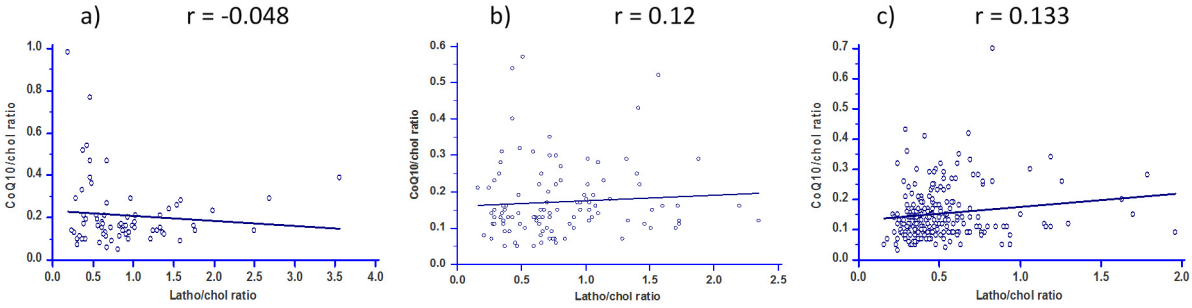


Figure 7.15 Correlation between cholesterol normalized CoQ10 in plasma and the estimated rate of cholesterol biosynthesis (lathosterol/cholesterol ratio) in study populations.
a) Control subjects (n=69), **b)** Case subjects (n=108) and **c)** Statin control subject (n=237). The correlation coefficient (r) and significance value were calculated using Spearman rank correlation testing, p = not significant in all groups.

7.4 DISCUSSION

One of the most common reasons for non-adherence to lipid-lowering therapy with HMG-CoA reductase inhibitors is the onset of associated muscle symptoms, with or without a significant elevation of creatine kinase (Bitzur *et al.*, 2013). Patient centred reports have documented a negative association between muscle related problems and quality of life which can lead to discontinuation of pharmacological interventions and poor control of LDL-cholesterol (Cham *et al.*, 2010). Minor muscle symptoms are not uncommon, and placebo based clinical trials suggest they may not be pharmacological in origin as they occur with similar frequency in both the test and control cohorts (Joy *et al.*, 2014). Symptoms in patients with true statin-associated muscle symptoms quickly resolve on discontinuation of the statin and recur when re-challenged. Nonetheless, as the benefit of a regular statin regime in reducing cardiovascular morbidity and mortality is well established, patients reporting signs of intolerance should be re-challenged with at least three different statins of lower dose or reduced potency before being considered for alternative lipid-lowering therapies (Stroes *et al.*, 2015).

In order to determine if altered profiles of metabolites downstream of the site of action for statins in the mevalonate pathway contribute to the myopathic symptoms experienced by some patients, the profiles in patients referred to the specialist lipid clinic were investigated. In total, plasma from 108 cases with defined SAMS grade 2 and 237 age matched controls were examined, together with 69 patients not on statin medication.

The cases had a higher proportion of females to males which is consistent with previously reported findings that the female sex, together with increased age and low body mass index, increase susceptibility to SAMS (Harper and Jacobson, 2010).

In comparison to controls, the cases had approximately 20 % higher total plasma cholesterol concentrations and marginally elevated triglycerides with an associated increase in non-HDL cholesterol. No difference in CK levels were observed between the two groups prescribed statins and all values were less than 4 times the upper limit of normal.

As the non-cholesterol sterols and CoQ10 are carried by lipoproteins the concentrations were expressed relative to cholesterol to correct for the different number of lipoprotein acceptor particles.

7.4.1 Cholesterol absorption markers

Although not statistically significant, absolute molar concentrations of the individual absorption markers (sitosterol, cholestanol and campesterol) were 5 – 25 % lower in cases than in statin controls. When corrected for cholesterol concentrations these differences were amplified to 50.3, 33.7 and 63.5 % respectively, with a combined reduction of 32.8 %. Compared to controls, a 25.8 % reduction in absorption markers was apparent in the cases. This data suggests absorption of cholesterol was significantly increased in patients with good statin tolerance but decreased in subjects with SAMS. This is in some way contradictory to previous evidence which associates high absorption marker concentrations with increased adverse events including cardiovascular disease risk and myocardial infarctions (Matthan *et al.*, 2009).

