

**METABOLIC REGULATION OF
ENDOTHELIAL DYSFUNCTION AND
CARDIOVASCULAR RISK**

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Abstract

Despite the use of current therapies for lipid modification, glucose regulation, and blood pressure control, residual cardiovascular risk persists, creating a need for additional treatment modalities. The endothelium is essential for the regulation of vascular tone and the maintenance of vascular homeostasis. Endothelial dysfunction is instrumental in the pathogenesis of vascular disease, and independently predicts cardiovascular risk. The endothelium is thus an important treatment target in addition to traditional cardiovascular risk factors.

This thesis describes a series of clinical studies that were designed to address further strategies for cardiovascular risk reduction. Coenzyme Q₁₀ is an obligatory cofactor in the mitochondrial electron transport chain and a lipophilic antioxidant that may have a complementary role in modifying cardiovascular risk. Statins have well proven benefits on endothelial function and cardiovascular risk reduction, but are yet to be established for non-ischemic heart failure. In parallel, strategies are required to improve statin tolerance and thus cardiovascular risk management.

In the first study, 12 weeks of coenzyme Q₁₀ supplementation resulted in significantly improved endothelial function in statin-treated patients with the metabolic syndrome. In the second study, 12 weeks of coenzyme Q₁₀ therapy did not lead to clinically significant reductions in systolic or diastolic 24-hr ambulatory blood pressure in patients with the metabolic syndrome and inadequately treated hypertension, despite standard therapies; but there was a significant reduction in daytime diastolic blood pressure loads. In the third study, 12 weeks of supplementation with coenzyme Q₁₀ in combination with upward titration of simvastatin therapy from 10 mg/day to 40 mg/day was not associated with improved simvastatin tolerability, nor reduced muscle symptoms in patients with a history of statin-related myalgia compared to statin monotherapy. In the fourth study, genetic variation of *COQ2* and *AMPD1* were found to be associated with the risk of statin-induced myopathy in two independent cohorts of cases with a history of statin-induced myopathy and statin tolerant controls. A significant gene interaction was observed between *COQ2* SNP-2 and *AMPD1* Q12X, highlighting an increased risk of statin myopathy with affected alleles. The fifth study, demonstrated that chronic high-term, long-high statin therapy does not lead to subnormal

plasma coenzyme Q₁₀ concentrations in patients with phenotypic or genotypic familial hypercholesterolemia compared to untreated controls, although low coenzyme Q₁₀ levels were associated with increased arterial stiffness in patients with familial hypercholesterolemia. In the final study, short-term treatment with atorvastatin resulted in marked improvements in endothelial function in patients with non-ischemic heart failure that were largely independent of LDL-cholesterol reductions. This was not mediated via reductions in plasma asymmetric dimethylarginine levels.

Coenzyme Q₁₀ supplementation may therefore have an important role in reducing residual cardiovascular risk through augmentation of endothelial function in statin-treated patients. Coenzyme Q₁₀ may still have beneficial effects on blood pressure and statin tolerance in specific populations. Further determination of genetic risk factors for statin intolerance could allow individualisation of lipid modifying therapy. The potential benefits of statin therapy on cardiovascular risk reduction in chronic heart failure requires further evaluation.

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1 Introduction

This thesis was designed to explore strategies for cardiovascular risk reduction in patients, where absolute risk remains high, despite standard therapies. The vascular endothelium has been recognised as an important treatment target in addition to traditional cardiovascular risk factors, such as lipid modifying and blood pressure lowering. Coenzyme Q₁₀ (CoQ₁₀) may have a complementary role in augmenting endothelial function and modifying cardiovascular risk, but this requires further investigation. Statins have well proven benefits on endothelial function and cardiovascular risk reduction, but additional strategies are required to improve statin tolerance.

The introductory chapter describes the healthy endothelium, demonstrating its important roles in the regulation of vascular tone and maintenance of vascular homeostasis. Risk factors and pathological consequences of endothelial dysfunction are outlined. The second part of the introductory chapter reviews metabolic therapies targeting endothelial dysfunction and cardiovascular risk. Specific emphasis is placed on statins and their effects on modifying endothelial dysfunction and cardiovascular risk in primary and secondary prevention, and related adverse side effects. The key functions of CoQ₁₀, its biosynthesis, absorption and metabolism, and factors affecting plasma CoQ₁₀ concentrations are reviewed, in addition to the potential role of CoQ₁₀ supplementation in modulating cardiovascular risk. Finally, the issue of residual cardiovascular risk in patients despite standard treatments, and the requirement for additional modalities and improved adherence to current therapeutic agents is discussed.

The development and validation of two commonly used techniques to measure *in vivo* endothelial function, brachial ultrasound and invasive strain-gauge plethysmography, in addition to pulse wave analysis, a widely used technique to assess characteristics of the central pulse pressure waveform, form the basis for the research presented in chapters 3, 7 and 8. The history, underlying principles and protocols for these techniques are described in the methodology chapter. Protocols for the first brachial ultrasound methodology and the pulse wave analysis methodology were specifically established and validated for the purposes of this thesis and are therefore discussed in more detail.

The subsequent chapters describe a series of clinical studies that were designed to: explore the effects of therapies on modifying endothelial dysfunction and cardiovascular risk, elucidate potential mechanisms of improvement in endothelial function and explore genetic determinants of drug intolerance.

1.1 Vascular endothelium

1.1.1 Structure of the endothelium

All arteries have a three-layered structure: the tunica adventitia, tunica media and tunica intima (Figure 1.1). The tunica adventitia is a connective tissue sheath, which secures the blood vessel in place and provides nutrients to the media. The tunica media is made up largely of vascular smooth muscle cells that regulate blood flow. The tunica intima is composed of a continuous monolayer of endothelial cells which overlies a thin layer of connective tissue and lines the vascular lumen throughout the arterial tree (1). Endothelial cells are heterogeneous with respect to their cell structure, protein expression and physiological functions (2). Endothelial cell structure varies in size, shape, permeability and thickness throughout the vascular tree and can adapt to physiological changes (3). The endothelium is mechanically and metabolically strategically located, separating the vascular wall from the circulation and the blood components (4).

1.1.2 Functions of the endothelium

The vascular endothelium plays an important role in the maintenance of vascular homeostasis, not only by acting as a barrier, but also by utilising autocrine, paracrine, and classical endocrine signalling, stimulating the release of circulating agents that modify vessel wall phenotype (5, 6). In this role, the endothelium releases a number of agonistic and antagonistic molecules, including vasodilators and vasoconstrictors, pro-coagulants and anti-coagulants, inflammatory and anti-inflammatory molecules, fibrinolytics and anti-fibrinolytics, oxidants and anti-oxidants, and many others (5) (Table 1.1). As well as maintenance of vascular tone, the endothelium effects include inhibition of platelet aggregation and adhesion, smooth muscle cell proliferation and leukocyte adhesion (7). Under homeostatic conditions, the endothelium maintains its function, preserving vascular tone, and blood fluidity, in addition to little or no expression of pro-inflammatory mediators (8). Nitric

oxide (NO) is arguably the most important molecule derived from the endothelium, with a large body of evidence supporting its multiple anti-atherogenic properties (9). In addition to its vasodilatory effects, NO prevents leukocyte adhesion and migration into the arterial wall, smooth muscle cell proliferation, and platelet adhesion and aggregation, key events in the development of atherosclerosis (9).

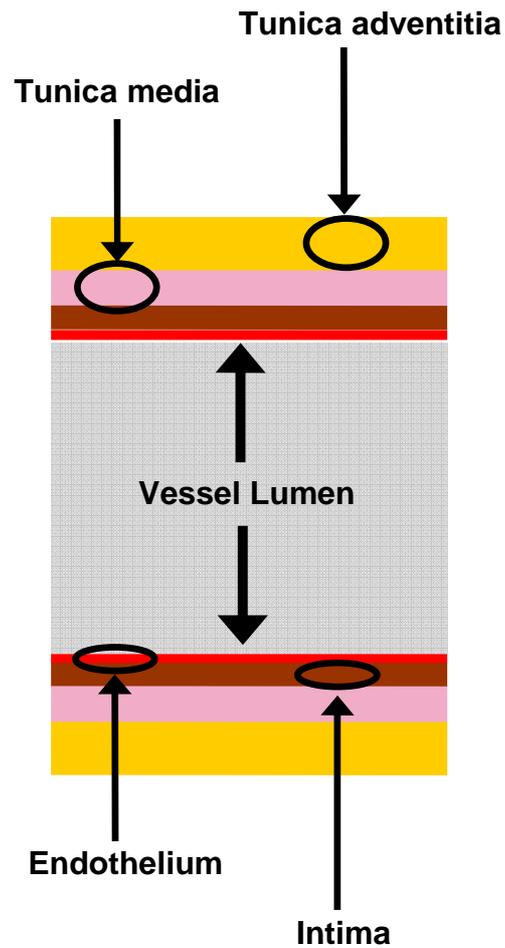


Figure 1.1 Structure of the arterial wall. Endothelial cells form a continuous monolayer lining the intima throughout the vasculature