7.4.2 Cholesterol synthesis markers

Lanosterol, the early precursor for cholesterol synthesis was 43 % lower in the subjects who tolerated statins without incidence compared to the controls. This is in keeping with documented reports signifying statins can effectively inhibit HMG-CoQ reductase by 45 - 95 % depending on bioavailability (Mas and Mori, 2010). Moreover, the statin control group investigated here showed a significant reduction (34 %) in total cholesterol synthesis markers comparative to both the controls and the cases. Additionally, the ratio of lathosterol to cholesterol as an independent indicator of whole body cholesterol synthesis, demonstrated statin tolerant subjects had on average a 39 % lower rate of endogenous cholesterol synthesis. This is in good accordance with previous reports of a similar decline in lathosterol to cholesterol ratio following an 18 week regime of lovastatin (Uusitupa *et al.*, 1992).

Defined case subjects however did not differ in their rate of cholesterol synthesis from the control group when evaluated using lathosterol or lanosterol alone but did indicate a 5 % marginally significant lower rate when the ratio of all measurable cholesterol precursors was evaluated. This may be a reflection of non-adherence to a strict statin regime by subjects in

the cases group, whereas the desired effect of suppressing cholesterol synthesis is clearly apparent from the statin control cohort.

7.4.3 Bloch and Kandutsch-Russell Pathways

In vitro analysis has demonstrated that of all the cholesterol precursors, 24-dehydrolathosterol has the highest substrate affinity for DHCR24, suggesting the Bloch pathway is initially preferentially used during cholesterol synthesis until a shift occurs via lathosterol to the KR pathway (Ačimovič and Rozman, 2013). The linear correlations seen here between the intermediates of either the Bloch or the KR pathways and cholesterol suggests that the rate of transition from lanosterol to cholesterol via the two proposed routes are closely related.

In the statin controls, intermediates from the Bloch pathway showed a higher degree of correlation to cholesterol than the KR intermediates (r = 0.566 vs 0.306 respectively). Conversely, statin naive controls had improved correlation with the KR intermediates (r = 0.548 for KR vs 0.421 Bloch). However, the similar coefficients calculated with either the cholesterol corrected Bloch (r = 0.469), or KR intermediates (r = 0.47) and the relatively high degree of correlation (r = 0.735) observed between total components from the two routes in the case subjects, does suggest a possible shift in preference in this group of patients.

The contribution of each precursor to the total pool of cholesterol synthesis markers indicates levels of lanosterol and zymosterol, which are both early indictors in the pathway with a retained C24 double bond, are equally represented across all groups. There is some indication to suggest that in subjects who tolerated regular statin intake DHCR24 possibly acts very early in the process as this group did have a higher lanostenol contribution (0.78 % vs 0.25 % cases and 0.16 % controls). However, it should be noted that this analyte had very low concentrations ranging from 0 – 1.154 μmol/L and was actually undetectable in more than 70 % of samples analysed. Therefore, caution needs to be taken with interpretation of this significance.

A significant finding was observed in relation to zymostenol, lathosterol and desmosterol. Lathosterol is the predominant precursor in the KR pathway and is produced by either isomerisation of zymostenol or reduction of 24-dehydrolathosterol (Ačimovič and Rozman,

2013). Desmosterol is the penultimate component of the Bloch pathway. Subjects with evidence of inhibited cholesterol synthesis through well tolerated statin use, had a significant shift towards maintaining flow via the Bloch pathway. The elevated contribution of desmosterol, and reduced zymostenol and lathosterol content in the total pool of synthesis markers, both suggest the reducing action of DHCR24 is delayed in this cohort. Although the reverse could be inferred for patients with statin related myopathy, this group however did not show any significant difference in the preferential flow through either of the post-lanosterol cholesterol synthesis pathways comparative to control subjects.

The one exception to this was observed with 7-dehydrocholesterol. In controls, this was marginally lower, accounting for approximately 6.2 % of all cholesterol precursors compared to 7.9 % in cases and 8.5 % statin controls.

7.4.4 Co-enzyme Q10

The inhibitory action of statins on HMG-CoA not only has the desired effect of reducing cholesterol concentration but arguably has an influence on other metabolites downstream in the mevalonate pathway (Ghirlanda *et al.*, 1993; Massy and Guijarro, 2001). CoQ10 is one such compound that was warranted investigatory links with statin use. Transported in similar fashion in lipoproteins to cholesterol, CoQ10 is a key component of the mitochondrial respiratory chain and an active antioxidant in protection of membrane lipids and LDL-cholesterol (Bentinger *et al.*, 2010).

Congenital deficiencies in CoQ10 have been linked to mitochondrial dysfunction and myopathy (Quinzii and Hirano, 2010) and supplementation with oral CoQ10 has shown to ameliorate these effects, principally through decreased production of ROS (Cornelius *et al.*, 2017). Whilst several studies have reported a decrease of between 15 – 54 % in plasma CoQ10 with statin therapy (Ghirlanda *et al.*, 1993; Altekin *et al.*, 2002; Hargreaves *et al.*, 2005), and demonstrated the benefit of oral supplementation in reversing these decreases (Bargossi *et al.*, 1994), evidence for causality of statin associated muscle symptoms or benefit as an adjunct therapy is discrepant (Young *et al.*, 2007a; Bookstaver *et al.*, 2012; Zlatohlavek *et al.*, 2012).

As alluded to in section 1.6, most of the reported studies have involved relatively small sample sizes. In contrast, over one hundred patients with defined SAMS grade 2 and 237 matched

controls were enrolled on this study. As with the sterol intermediates, there was considerable variation in the CoQ10 concentrations measured within each group which did not follow a normal pattern of distribution. Median values tended to support the notion of a decrease in CoQ10 concomitant with statin treatment, as evidenced by a 14 % decrease in the statin control group compared to controls. This decrease remained significant even when normalised to cholesterol. However, viewed in combination with the median duration on statin in these patients to be 24 months, this is a relatively small decrease compared to published reports. One patient assigned to the statin controls had a measured CoQ10 of 5.49 μmol/L which was significantly above the normal range established by this method (0.324 – 2.58 μmol/L). Although no reports of supplemental CoQ10 were noted, exogenous intake, either pharmacological or dietary cannot be excluded. Nonetheless, exclusion of this patient did not significantly alter the data.

Plasma CoQ10 levels did not statistically differ between controls and case subject, but absolute concentrations did show an increased trend in the cases (median increase 19.7 %, $p = 0.098$). Normalisation to cholesterol diminished any variation suggesting the perceived increase is likely related to an increased number of low density lipoproteins. Surprisingly, data from the control subjects did not identify a correlation between circulatory CoQ10 and cholesterol concentrations although this relationship was evident in the other study groups. The reason for this is unclear. Conversely, the lack of correlation seen here between CoQ10 and either lathosterol/cholesterol ratio or absorption markers supports the view that CoQ10 changes are independent of cholesterol synthesis and absorption (Berthold *et al.*, 2006).

7.4.5 Limitations of the study

It was not possible to measure the full range of non-cholesterol sterols in cholesterol synthesis. This was due to lack of commercial availability of standard compounds and to the limitations of the instrumentation available. A more comprehensive range and improved recovery, in particular for lanostenol, may have been possible using a mix of GCMS and HPLC techniques (McDonald *et al.*, 2012). Moreover, quantitation was performed using a single internal standard and calibration curves as the cost of stable isotopes for each compound was not financially possible. However, the method used here was relatively simple and robust and has proved to be sufficiently accurate for use in a clinical setting.

Similarly, a deuterated standard was not readily available for measurement of CoQ10 thus quantitation relied on comparison to a CoQ9 analogue. Whilst the expected endogenous content of CoQ9 in human plasma is expected to have a negligible impact on quantitation of CoQ10 it is acknowledged that a stable isotope method may have improved accuracy. Furthermore, this study measured circulating CoQ10 in plasma which does not necessarily reflect tissue concentrations (Duncan *et al.*, 2005). It is possible that measurement of CoQ10 in muscle biopsies or mononuclear cells may have altered the lack of association seen here between subjects with myopathy and controls.

Whilst it is acknowledged that dietary intake of phytosterols and CoQ10 rich foods was not specifically addressed in study participants there is no reason to suggest intake would have been higher in one group or the other.

The degree of inhibition of cholesterol synthesis was more pronounced in the subset of patients taking statins without any muscle symptoms compared to the cases. It is highly likely these patients were taking a higher regular dose of statin as adverse effects are known to result in low compliance with statin therapy. Thus, pharmacokinetic properties cannot be completely excluded as responsible for the altered balance of cholesterol homeostasis. However, the large sample sizes examined here should be sufficient to cover the variation in dosing regimens.