Table 1.1 Functions of the endothelium

Role	Agonist and antagonist mediators
1. Vascular Tone	Angiotensin II, Endothelium-Derived Hyperpolarizing Factor, Endothelium-Derived Constricting Factor, Endothelin-1, Nitric Oxide, Prostacyclin, Prostaglandin H ₂ , Thromboxane, Asymmetric Dimethylarginine
2. Cell growth	Angiotensin II, Endothelin, Heparan Sulfate, Nitric Oxide, Platelet Derived Growth Factor, Prostacyclin
3. Coagulation	Dermatan Sulfate, Fibrinogen, Heparin Sulfate, Nitric Oxide, Prostacyclin, Thrombomodulin, Tissue Factor, Thromboxane, Von Willebrand's Factor
4. Erythrocyte adherence	Integrins
5. Fibrinolysis	Plasminogen Activator Inhibitor, Tissue Plasminogen Activator
6. Inflammation	P and E Selectin, Nuclear Factor Kappa Beta, Interleukin 8, Intracellular Adhesion Molecules, Nitric Oxide, Vascular Cellular Adhesion Molecules
7. Permeability	Receptor for Advanced Glycosylated End-Products
8. Thrombosis	Ecto-Adpase, Nitric Oxide, Prostacyclin
9. Vasculogenesis/angiogenesis	Platelet-Derived Growth Factor, Nitric Oxide, Transforming Growth Factor-Beta, Vascular Endothelial Growth Factor

(From Stoner *et al.* (2011) (10))

1.2 Endothelial dysfunction

Endothelial dysfunction reflects an imbalance between the release of agonistic and antagonistic endothelium-derived factors. NO appears to be the most important of these mediators. Reductions in NO bioavailability are either due to a decrease in NO synthesis or inactivation of NO resulting from increased production of endothelial reactive oxygen species (11). Elevations in oxidative stress contribute to the uncoupling of endothelial NO synthase

(eNOs), subsequently reducing NO, along with increased superoxide generation, as a result of oxidation of the cofactor, tetrahydrobiopterin (12). It is possible that elevated levels of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of eNOS may also contribute to further reductions in NO generation (13). This sets up a vicious cycle of vascular oxidative stress, whereby the products of mitochondrial respiration form superoxide and peroxynitrite, which further consumes NO and increases oxidative stress (14).

1.2.1 Risk factors for endothelial dysfunction

A number of factors have been identified that contribute to vascular endothelial dysfunction, including: genetic (15), lifestyle related (16), and age-related factors (17), and the consequence of particular disease states (Figure 1.2). The following pathological conditions have been reported to be risk factors for endothelial dysfunction, and associated with impaired endothelial responses: type 2 diabetes mellitus (18), obesity (19), hypertension (20), hypercholesterolaemia (21), dyslipidaemia (22, 23), the metabolic syndrome (24), and heart failure (25, 26). Oxidative stress appears to play a pivotal role in the alteration of endothelial function that typifies all the above risk factors (8).

1.2.2 Measurement of *in vivo* endothelial function

In vivo assessments of endothelial function are typically considered an index of NO bioavailability. Endothelial function is measured in the periphery by assessing the NO-agonist induced vasodilator responses of conduit and resistance arteries. Flow-mediated vasodilation (FMD) of the brachial artery can be measured following an induced period of local ischaemia using high-resolution ultrasonography (27) (reviewed in Chapter 2). Venous occlusion strain-gauge plethysmography allows measurement of the blood flow changes in the forearm microcirculation following reactive hyperaemia, or intra-arterial infusion of NO agonists (28) (reviewed in Chapter 2). Endothelial function can also be measured in the coronary circulation by use of quantitative angiography to measure vessel diameter changes in response to shear stress or pharmacological agonists or stimuli (29). A number of other techniques can be used to assess endothelial function, such as positron emission tomography (30), iontophoresis (31), laser Doppler flowmetry (32), and pulse wave analysis (33) (reviewed in Chapter 2). Biomarkers in the circulation may be measured as indirect indicators of endothelial cell damage, activation and inflammation (34).

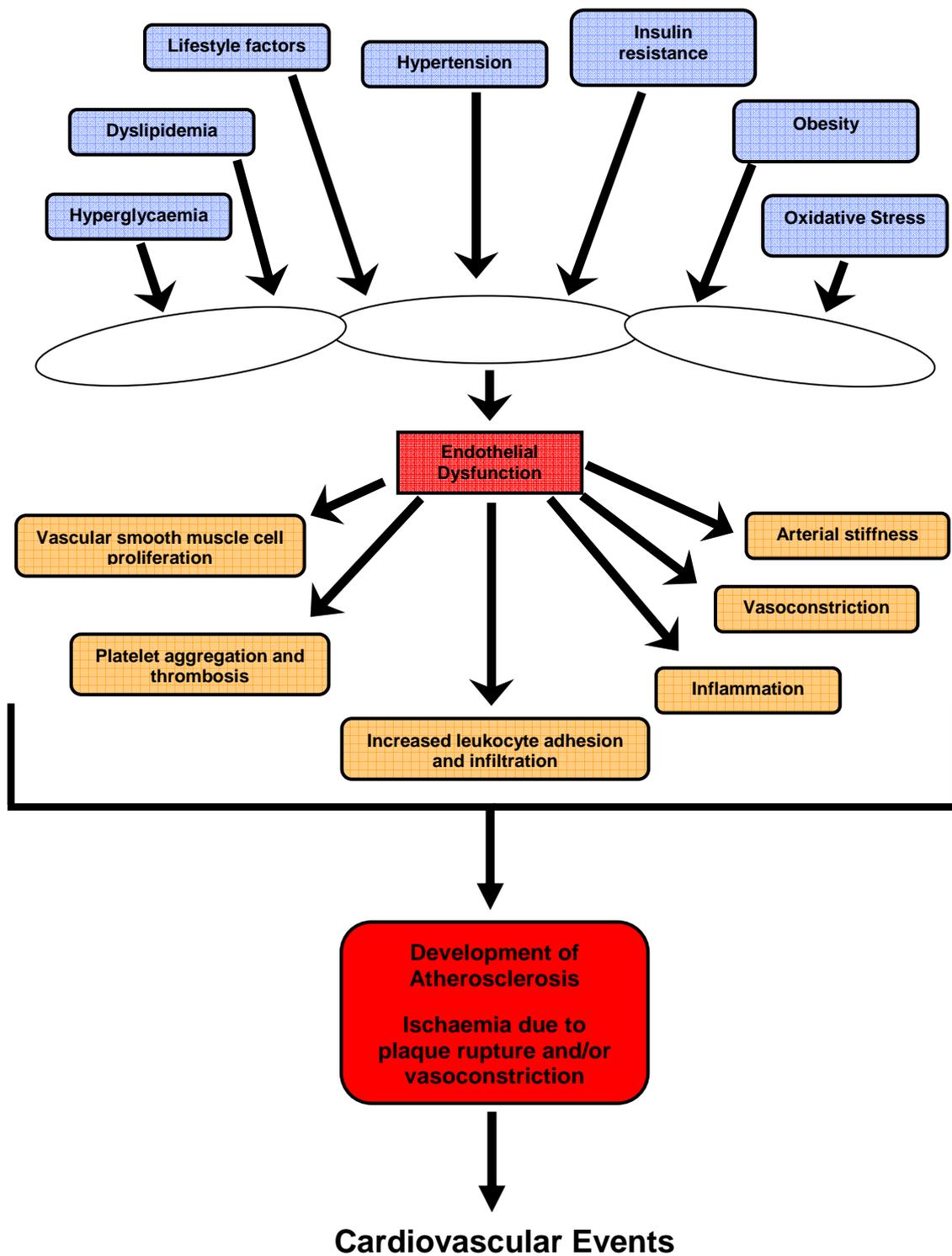


Figure 1.2 The role of endothelial dysfunction in the pathogenesis of cardiovascular disease events. Cardiovascular risk factors disrupt endothelial functions and contribute to the development, progression and clinical expression of atherosclerosis

1.2.3 Consequences of endothelial dysfunction

The pathological complications of atherosclerosis, namely, myocardial infarction and stroke, remain the leading cause of mortality in the developed world (35). Disruption of the endothelial cell monolayer is thought to occur early in the pathogenesis of cardiovascular disease. Functional impairment of endothelial response has been found to occur early in the course of atherogenesis, preceding evidence of the atherosclerotic plaque (5). An impaired endothelial vasomotor response has been demonstrated in children as young as seven years old with familial hypercholesterolaemia (FH) (36). Disruption of the functional integrity of the endothelium plays an important role in all stages of atherogenesis, ranging from the initiation of lesions to plaque rupture. Endothelial dysfunction leads to increased permeability to lipoproteins, foam cell formation, T-cell activation, and smooth muscle migration into the arterial wall (Figure 1.3) (37). The first step in the formation of plaque occurs upon activation of an inflammatory response and fatty streaks appear. With persisting conditions, the fatty streaks progress and the plaques become vulnerable to rupture (37).