Although significant differences were observed based on median values for the data, a considerable degree of crossover was noted between groups for most of the parameters studies. There was no clear distinction between any variables that could definitively be used to identify a possible risk for statin associated myopathy

7.4.6 Conclusion

A large scale comparison of mevalonate derived analytes produced downstream of the site of action of statins has been performed in patients with or without statin related myopathy. Ubiquinone and eleven markers relevant to cholesterol synthesis and absorption were analysed.

Alterations in CoQ10 concentration were not associated with an increased incidence of myopathy during statin treatment for hypercholesterolaemia, although data did support a decrease concomitant with reduced cholesterol synthesis.

Similarly, although data presented here suggest the compliant use of statins results in a balance shift weighted towards intermediates of the Bloch pathway instead of the expected crossover to the Kandutsch-Russell route at the level of lathosterol, no association between non-cholesterol sterols and myopathic symptoms was observed. Thus altered cholesterol homeostasis is not indicated to play a possible role in statin related myopathy.

Discussion

Chapter 8. Discussion

8.1 GENERAL DISCUSSION

8.1.1 Statins

The statins are the mainstay of the treatment of hypercholesterolaemia, atherosclerosis and coronary artery disease (CAD). High circulating concentrations of atherogenic lipids including low-density lipoprotein cholesterol are associated with increased risk of CAD and constitutes the single most important risk factor (Cholesterol Treatment Trialists' Collaboration *et al.*, 2010). Furthermore, CAD is the most common cause of morbidity and mortality in the developed world. Statins are very effective at lowering LDL-C because they are potent inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase, the rate limiting step of cholesterol biosynthesis (Brown and Goldstein, 1980). Inhibition of hepatic cholesterogenesis, the main source of LDL-C, leads to upregulation of hepatic LDL receptors with resultant increased hepatic uptake and clearance of LDL from the blood stream (Schachter, 2005).

8.1.2 Statin associated muscle symptoms

Although the statins are generally well-tolerated, a minority of patients can develop side-effects, particularly those involving muscle. This problem was brought into sharp focus with the introduction of cerivastatin which resulted in around 100 deaths associated with rhabdomyolysis, before it was withdrawn from use (Seehusen *et al.*, 2006). Statin-related myopathy is now well-recognised and a recent review documents the scale of the problem, however it is probable that milder signs and symptoms of muscle involvement (myalgia) are under-reported and as many as 29 % of treated patients may be affected (Stroes *et al.*, 2015). There are at least seven different statins which vary primarily in their lipophilicity and therefore in their ability to penetrate cells. All statins have been reported to produce unwanted muscle-related side-effects which are dose dependent but which are also dependent on some predisposing factors (Rosenson, 2004). Most of these factors affect the bioavailability or metabolism of statins such as the recently reported strong association with a single nucleotide polymorphism in *SLCO1B1*, the gene coding for the organic anion transporting polypeptide OATP1B1 which has been shown to regulate the hepatic uptake of statins (Group, 2008). However, many other medications and conditions may precipitate or contribute to myopathy either directly, such as steroids, alcohol and strenuous exercise, or

indirectly by competing with elimination pathways of statins (CYP3A4 or CYP2C9 depending upon the statin in question) (Chatzizisis *et al.*, 2010).

Much attention has been paid to the relationship of the pharmokinetics of statins to myopathy, however, and crucially, the exact mechanisms of statin myotoxicity remains largely unknown. Statins were introduced to control endogenous, primarily hepatic, cholesterol biosynthesis. Limited research has focused on their effects in relation to the biomarkers of cholesterol synthesis and absorption. Moreover, it is possible that biological variation in the timing of DHCR24 action, causing shifts in the contribution of specific Bloch or Kandutsch-Russell intermediates may be associated with an increased risk of adverse effects.

Furthermore, in addition to the desired decrease in cholesterol, there are several other mevalonate-derived isoprenoids whose synthesis would also be attenuated by inhibition of HMG-CoA reductase, such as ubiquinone, a key component of the mitochondrial respiratory chain, and the dolichols and farnesyl and geranylgeranyl pyrophosphates which are involved in the post-translational modification of proteins. Some key observations suggest that it is not the lowered concentration of cholesterol *per se* which causes myopathy but the effects on other isoprenoids.

8.1.3 CoQ10 depletion

CoQ10, an end product of the mevalonate pathway, transfers electrons from complexes I and II to complex III of the respiratory chain and is therefore an essential component of energy transduction. It is also an effective antioxidant (Bentinger *et al.*, 2007). The active homologue is obtained both from the diet and endogenous synthesis, and whilst evidence is fairly supportive that statins reduce the plasma concentration of CoQ10 (Ghirlanda *et al.*, 1993; Berthold *et al.*, 2006), a reduction in skeletal muscle levels remains debatable (Littarru and Langsjoen, 2007; Mas and Mori, 2010). Thus the association of CoQ10 depletion and myopathy is unclear, particularly as CoQ10 levels have not been studied on a large scale in patients who develop myopathy.

This study aimed to investigate isoprenoid metabolism in a large cohort of patients with statin-related myopathy to test if modulated isoprenoid metabolism is involved in the aetiology of myopathy and to establish if biomarkers in plasma could be used to help predict the risk of

adverse symptoms with statin therapy. As statin-induced depletion of CoQ10 could contribute to myopathy via abnormal mitochondrial function, the role of attenuated CoQ10 levels, either statin induced or by selective inhibition, on basic mitochondrial function was investigated *in vitro*. In addition, the distribution of CoQ10 within lipoprotein particles was considered as a possible key component associated with endothelial dysfunction.

8.1.4 CoQ10 analysis

A new method was developed for the analysis of CoQ10 in various biological matrices, such as plasma, skin fibroblasts and skeletal muscle. A modification of the Folch liquid-liquid extraction method was performed using hexane to partition the lipids into the upper layer. Reversed phase HPLC using an isocratic gradient was used to separate key lipids for quantitation by MSMS in MRM mode. The method is highly sensitive and potentially allowed for the detection of CoQ10 in dried blood spots, using equivalent to 12 µl whole blood, and also for direct flow injection analysis, which had added cost saving benefits, although more validation is required in these areas.

8.1.5 Non-cholesterol sterol analysis

A method was developed for the analysis of an extended panel of cholesterol precursors to enable in-depth evaluation of the two proposed alternative routes of cholesterol synthesis, the Bloch and Kandutsch-Russell pathways. In total, 8 synthesis markers, including cholesterol, and 3 absorption markers, were detectable in a single analytical run using GCMS in SIM mode. Extraction was performed using hexane extraction of lipids following alkali hydrolysis of a small volume of plasma. Two pairs of intermediates, sitosterol and lanosterol, and lathosterol and zymosterol, could not be chromatographically resolved but their mass spectral patterns were sufficiently different to allow separation using the mass spectrometer.

The methods developed in this study were used to analyse samples from patients with evidence of statin associated myopathy as well as age matched controls on statin medication and statin naive controls. In total data was obtained for a large cohort of 108 patients with myopathy, and 237 statin tolerant controls. Additionally, the CoQ10 method was adapted to allow analysis in separated lipoprotein fractions from dyslipidaemic patients to enable insight into its distribution in the circulation and availability for transfer to peripheral tissue.

8.1.6 CoQ10 distribution

Lipoprotein fractions from 51 dyslipidaemic patients, sub-divided based on the Fredrickson classification, were analysed for their CoQ10 content. No difference in total CoQ10 concentrations were found between classes. In normolipidaemia, approximately 60 % of the total CoQ10 in plasma is estimated to be transported in LDL (Tomasetti *et al.*, 1999). This was also demonstrated to hold true for patients in this study with type II hypercholesterolaemia. However, the results suggested that hypertriglyceridaemia was associated with an altered distribution of CoQ10, with concentrations being equally distributed between the LDL and VLDL particles. In terms of oxidative protection of lipoproteins, this essentially suggested LDL-C, a known target for oxidation associated with increased cardiovascular disease, was better protected in patients with high triglyceride levels. Conversely, VLDL-C, which is also atherogenic, had less protection in the form of CoQ10. Of course, it is acknowledged that this study did not examine the presence of other antioxidants, such as vitamin E, which also confer antioxidant protection to lipoproteins.