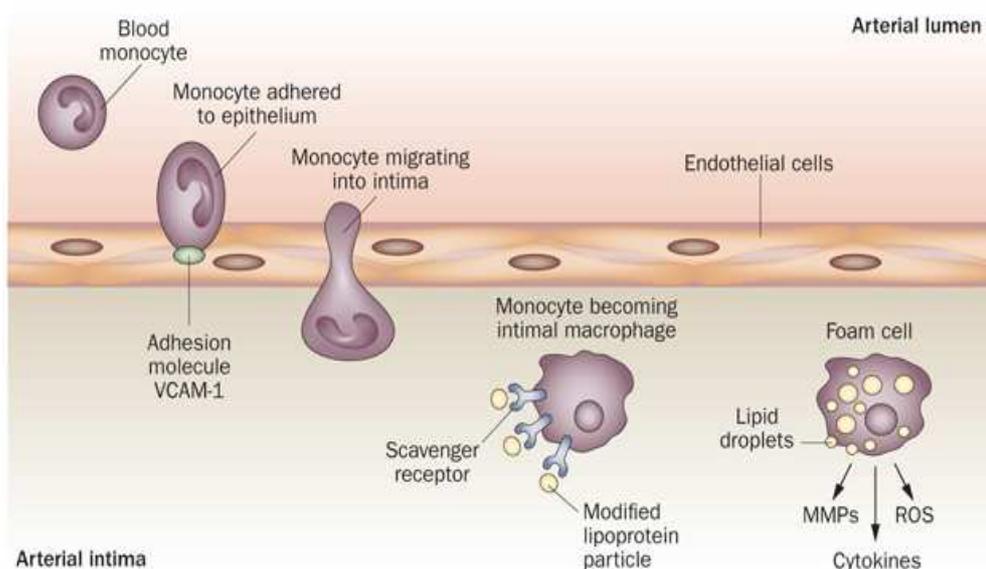


Figure 1.3 Endothelial dysfunction in atherosclerosis. Reprinted by permission from Macmillan Publishers Ltd: *Nat. Rev. Rheumatol.* 6 (5): 253-61. Khan, F et al. © (2010)

1.2.4 Predictive value of endothelial dysfunction

There is strong evidence to indicate that coronary and peripheral endothelial dysfunction is an independent predictor of future cardiovascular events, including myocardial

infarction and stroke, in patients with established cardiovascular disease and in asymptomatic subjects (38-44) (detailed in Chapter 2). The prognostic capacity of these endothelial function tests has potential relevance for risk stratification and improved cardiovascular risk management.

1.3 Metabolic regulation of endothelial dysfunction and cardiovascular risk

While many therapeutic modalities exist for the treatment and prevention of cardiovascular disease, the following sections will focus on therapies relating to lipid modification, glycaemic control, blood pressure reduction and antioxidant supplementation and their effects on modulation of endothelial function and cardiovascular risk.

1.3.1 Lipid modifying agents

1.3.1.1 Statins

Statins (hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors) are the most widely prescribed cholesterol-lowering drugs and are first-line agents for the treatment of hypercholesterolaemia and prevention of coronary heart disease (CHD). The first known statin, compactin, was discovered in the early 1970s by Akira Endo, a Japanese microbiologist (Sankyo) experimenting with *Penicillium citrinum* for potential antimicrobial agents (45). This was shortly followed by the extraction of lovastatin from *Aspergillus terreus* (46), which became the first commercially used statin in 1987 (47). Seven statins have since been introduced, the earlier agents being fungal extracts (simvastatin, pravastatin), and newer agents being synthetically manufactured (fluvastatin, cerivastatin [withdrawn in August 2001], atorvastatin, rosuvastatin, pitavastatin), which vary with respect to their pharmacokinetic profiles with differences in absorption, half-life, bioavailability and metabolic pathways (48).

1.3.1.1.1 Lipid modifying effects

Statins act by competitively and reversibly inhibiting HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, which catalyses the reduction of HMG-CoA to mevalonic acid (49, 50) (Figure 1.4). This leads to a decrease in hepatic cholesterol synthesis

and upregulation of LDL receptors, resulting in increased LDL-cholesterol hepatic uptake, and reduction of circulating LDL-cholesterol levels (51-53). The more potent agents have been shown to reduce LDL levels by up to 55%. Although LDL receptor upregulation is the primary mechanism of action, statins also decrease triglyceride levels by up to 20%, through the inhibition of its synthesis in the liver and enhancement of lipoprotein lipase enzyme activity in the adipocytes (54, 55). In addition, statins are known to modestly increase levels of HDL-cholesterol (up to 10%), although the precise mechanism is not known (56).

1.3.1.1.2 Primary and secondary prevention of cardiovascular events

The benefits of statin therapy in primary prevention, especially in high risk patients has been well documented. The West of Scotland Coronary Prevention Study (WOSCOPS) was the first major primary prevention study to evaluate the effects of statin treatment on CHD events in approximately 6,500 patients with hypercholesterolaemia and no previous history of MI (57). After a 5-year follow-up, pravastatin was associated with significant reductions in nonfatal MI by 31% and cardiovascular mortality by 32% (57). More recently, the Management of Elevated Cholesterol in the Primary Prevention Group of Adult Japanese (MEGA) trial confirmed this finding (58). Statins also have a role in primary prevention of cardiovascular events in patients with moderate to low cholesterol levels, as demonstrated by the Air Force/Texas Coronary Atherosclerosis Prevention (AFCAPS/Tex-CAPS) trial (59), the Asymptomatic Carotid Artery Progression Study (ACAPS) (60), and the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOTLLA) (61). A meta-analysis of 29 randomised primary prevention trials involving 80,711 patients has shown that statin therapy reduces the risk of MI by 36%, stroke by 19%, and overall mortality by 10% (62). The cardiovascular benefits of statins in asymptomatic patients have also been investigated by the Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER), which enrolled nearly 18,000 healthy subjects with normal cholesterol levels and elevated CRP levels (63). In this trial, rosuvastatin treatment was associated with significant reductions in MI (54%), stroke (51%) and total mortality (20%) (63). However, a later analysis of the JUPITER study found that only 25% of patients were considered low risk, and when stratified according to underlying risk, no benefit was observed in the low-risk patients (64). Further investigations are therefore required to ascertain the potential benefits of statin therapy in low risk individuals.

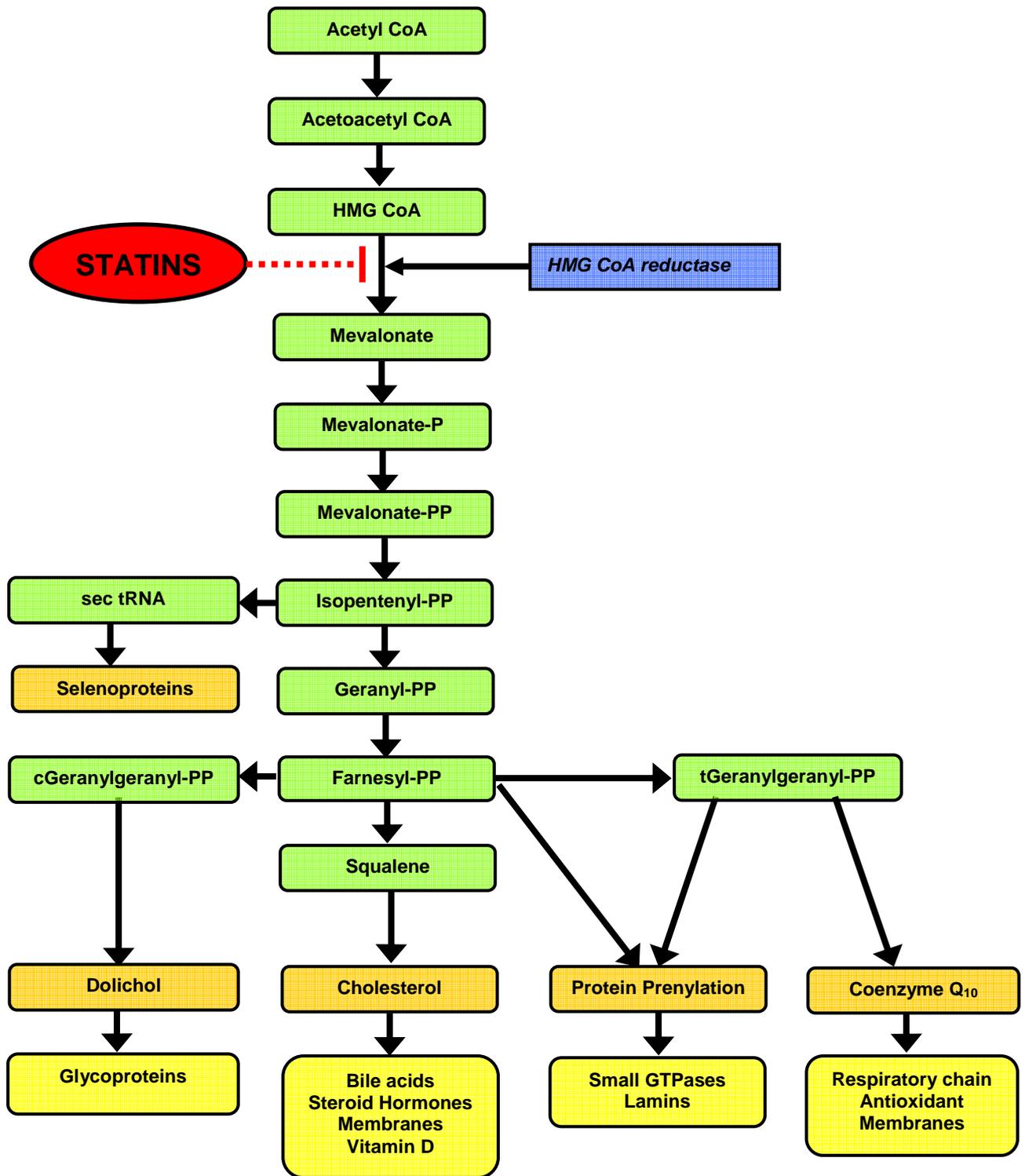


Figure 1.4 Effect of statin therapy on metabolites of the mevalonate pathway

The clinical benefits of statins in secondary prevention have also been well established. The Scandinavian Simvastatin Survival Study (4S) was the first large randomised controlled trial to demonstrate a beneficial effect of statin therapy in patients with established CHD (65). After 5 years follow-up, simvastatin resulted in significant reductions in the rates of overall