This finding of an altered CoQ10 distribution could suggest that it is not reduced levels in plasma *per se* that contribute to the phenotypes associated with endothelial dysfunction in hypertriglyceridaemia (Gaenzer *et al.*, 2001), but that a high proportion of the total plasma CoQ10 is likely recycled back to the liver in VLDL and a significantly reduced concentration is available for transfer to endothelial cells via LDL. Thus oxidative stress mediated through the endothelial nitric oxide pathway and ROS production is evident in these patients.

8.1.7 Statins and CoQ10

In the investigations performed in skin fibroblast cells, the use of a fluorescent dye specific for mitochondrial superoxide demonstrated the potential of statins to cause increased oxidative stress in mitochondria. A previous report proposed mitochondrial function may be preserved during statin induced CoQ10 reductions at the expense of a decrease in the cytosolic pool of CoQ10, owing to the existence of independent enzymes, Coq2 and Ubiad1, responsible for controlling synthesis in the mitochondria and cytosol respectively (Mugoni *et al.*, 2013). The increase in mitochondrial ROS and corresponding decrease in CoQ10 seen here with statin treatment was comparable to that observed by selective inhibition of CoQ10 synthesis using a 4-hydroxybenzoate analogue, 4NB. Although not conclusive this supports a link between

statin induced CoQ10 reduction and increased production of mitochondrial ROS which could result in mitochondrial dysfunction.

Further analysis using co-treatment to counteract the decreased CoQ10 prior to flow cytometry analysis of ROS would have helped to confirm this link. However, in contrast to 4NB inhibition, residual CoQ10 levels of approximately 50 % could not be restored in cells treated with statins using exogenous 4HB, suggesting the mevalonate derived isoprenoid chain was the limiting factor in these cells. Thus it cannot be certain that the increased ROS seen was solely due do the decrease in CoQ10 and not to some other pleotropic influence of statins. Co-incubation in the presence of supplemented CoQ10 may be a viable option although penetration of ubiquinone into the mitochondria is low (Quinzii *et al.*, 2012). On the other hand, co-treatment with 4NB and 4HB did restore CoQ10 concentrations so this combination should be tested and used as a substitute indicator of the link between CoQ10 and ROS production.

Using fluorescent markers such as TMRM and CellTracker Green it was possible to examine the relationship between cell number and mitochondrial mass using microscopy and live cell imaging. Mitochondrial mass is a marker for mitochondrial biogenesis and the experiments performed indicate statins significantly increased the volume of mitochondria per cell as calculated by ImageJ software. This change was not evident in cells subjected to selective inhibition of CoQ10 using 4NB. The reason for the increased mitochondrial mass, which can be caused by inhibition of mitophagy, inhibition of fission or increased fusion, is not clear. There was a noteworthy decrease in cell number with statin treatment which suggests evidence of toxicity, however a significant number of cells were viable and the increased mitochondrial mass per cell seen does appear to be a true effect of statin treatment. ROS, in controlled amounts, are needed to maintain normal cellular functions. As mitochondria are responsible for a significant proportion of ROS generation through electron leaks, mitochondrial numbers should be as low as possible to limit ROS production to an acceptable level. Enhanced mitochondrial mass could be responsible for the increased detection of ROS seen in the flow cytometry experiments.

It is acknowledged that a limitation of this project is that results of the live cell imaging are based on a single experiment. Ideally, it would have been preferred to obtain a comparable

set of results in a duplicate experiment. Unfortunately, on one occasion, a fault with the confocal microscope meant it was not possible to image the cells after the staining had been performed so this experiment had to be abandoned and the data discounted. Time restraints for the project limited further evaluations. However, this does warrant further investigation using dose dependant concentrations of statin with concomitant treatment of cells with 4NB and comparisons of the changes in CoQ10 levels with mitochondrial mass.

8.1.8 CoQ10 and sterols in statin related myopathy.

This project was a large scale comparison of mevalonate derived ubiquinone and cholesterol biomarkers as well as cholesterol absorption markers in patients with evidence of statin related myopathy. The number of cholesterol precursors measured made it possible to distinguish between flow through the two potential routes of cholesterol synthesis to evaluate firstly, if statin use altered this distribution and secondly, to test a hypothesis that a preference for either the Bloch or KR pathway may influence a patients susceptibility to statin related myopathy.

This project provided evidence that inhibition of the mevalonate pathway by statins can result in a decrease in plasma CoQ10 concentrations. The results, however could not substantiate a link between statin induced inhibition of CoQ10 and myopathy. Similarly, although the evidence suggests statin use can delay the action of DHCR24 to favour an increase in cholesterol synthesis via the C24 unsaturated intermediates of the Bloch pathway, no association with increased reports of muscle symptoms was established. Therefore, evidence provided here suggests the cause of statin associated muscle symptoms lies elsewhere in the possible myriad of consequences that result from inhibition of the mevalonate pathway.

8.2 Future work

This investigation only focused on limited influences of statins, or CoQ10 reduction, on mitochondrial function. Examination of changes to the bioenergetics would be beneficial to fully understand the possible effects. Oxidative phosphorylation and glycolysis could be investigated in statin treated and Coq10 inhibited fibroblast cells using the Seahorse XF-24 analyser (Seahorse Bioscience) and a combination of oligomycin, which inhibits ATP synthase thus enables measurement of respiration due to proton leak, FCCP to uncouple the proton

circuit and measure maximal respiration, and rotenone and antimycin which inhibit complex 1 and complex 3 respectively to give a measure of non-mitochondrial respiration.

The effect of the statin on mitochondrial mass certainly warrants further investigation. In this study only one concentration was tested which resulted in a significant increase in the number of mitochondria per cell, but which also resulted in diminished cell growth. Lower concentrations of statins produced a similar percent reduction in CoQ10 so live cell imaging using a lower concentration of statin could confirm the validity of this perceived consequence. If this result is confirmed it could be reasoned that the increase in mitochondrial mass may be responsible for increased ROS production which can have a detrimental effect on multiple cell functions.

Manipulation of the mevalonate pathway at the level of HMG-CoA reductase potentially affects multiple downstream analytes and consequently several cellular processes can be altered. There is evidence that inhibition of cholesterol biosynthesis at squalene synthase or squalene epoxidase, enzymes involved in the final stages of sterol biosynthesis, does not affect cell viability, and suggests the levels of the isoprenoids, farnesyl- and geranylpyrophosphates, and their subsequent prenylated proteins may participate in myotoxicity to some degree, (Flint *et al.*, 1997; Holstein and Hohl, 2004). The Ras and Rab small GTPases are involved in signalling pathways mediating cell growth, organelle biogenesis and intracellular vesicular trafficking and are either farnesylated (Ras) or geranyl geranylated (Rab) (McTaggart, 2006). Measurement of the intracellular isoprenoid levels in patients with confirmed SAMS may be the next step in determining the cause of statin related symptoms.

Although this study did not suggest a direct link between plasma CoQ10 and muscle symptoms, the distribution of CoQ10 in lipoproteins in different hyperlipidaemia classes, has raised an interesting question; could the availability of CoQ10 be a contributing factor? It would be interesting to examine the distribution of CoQ10 in patients with myopathy to see if variation exists with patients in whom in sign of statin intolerance was noted. Although it is acknowledged that plasma CoQ10 is not a true reflection of intracellular levels, the practicalities of obtaining muscle biopsies from such are large cohort are not without problems. However, mononuclear cells have been shown to be good representatives of

intracellular levels (Duncan *et al.*, 2005) thus analysis in this sample type would give a clearer indication of the role of CoQ10 in muscle pathologies.

8.3 Conclusion

It is clear that for some patients the development of muscle symptoms casually linked to the use of statin therapy is significant, and that the provision of alternative or concomitant therapy would lower the incidence of statin discontinuation, reducing cardiovascular morbidity and mortality due to untreated hyperlipidaemia. Unfortunately the exact metabolic basis for the risk factors predisposing to statin-related myopathy are unknown. Methods were developed to evaluate two of the major products and their intermediates related to the mevalonate pathway that are potentially influenced by the inhibition of HMG-CoA reductase. Ubiquinone depletion was noted to occur secondary to statin use and was associated with increased mitochondrial ROS production and increased mitochondrial mass. Analysis of the precursors of cholesterol synthesis suggested a preference for Bloch intermediates occurred with statin treatment. However, none of these effects could be directly related to an increased risk of statin related myopathy. Further work is needed, covering a wide range of mevalonate derived metabolites, to conclusively identify patients at possible risk of statin related myopathy.

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