mortality by 30%, coronary mortality by 42%, major coronary events by 34% and cerebrovascular events by 37% (65). The Cholesterol and Recurrent Events (CARE) (66) study showed that 5 years of pravastatin therapy lowered major CHD events by 24% in MI patients with average cholesterol levels, and demonstrated greater benefit was conferred in women than men and in those with higher baseline LDL-cholesterol (66). The Long-Term Intervention with Pravastatin in Ischemic Disease (LIPID) study observed significant reductions in overall mortality and coronary death (67), and these findings were corroborated by The Heart Protection Study (HPS) (68). In HPS, the magnitude of benefit was shown to be dependent on the overall risk of the individual, rather than lipids alone (68).

Several trials have demonstrated that more intensive statin regimes result in a greater reduction in atherosclerosis progression (69) and cardiovascular events (70, 71), even in high-risk individuals with normal LDL-cholesterol levels (72). A recent meta-analysis by the Cholesterol Treatment Trialists' (CTT) collaborators that included 90,000 patients from 14 randomised-controlled statin trials, reported a relative reduction of approximately 20% in major vascular events (nonfatal MI, coronary mortality, coronary revascularisation and ischaemic stroke) per mmol/L reduction in LDL-cholesterol levels (73). Coronary and overall mortality was also decreased by 20% and 12% per mmol/L LDL-cholesterol reduction (73). A further meta-analysis of 76 randomised controlled trials and a total of 170,000 patients also revealed significant reductions in all major vascular events with statin therapy (74). Moreover, there is increasing evidence to support the use of statin therapy in patients with other high risk cardiovascular risk conditions such as diabetes mellitus (75, 76), the metabolic syndrome (77), peripheral vascular disease (78) and chronic kidney disease (79), however, evidence in CHF is currently lacking (80, 81).

1.3.1.1.3 Pleiotropic effects of statin therapy

There is increasing evidence from analyses of major statin trials and experimental studies to suggest that some benefits of statins may be due to pleiotropic effects, independent of direct reductions in LDL-cholesterol (82-84). Such effects include improved endothelial function, decreased vascular smooth muscle cell proliferation and migration, anti-oxidation, attenuation of vascular inflammation, inhibition of platelet aggregation, and stabilisation of atherosclerotic plaque (83, 85). Pleiotropic effects may play an important role in reducing cardiovascular morbidity and mortality (84). Inhibition of HMG-CoA reductase not only reduces cholesterol synthesis, but also other metabolites in the mevalonate pathway, such as

farnesyl pyrophosphate (F-PP) and geranylgeranyl pyrophosphate (GG-PP), which are involved in protein prenylation of intracellular signalling molecules, such as Ras, Rho and Rac (86). Statin-induced reductions in prenylated proteins have been shown to have important effects on vascular function (Figure 1.5). Discrimination between the pleiotropic effects from LDL-cholesterol lowering effects may be more evident during the early phase of statin treatment since plasma mevalonate levels drop up to 70% within 1 – 2 hr while a reduction of LDL-cholesterol becomes significant after 6 – 7 days, and thus it is possible that statins may exert their pleiotropic effects through direct inhibition of these isoprenylated proteins (87).

A multitude of studies have demonstrated that statin therapy improves endothelium-dependent vasodilation in the coronary (88, 89) and peripheral arteries (85, 90), due to increased availability of NO (91). Reriani *et al.* (2011) (85) conducted a recent meta-analysis of 46 randomised placebo-controlled trials in 20,706 patients with and without established cardiovascular disease, and demonstrated that statin treatment was associated with significant improvements in both macro- and microvascular endothelial function.

Treatment with statins has been shown to stabilise and upregulate eNOS, resulting in increased eNOS activity, by reducing prenylation of Rho (92-94). Statins also prevent downregulation of eNOS expression and activity by oxidised LDL (93, 94). Post-translational activation of eNOS has been attributed to activation of the phosphatidyl inositol 3-kinase (PI3K) and the serine/threonine kinase, Akt (PI3K-Akt) signalling pathway (95). This has also been shown to increase the binding affinity of eNOS for calmodulin, resulting in a displacement of the inhibitory partner, caveolin 1 (86). Statins reduce caveolin 1 levels, decreasing its inhibitory effects on NO synthesis (96). Statin-mediated inhibition of Rho has been shown to inhibit both vascular muscle cell proliferation and progression through the cell cycle (97). Statins also exhibit antioxidant effects that may improve endothelial function (98), as reactive oxygen species are known to limit the bioavailability of NO. Statins have been shown to reduce oxidative stress by inhibiting Rac-1 mediated NAD(P)H oxidase activity and by down regulating the angiotensin AT1-receptor, which leads to a reduction in free radical generation (99-101).

Statins may also ameliorate endothelial dysfunction by modulating inflammation, mainly through inhibition of Ras prenylation (102, 103). Studies have demonstrated that Ras inhibition reduces activity of nuclear factor kappa beta (NFkB), which is involved in a wide range of inflammatory signalling pathways (104, 105). Down regulation of the NF-kB

pathway also leads to increased activity of eNOS. In addition, statins decrease the activation of activator protein-1 (AP-1), a dimeric protein composed of Jun, Fos and activating transcription factor (ATF) (104), which is mediated by inhibition of farnesylation of Ras and geranylgeranylation of Rho (104). Reductions in plasma concentrations of C-reactive protein that are largely independent of LDL-cholesterol reductions, provide support for the anti-inflammatory pleiotropic effects of statins (106).

The mechanisms by which statins modulate platelet function are not well understood (107). However, given the well documented effects of endothelial NO on inhibition of platelet aggregation (108), statin-induced upregulation of eNOS (109), may contribute to reductions in platelet reactivity (110). Statin-mediated decreases in the production of thromboxane A₂ (111), and modifications in the cholesterol content of platelet membranes (111), may also reduce platelet reactivity.

The plaque-stabilising properties of statins are thought to be mediated by decreased macrophage accumulation in atherosclerotic lesions and by inhibition of matrix metalloproteinase generation, which can degrade the fibrous cap by activating macrophages (112). Statins have been shown to inhibit the expression of matrix metalloproteinases and tissue factor by both LDL-independent and dependent pathways, however direct macrophage effects occur at an earlier stage (112). These properties of statins may reduce the incidence of acute coronary syndromes by reducing the likelihood of plaque rupture (113).

To what extent the pleiotropic statin effects translate into clinically meaningful benefit however, remains a matter of debate (114). There is currently a lack of direct clinical evidence that distinguishes the LDL-cholesterol lowering-dependent effects from the LDL-cholesterol lowering-independent effects of statins (113). It also remains to be established whether LDL-cholesterol-independent functions depend on their inhibition of HMG-CoA reductase rather than on direct interference with pro-inflammatory, pro-thrombotic or endothelial dysfunction pathways (115).

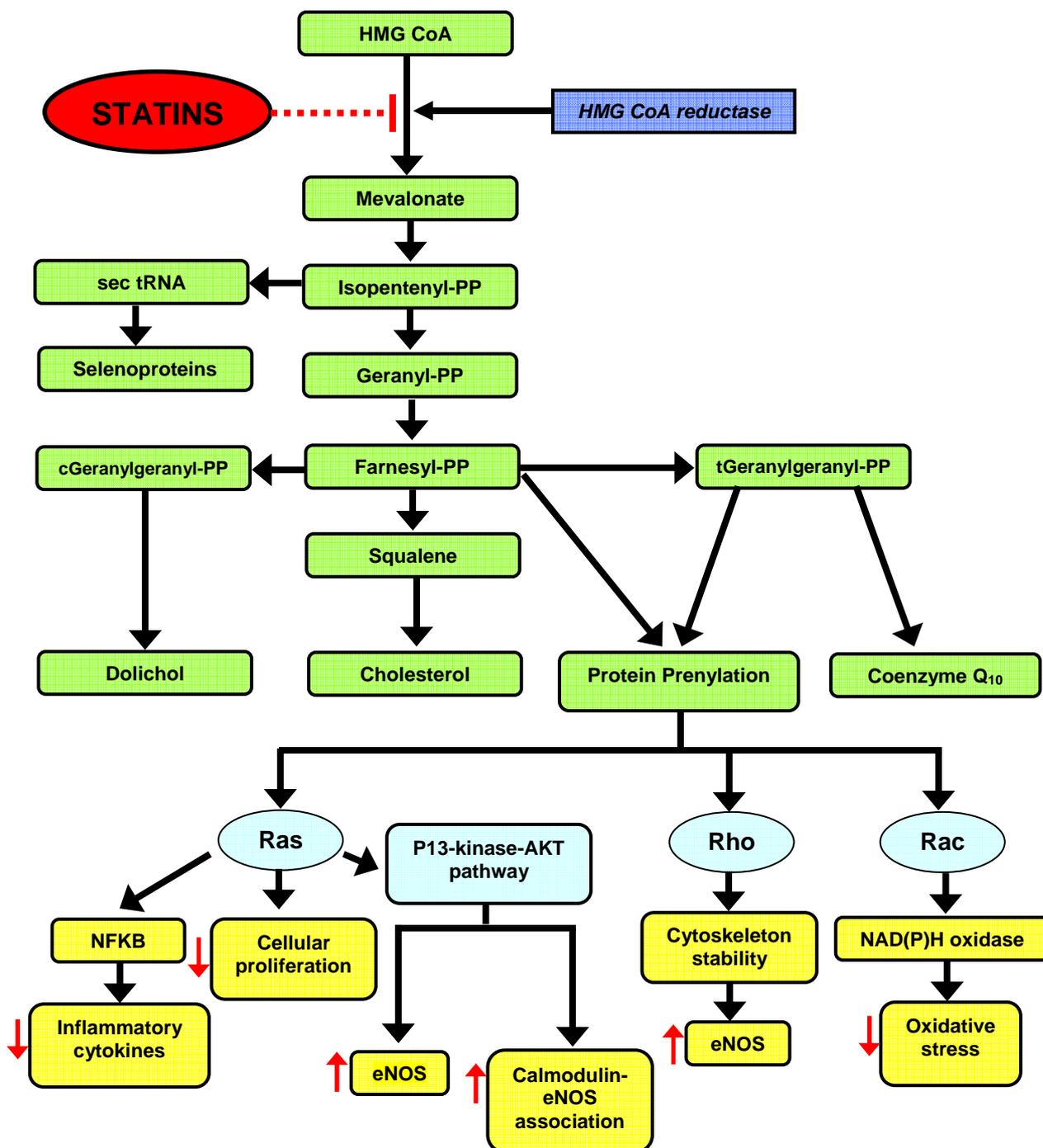


Figure 1.5 Statin modulation of nitric oxide (NO) and other pleiotropic effects. HMG-CoA reductase inhibition increases NO bioavailability by a number of mechanisms including PI3-kinase/AKT phosphorylation of endothelial nitric oxide synthase (eNOS) and increases in calmodulin-eNOS association, Rho-mediated increases in eNOS mRNA half-life, and Rac-mediated decreases of reactive oxygen species generation, thus increasing NO availability. Statins also improve vascular function through Ras-mediated decreases in cellular proliferation and inflammatory cytokines

1.3.1.1.4 Adverse side effects of statin therapy

1.3.1.1.4.1 Myopathy

Statins are generally fairly well tolerated. However, targets for cholesterol reduction have become progressively lower (72, 116, 117), necessitating statin higher doses, thereby increasing the frequency of adverse side effects, in particular muscle complaints ranging from clinically benign myalgia to rare life threatening rhabdomyolysis (118, 119). Statin myopathy represents a clinically important cause of statin intolerance and discontinuation (120-123). The clinical spectrum of statin-induced myopathy includes myalgia, myositis (creatinine kinase (CK) <10xULN), rhabdomyolysis (CK>10xULN), and asymptomatic increases in CK levels, based on the American College of Cardiology/American Heart Association/National, Heart, Lung, and Blood Institute (ACC/AHA/NHLBI) Clinical Advisory (124) (Table 1.2). Although most clinical studies use the ACC/AHA/NHLBI classification, there is a lack of consensus in the definition of statin myopathy. The National Lipid Association (NLA) Muscle Safety Expert panel and the U.S. Food and Drug Administration (FDA) have proposed different definitions on the basis of the degree of CK elevations and muscle complaints (118, 119) (Table 1.2). Although serious rhabdomyolysis is very rare (125-127), prospective post-marketing studies indicate that up to 10 to 15% of patients develop myalgia with statin therapy (128-130). These figures indicate a higher prevalence of statin myopathy than reported in randomised controlled trials (127, 131, 132), probably due to the fact that susceptible patients have often been excluded from such trials (133).

1.3.1.1.4.2 Cancer risk

Controversial data have been published on statins and the risk of cancer. Several trials reported an increased incidence of cancer associated with pravastatin use (66, 134). In the SEAS trial, incident cancers were significantly more frequent in the simvastatin/ezetimibe than in the placebo arm (135), but when the data were combined with the SHARP and IMPROVE-IT trials, there was no evidence of an adverse effects on cancer risk (136). However, a 10-year follow-up of the 4S study found no difference in cancer mortality and cancer incidence between those originally treated with simvastatin and placebo (137), and this was confirmed by the CTT collaborators in a safety analysis of statin trials (73). A large meta-analysis showed no effect of statins on cancer incidence and cancer death (138), thus, current data do not support the notion that statin therapy alters cancer risk (139).

Table 1.2 Proposed definitions for statin-associated myopathy

	ACC/AHA/NHLBI (124)	NLA (119)	FDA (118)
Statin myopathy	Any muscle symptoms related to statin use (proximal muscles more likely to be involved)	Symptoms of myalgia, weakness or cramps, or cramps and CK >10 x ULN	CK \geq 10 x ULN
Myalgia	Muscle aches or weakness without CK rise	NA	NA
Myositis	Muscle symptoms with CK <10 x ULN	NA	NA
Rhabdomyolysis	Severe muscle damage with CK >10 times ULN and CK rise. Usually with brown urine and myoglobinuria	CK > 10,000 IU/L or CK >10 x ULN and elevation in creatinine or medical intervention with intravenous hydration	CK >50 x ULN and evidence of organ damage

ACC/AHA/NHLBI, American College of Cardiology/American Heart Association/National Heart, Lung, and Blood Institute; FDA, Food and Drug Administration; NLA, National Lipid Association; CK, creatine kinase; NA, not available; ULN, upper limit of normal.

1.3.1.1.4.3 Diabetes mellitus

Two recent meta-analyses have revealed that statin therapy carries a small, but significant risk of developing diabetes (140) (74). Sattar *et al.* (2010) identified 13 major randomised controlled cardiovascular outcome trials, each with over 1,000 participants and a follow-up period of more than one year (140). Statin therapy was associated with a 9% increased risk for incident diabetes (140). In a subsequent meta-analysis of 76 randomised controlled trials, Mills *et al.* (2011) analysed data on incident diabetes available from 17 studies enrolling 111,000 individuals (74). A 9% relative increase in diabetes risk was observed in association with statin treatment (74), confirming the findings of Sattar *et al.* (2010) (140). Although the risk of incident diabetes is outweighed by the cardiovascular benefits of statin therapy, these findings do highlight the need to monitor patients for the development of diabetes, especially those on intensive-dose therapy.

1.3.1.1.5 Clinical features of statin-induced myopathy

Symptoms of statin-induced myopathy include fatigue, muscle pain, muscle tenderness, muscle weakness, nocturnal cramping, and tend to be proximal, and worse with exercise (129). Muscle symptoms have been reported to occur within weeks of commencing statin therapy, and less commonly up to 12 months later (129, 141). The time to resolution of myopathy after statin withdrawal is also variable, with one study reporting a mean duration of 2.3 months for symptom resolution after discontinuation of statin therapy (141).

1.3.1.1.6 Risk factors for statin-induced myopathy

A number of risk factors for statin-induced myopathy have been identified, including patient characteristics, statin properties and dose, and concomitant medications (48) (Table 1.3). Risk factors are thought to play a pivotal role in statin myopathy by increasing either systemic statin bioavailability or susceptibility to elevated statin blood levels (48).

1.3.1.1.6.1 Patient characteristics

Demographic characteristics associated with an increase of myopathy include advanced age (i.e. >70 years or >80 years), female gender, small body frame and frailty (119, 124). Myopathic risk factors proposed by the NLA (119) and ACC/AHA/NHLBI (124) include comorbid conditions, such as diabetes and hypothyroidism, impaired renal function and/or liver function. Statins can exacerbate exercise induced skeletal muscle injury (129). Statin-induced

myopathy has been observed in the setting of extensive surgical procedures (142), thus a short-term cessation of myopathy to minimise risk of myopathy during the peri-operative period is recommended (124). The Prediction of Muscular Risk in Observational conditions (PRIMO) study found patients with a personal or family history of myopathy, and those with a personal or family history of myopathy with lipid-lowering therapy were at increased risk of myopathy (129). Genetic predisposition and inter-individual variability in susceptibility to statin-induced muscle effects have been reported. A number of candidate gene variants affecting statin pharmacokinetics (143-149), and pharmacodynamics (150), CoQ₁₀ synthesis, and inherited metabolic muscle diseases (151-153) have been implicated.

1.3.1.1.6.2 Statin properties and dose

Although the therapeutic effect of statins is related to reductions in LDL-cholesterol, the risk of adverse muscle effects is dose-related, regardless of the degree of LDL-cholesterol decrease (129). A meta-analysis of four randomised-controlled trials of intensive versus low dose statin therapy showed a significant increase in the risk (odds ratio 9.97, $P=0.028$) for CK elevations greater than 10 x ULN with intensive treatment (154). In the PRIMO study, marked differences were observed in the risk of muscle symptoms with individual statins; fluvastatin treatment was associated with the lowest rate of myopathy (5.1%), while patients receiving high-dose simvastatin showed the highest rate which likely reflects differing pharmacokinetic and physiochemical properties between statins (129). Recently, a disproportionate increase in the incidence of rhabdomyolysis with high-dose simvastatin compared with superior LDL-cholesterol lowering doses of rosuvastatin and atorvastatin was reported (155), and accordingly the FDA made recommendations to restrict the use of the 80mg dose of simvastatin to patients who have been taking it for 12 months or longer without signs or symptoms of muscle toxicity.

1.3.1.1.6.3 Statin-drug interactions

Interaction of statins with other drugs has been shown to increase the risk of myopathy. In fact, approximately 60% of cases of statin-induced rhabdomyolysis are related to drug interactions (131). Drug interactions usually have a pharmacokinetic basis involving intestinal absorption, distribution, metabolism, protein binding or excretion of statins (48). The majority of cases are related to competition at the level of liver metabolism, since over half of the currently available drugs are metabolised by the CYP3A4 isoenzyme; inhibition of the CYP activity by concomitant medications, increases the risk of myopathy (156). Simvastatin,

lovastatin and atorvastatin, are primarily metabolised through CYP3A4 (157). Inhibitors of the CYP3A4 pathway decrease statin metabolism and therefore increase their serum levels and the likelihood of myopathy (48). CYP3A4 inhibitors known to interact with statins include protease inhibitors (ritonavir, nelfinavir, indinavir), cyclosporine, amiodarone, fibrates (especially gemfibrozil), azole antifungals (itraconazole, ketoconazole, fluconazole), macrolide antibacterials (erythromycin, clarithromycin), calcium channel antagonists (diltiazem, verapamil) and the consumption of large quantities of grapefruit juice, exceeding approximately one litre daily (156, 158-163). Notably, fluvastatin and rosuvastatin are primarily metabolised by CYP2C9, and pravastatin is not metabolised by the P450 system, but undergoes renal metabolism (157). These statins may have a lower risk of myopathy, especially with respect to concomitant medications (129).

Table 1.3 Risk factors for statin-induced myopathy

Age
Sex – females at increased risk
Weight
Drug interactions
other lipid lowering agents including fibrates
protease inhibitors
amiodarone
cyclosporine
azole antifungals
macrolide antibiotics
calcium channel antagonists
Medical disorders
untreated hypothyroidism
renal dysfunction
liver dysfunction
multisystem disease
Inter-current infections
Major surgery or trauma
Family history of myopathy
Excessive exercise
Excessive grapefruit juice
Genetic predisposition

1.3.1.1.7 Pathophysiology of statin-induced myopathy

The precise mechanisms underlying statin myopathy are not yet defined. Proposed mechanisms for statin myopathy include reduction of mevalonate pathway products, including metabolites of isoprenoids (Figure 1.4), mitochondrial dysfunction, and genetic predisposition.

1.3.1.1.7.1 Reduction in cholesterol content of myocyte membranes

Decreased cholesterol content of skeletal myocyte membranes inducing membrane instability has been proposed as a potential mechanism. Statins have been shown to affect ionic channels such as the sodium, potassium and chloride channels, inducing instability which may result in the development of myopathy (164). However, this pathway is unlikely to be an important mechanism, since inherited disorders of the cholesterol biosynthesis pathway that reduce cholesterol levels are not associated with myopathy (165), and in experimental models non-statin lipid lowering agents induce myopathy through distinctly non-overlapping pathways (166). Furthermore, decreasing cholesterol synthesis by inhibiting squalene synthase does not induce myopathy (167, 168).

1.3.1.1.7.2 Vitamin D deficiency

Deficiency of vitamin D, a metabolite of cholesterol synthesis, has been implicated in statin-induced myopathy. Ahmed *et al.* (2009) (169) observed a significant association between low serum 25 (OH) vitamin D (D2 + D3) (32 ng/mL) and myalgia in statin-treated patients (169). Interestingly, vitamin D supplementation largely reversed statin-related myalgia observed in this patient group, leading to speculation that vitamin D deficiency induces statin-myalgia (169).

1.3.1.1.7.3 Dysprenylation of GTP-binding proteins

It has been proposed that statins may induce myopathy by reducing the availability of the isoprenoid co-metabolites, farnesyl pyrophosphate (F-PP) and geranylgeranyl PP (GG-PP), leading to the dysprenylation of GTP-binding proteins, which control apoptosis (170). Enhanced apoptosis occurs in muscle cells exposed to statins (171). A reduction in farnesylation or geranylgeranylation of GTP binding proteins is thought to lead to elevated levels of cytosolic calcium and activation of mitochondrial-mediated apoptotic signalling (171). Interestingly, supplementation with isoprenoids, including F-PP and G-PP has been

shown to prevent statin-induced apoptosis in vascular smooth muscle cells (172, 173). Furthermore, mevalonate administration restored protein synthesis and reversed the myopathic changes induced by statins in rat skeletal myotubes (174). This finding was confirmed by Flint *et al.* (1997) who reported that administration of farnesol and geranylgeraniol reversed the inhibition of protein synthesis and loss of differentiated myotubes (167).

1.3.1.1.7.4 Dysprenylation of lamins

Lamins are the main component of the intermediate filament lamina, which line the inner nuclear membrane, and are involved in chromatin organisation and gene expression (175). *In vitro* studies have reported blocked prenylation-dependent processing of lamin A (176), and prevention of its assembly into the nuclear lamina (177). However, statin concentrations required to inhibit prenylation of prelamin A were several fold higher than those needed to inhibit endogenous cholesterol synthesis (178). It is thus possible that statin-induced lamin dysprenylation may underlie statin-associated myopathy, particularly in the setting where statins interact with other medications, and consequently high concentrations are reached (179). Dysprenylation of lamins by statins may lead to nuclear fragility, with a defective nucleoskeleton that is unable to sustain mechanical forces conducted during muscle contraction, in addition to defective nuclear mechanotransduction and impaired gene transcription, and thereby contribute to myopathy (180).

1.3.1.1.7.5 Inhibition of dolichol synthesis and N-linked glycosylation

Dolichols are derivatives of isopentyl-pyrophosphate and F-PP. Dolichols mediate *N*-linked glycosylation, which is essential for proper biologic functioning of proteins (179). *In vitro* studies in adipocytes showed that statins affect protein *N*-glycosylation, and consequently processing of insulin-like growth factor receptors and insulin receptors which may have synergy with dysprenylation of downstream intermediates, such as Ras proteins (181). It has been therefore been proposed that an impaired response to growth factors may underlie statin-induced myopathy (179), although most cell surface receptors are not dependent on dolichol mediated *N*-glycosylation (179).

1.3.1.1.7.6 Inhibition of selenoprotein synthesis

A negative effect of statins on selenoprotein synthesis has been postulated to contribute to statin-induced myopathy (182). Moosmann *et al.* (2004) (182) hypothesised that statins

interfere with the enzymatic isopentenylation of selenocysteine-tRNA^{[Ser]^{Sec}} (Sec-tRNA) and prevent its maturation into a functional tRNA molecule, resulting in a fall in selenoproteins. Isopentenylation of Sec-tRNA is undertaken by tRNA isopentenyl transferase, which utilises isopentenyl pyrophosphate (IPP) as a substrate (183). Statins block IPP, a direct metabolite of mevalonate (184). Cell culture studies have shown that selenoprotein synthesis is reduced with the addition of lovastatin (185). Furthermore, individuals with statin-induced myopathy have demonstrated similar clinical and pathological features to patients with syndromes that have been associated with severe selenoprotein deficiency (182).

1.3.1.1.7.7 CoQ₁₀ deficiency and mitochondrial dysfunction

Another proposed mechanism for statin-induced myopathy is mitochondrial dysfunction, resulting from reductions of CoQ₁₀. CoQ₁₀ is an intermediate in the mevalonate pathway (Figure 1.4), and consequently statin therapy has been shown to lower plasma CoQ₁₀ concentrations (186-203), and impair mitochondrial function in some trials (151, 194, 204). Since CoQ₁₀ is an essential cofactor in the mitochondrial electron transport chain (section 1.3.3.3.1.1) and given the fundamental role of mitochondria in skeletal muscle function, this has led to speculation that statin-induced CoQ₁₀ depletion may contribute to statin myopathy (further explored in section 1.3.3.3.7.3).

1.3.1.1.7.8 Genetic predisposition

The PRIMO study found that family history of statin intolerance was more common in subjects with myopathy (129), suggesting that genetic variation may play an important role in the predisposition to statin adverse effects. Candidate genetic variants relating to coenzyme Q₁₀ (CoQ₁₀) synthesis (205), statin pharmacokinetics (143-149), and pharmacodynamics, (150), vascular function (206), pain perception (207), and inherited metabolic muscle diseases (151-153) have been implicated in statin myopathy (detailed in Chapter 6). However, further investigations are needed to define the pathogenesis of statin-induced myopathy, including exploration of genetic determinants of statin intolerance.

1.3.1.2 Fibrates

Fibrates are agonists of the peroxisome proliferator-activated receptor (PPAR) alpha, and are involved in the transcriptional regulation of genes that control lipid metabolism (208) and vascular biology (209). Fibrate therapy can reduce plasma triglycerides by up to 50%, and LDL-cholesterol by 20% (210). Fibrates also increase HDL-cholesterol by up to 20%, and

promote the formation of large, less dense LDL particles (210). Fibrates are generally well tolerated, the most serious adverse effects being muscle toxicity (211), and subsequent rhabdomyolysis. These side effects have been mainly observed with gemfibrozil, particularly when combined with statin therapy (212). In several clinical trials, fenofibrate therapy has been associated with significant improvements in endothelial vasomotor function in patients with hypertriglyceridaemia (213, 214). A recent meta-analysis of 45,058 patients from 18 trials observed a 10% relative risk reduction for major cardiovascular events and a 13% relative risk reduction for coronary artery events with fibrate therapy, but no effects on all cause mortality or cardiovascular mortality (215). Recent evidence suggests that fibrates may play an important role in individuals with type 2 diabetes mellitus and in those with combined dyslipidaemia. For example, in the Fenofibrate Intervention in Event Lowering in Diabetes (FIELD) study the reduction in total cardiovascular events was significantly greater in those with diabetic dyslipidaemia than those without dyslipidaemia (216). The Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial examined the additive effects of fenofibrate in statin-treated type 2 diabetes patients with cardiovascular disease or risk factors, and good glycaemic and blood pressure control (210). Although combined fenofibrate and statin therapy did not significantly reduce the primary outcome, in a pre-specified analysis, there was a 31% reduction in cardiovascular risk in diabetic patients with triglycerides >2.3 mmol/L and HDL-cholesterol <0.9 mmol/L (210).

1.3.1.3 Nicotinic acid (Niacin)

Niacin raises HDL-cholesterol by up to 35%, although its mechanism of action is yet to be defined (217). Niacin also exerts other beneficial effects on the lipid profile including reducing triglycerides and LDL-cholesterol levels, and promoting the formation of larger, less dense atherogenic LDL, in addition to lowering lipoprotein (a) (217). Flushing is a significant dose-dependent side effect of niacin, which is reduced with niacin-ER and niacin combined with laropiprant, a prostaglandin D2 inhibitor. Niacin has been associated with worsening glycaemic control (218), which needs to be further addressed in long-term studies. Treatment with niacin has demonstrated increases in FMD and eNOs expression in patients, suggesting that HDL-cholesterol mediated upregulation of eNOs expression may contribute to enhanced vasodilation (219). Niacin monotherapy may decrease cardiovascular events and mortality in patients with coronary artery disease, as evidenced by the Coronary Drug Project and its post-trial follow-up (217, 220). Although niacin therapy has demonstrated beneficial effects on surrogate endpoints of cardiovascular disease in combination with statin (221) or ezetimibe

therapy, large cardiovascular outcome trials are awaited. The Atherothrombosis Intervention in Metabolic Syndrome with Low HDL-cholesterol/High Triglyceride and Impact on Global Health Outcomes (AIM HIGH), was recently discontinued due to clinical futility, with no difference in event rates between treated and control patients, despite significant improvements in HDL-cholesterol and triglyceride levels (222). The Heart Protection Study-2: Treatment of HDL to Reduce the Incidence of Vascular Events (HPS-2 THRIVE) is currently ongoing, and will help to address whether niacin-statin combination therapy confers additional cardiovascular benefit (223).

1.3.1.4 Ezetimibe

Ezetimibe is the first of a new class of cholesterol absorption inhibitors that potently inhibits dietary and biliary cholesterol absorption at the brush border of the intestine (224). Ezetimibe therapy results in the upregulation of hepatic receptors, thereby increasing the catabolism of LDL and possibly LDL ApoB (225). Ezetimibe decreases LDL-cholesterol by 10-20%, and has minimal effects on triglycerides and HDL-cholesterol. 'Dual inhibition' of cholesterol absorption and synthesis has complementary effects on the catabolism of ApoB-containing lipoproteins (226). For example, similar reductions in LDL-cholesterol levels have been obtained with 10mg ezetimibe plus 10mg simvastatin or atorvastatin versus 80mg of the respective statin in hypercholesterolemic patients (227). In a number of studies, Ezetimibe administration has been shown to improve endothelial function in patients with CAD, type 2 diabetes mellitus, and metabolic syndrome (228). However, others studies failed to demonstrate this effect (228). In one trial, addition of ezetimibe to a statin did not lead to reduction in cardiovascular events in patients with aortic stenosis (135). In contrast, the Study of Heart and Renal Protection (SHARP) trial reported a significant reduction in major vascular events in patients with kidney disease treated with simvastatin plus ezetimibe compared with placebo (229). The ongoing IMPROVE-IT (Examining Outcomes in Subjects with Acute Coronary Syndrome: Vytorin (Ezetimibe/Simvastatin) versus (Simvastatin) trial will help to address the potential benefits of ezetimibe on cardiovascular outcomes. This trial is investigating the effects of simvastatin (40mg/day) plus ezetimibe (10mg/day) with simvastatin monotherapy on cardiovascular morbidity in patients with acute coronary syndrome (230).

1.3.1.5 Omega-3 fatty acids

Omega-3 (n-3) fatty acids (eicosapentaenoic acid and docosahexaenoic acid) produce dose-dependent reductions in plasma triglycerides, which are more pronounced in patients with hypertriglyceridaemia (225). Treatment with n-3 fatty acids has also been shown to decrease small dense LDL particles and ApoB production (231, 232). Several mechanisms have been proposed for these effects of n-3 fatty acids, including reduced hepatic synthesis of triglycerides by inhibition of acyl CoA:1,2-diacyl-glycerol acyltransferase (233). n-3 fatty acids increase hepatic β -oxidation and upregulate fatty acid metabolism in the liver by stimulation of PPARs, leading to a subsequent decrease in the availability of free acids for triglyceride synthesis (234). Commercial preparations of n-3 fatty acids are available, such as Omacor. n-3 fatty acids have been associated with beneficial cardiovascular outcomes. In the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardio (GISSI) Prevenzione trial, n-3 fatty acid administration was associated with reductions in all-cause mortality and sudden death in post-MI patients (235). In the GISSI heart failure (GISSI-HF) study, treatment with n-3 fatty acids in addition to standard therapy resulted in reductions in all-cause mortality, and all-cause mortality or hospitalisations for cardiovascular disease in patients with CHF (236). Additional effects of n-3 fatty acids include improved endothelial function, blood pressure, anti-inflammatory and anti-arrhythmic effects, and plaque stabilisation, which may account for the benefit of these agents on cardiovascular risk (237).

1.3.1.6 Metformin

The main hypoglycaemic action of the biguanide, metformin is suppression of hepatic gluconeogenesis by activation of adenosine monophosphate-activated protein kinase pathways (238). Metformin may also stimulate the uptake of glucose in muscle (238). Metformin reduces HbA1c levels by between 1% and 2%, and is considered the treatment of choice for overweight people with type 2 diabetes mellitus. Major adverse effects of metformin are gastrointestinal disturbances, and lactic acidosis, although this is rare (239). Metformin therapy was shown to augment endothelium-dependent microcirculatory function and insulin sensitivity in a randomised, control trial of diet-treated patients with type 2 diabetes mellitus (240). In contrast, another clinical trial in patients with type 2 diabetes mellitus, failed to show any significant effects on endothelial function or insulin sensitivity, despite improved glycaemic control (241). Ten-year follow-up data from the United Kingdom

Prospective Diabetes Study (UKPDS) trial revealed a continued benefit for metformin with respect to myocardial infarction, all-cause mortality, and diabetes-related endpoints (242).

1.3.1.7 Sulphonylureas

Sulphonylureas are insulin secretagogues that lower glycaemia by binding to SUR1 receptors, resulting in the closure of pancreatic beta-cell potassium-dependent ATP channels and subsequent stimulation of pancreatic beta cell insulin secretion. Sulphonylureas lower HbA1C by approximately 1-2%, exhibiting similar efficiency to metformin (243). Major adverse effects of sulphonylureas are hypoglycaemia and weight gain. Several cross-over studies have failed to show any significant effects of sulphonylurea treatment on endothelial function in comparison to metformin or placebo (244, 245) or diet therapy alone (246). One double-blind, randomised, crossover trial in patients with type 2 diabetes mellitus indicated that glibenclamide reduced forearm blood flow responses to hyperaemia compared to gliclazide, which may reflect differential binding of these agents to sulphonylurea receptors (247). A further study did not however, observe any differences between these two drugs in the forearm vasodilator responses to acetylcholine (248). The effects of sulphonylurea therapy on cardiovascular end-points have been conflicting; however, recent analyses suggest there are worse long-term cardiovascular outcomes with sulphonylureas compared with metformin (249).

1.3.1.8 Meglitinides

The meglitinides, including repaglinide and nateglinide, are short-acting insulin secretagogues with a similar hypoglycaemic action to sulphonylureas. These agents have been shown to reduce post-prandial glycaemia, and are associated with HbA1c reductions of up to 2.1% (250). Side effects of meglitinides include hypoglycaemia and weight gain. In a randomised cross-over study, repaglinide but not glibenclamide therapy was associated with increased FMD of the brachial artery in patients with diet-controlled type 2 diabetes, and this improvement in endothelial function was associated with changes in post-prandial glycaemia (251).

1.3.1.9 Alpha-glucosidase inhibitors

Acarbose, is an alpha-glucosidase inhibitor, that targets postprandial hyperglycaemia, with its main action being to attenuate absorption of carbohydrate. Acarbose has been shown

to reduce HbA1c by approximately 0.8% in type 2 diabetic subjects (252). The utility of alpha-glycosidase inhibitors is limited by gastrointestinal side effects, which results in frequent (up to 45%) discontinuation of these agents (252). Shimabukuro *et al.* (2006) (253) showed that a single dose of acarbose attenuated post-prandial impairment of hyperaemic forearm blood flow response in a randomised, placebo-controlled cross-over trial of diet-controlled patients with type 2 diabetes mellitus (253).

1.3.1.10 Thiazolidinediones

Thiazolidinediones improve insulin sensitivity and reduce glycaemia by PPAR-gamma receptor-mediated effects on adipocytes, resulting in decreased hepatic glucose production and increased peripheral glucose uptake by skeletal muscle (254). These agents have been associated with a 0.5 – 1.4% reduction in HbA1c (252). In several clinical trials, rosiglitazone treatment has improved forearm blood flow responses to acetylcholine in patients with type 2 diabetes mellitus (241, 255). In a randomised, double-blind, placebo-controlled, cross-over study treatment with pioglitazone was also shown to improve FMD of the brachial artery in patients with type 2 diabetes mellitus (256). In addition, pioglitazone has been shown to reduce arterial stiffness and CRP levels in diabetic patients, independent of changes in glycaemic control (257). Major adverse effects of thiazolidinediones include oedema, heart failure, higher fracture rates, and weight gain. While recent meta-analyses have shown that pioglitazone is associated with a reduction in the incidence of mortality, myocardial infarction and stroke (258), similar analyses of rosiglitazone suggested an increased risk of myocardial infarction and heart failure (259, 260). On this basis, rosiglitazone has been withdrawn from most markets worldwide, but is currently still available in the US, although access is restricted to patients who do not achieve adequate control with other anti-glycaemic agents.

1.3.1.11 Insulin

Insulin is the most effect glucose lowering agent, with numerous preparations of therapy available depending on whether they are rapid, short, intermediate or long-acting in nature. Adverse effects of insulin include the need for subcutaneous administration, weight gain and risk of hypoglycaemia. In type 2 diabetic patients with ischaemic heart disease, insulin therapy has been associated with reductions in HbA1C levels and improvements in insulin-stimulated forearm blood flow response to acetylcholine in a randomised controlled trial

(261). Insulin may also elicit beneficial effects on the vasculature by increasing endothelial NO generation via phosphatidylinositol 3-kinase signalling pathways (262).

1.3.1.12 Emerging anti-glycaemic agents

Novel therapeutic agents that target the incretin pathway include the glucagon-like peptide 1 (GLP-1) agonists and the dipeptidyl peptidase-4 (DPP-4) inhibitors. GLP-1 agonists and DPP-4 inhibitors reduce glycaemia by stimulating insulin secretion, suppressing glucagon secretion and inhibiting gastric emptying and also induce satiety. DPP-4 inhibitors prevent rapid degradation of the incretins by DPP-4, and include sitagliptin, vildagliptin and saxagliptin. DPP-4 inhibition is associated with a 0.74% reduction in HbA_{1c} and is weight neutral (263). GLP-1 agonists include exenatide and more recently the long-acting formulation, liraglutide, which are administered by subcutaneous injection. GLP-1 agonists have been associated with a 1% lowering of HbA_{1c} as add on therapy in clinical trials and produce modest weight loss (264). The sodium glucose co-transporter 2 (SGLT2) inhibitors, such as dapagliflozin and canagliflozin, remogliflozin, are another class of antiglycaemic agents currently in development. SGLT2 inhibitors block 98% of renal re-absorption, leading to increased urinary glucose excretion, thereby improving glycaemic control (265). The benefits of these promising anti-glycaemic agents on cardiovascular risk reduction need to be elucidated in large randomised controlled trials, which are currently in progress.

1.3.2 Anti-hypertensive agents

1.3.2.1 Angiotensin-converting enzyme (ACE) inhibitors and Angiotensin receptor blockers (ARBs)

Angiotensin II has been demonstrated to increase the release of superoxides by stimulating activity of nicotinamide adenine dinucleotide phosphate (NADPH)/ nicotinamide adenine dinucleotide (NADH) (266). The improvement of endothelial function observed in patients treated with angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs) has been attributed to the increase of NO bioavailability and activity of superoxide dismutase, and the decrease of oxidative stress (267, 268). ACE inhibitors and ARBs can also reduce the breakdown of bradykinin. Reduction in the eNOS inhibitor, ADMA, has been reported with enalapril (269). ARBs have been shown to improve basal NO release and decrease the vasoconstrictor effect of endogenous endothelin-1 (270). A number

of clinical trials have demonstrated that these antihypertensive agents can improve endothelial function in the macro and micro circulation in patients with and without coronary artery disease (269, 271-274).

1.3.2.2 Beta blockers

Not all beta blockers have been shown to improve endothelial dysfunction (275, 276). While first-generation beta-blockers do not seem to benefit endothelial function, third generation beta-blockers, such as nebivolol (277) and carvedilol (278) consistently provide specific endothelium-mediated vasodilating effects. These occur through activation of the L-Arginine-nitric oxide pathway in the case of nebivolol (279) and via antioxidant actions of carvedilol (280). Placebo-controlled clinical trials have demonstrated improvements in both endothelium-dependent forearm blood flow (278) and coronary blood flow responses (281, 282), in patients receiving nebivolol or carvedilol.

1.3.2.3 Calcium channel antagonists

Calcium channel antagonists increase endothelium-dependent vasodilation in several vascular beds mainly by restoring nitric oxide availability through a mechanism probably related to an antioxidant effect (270, 283-285). The endothelium-ameliorating effects of calcium channel blockers have also been extended to patients with hypercholesterolaemia (286). For example nifedipine, a dihydropyridine calcium channel antagonist improves endothelium-dependent vasodilation to acetylcholine in hypercholesterolemic patients (286). However some studies have failed to show an effect of calcium channel antagonists on improvements in endothelial function (287).

1.3.3 Antioxidants

1.3.3.1 Vitamin E

Vitamin E (α -tocopherol) is a lipophilic antioxidant with the ability to scavenge lipid radicals and terminate oxidative chain reactions, and thereby protect other lipids from consumption (288). Following its oxidation, vitamin E can be recycled back to its native unoxidised form by other antioxidants, including vitamin C and ubiquinol; a process considered to be critical for its antioxidant activity (289). Vitamin E has been shown to

enhance various functions of the endothelium, including eNOS activity (290), and production of PGI₂ (291), although the effect of vitamin E therapy on endothelium-dependent impairment of vasodilation is unclear. Studies have reported improvements in endothelial function in patients with hypercholesterolaemia (292-294), angina (295), and in smokers (296, 297), while other studies have shown no effects (298-301). Despite evidence that vitamin E can augment endothelial function, numerous trials have failed to demonstrate a significant benefit of vitamin E on primary or secondary prevention of cardiovascular events (235, 302-307). In contrast, only a few trials have reported a reduction in cardiovascular risk with vitamin administration (308, 309). Overall, vitamin E supplementation has been associated with an increased risk of mortality in a recent meta-analysis (310). These effects of vitamin E could be explained by the ability of vitamin E to act as a pro-oxidant due to formation of the α -tocopheroyl radical (311). Furthermore, it has been proposed that the failure of vitamin E to show a beneficial effect is due to the fact that it has been indiscriminately administered to a large population (312). For example, when vitamin E was selectively administered to type 2 diabetes patients with the Hp 2-2 phenotype, supplementation resulted in significant reductions in cardiovascular events and overall mortality (313), and retrospective subgroup analyses according to Hp phenotype have shown similar results (314, 315).

1.3.3.2 Vitamin C

Vitamin C (ascorbic acid) is a hydrophilic antioxidant that is capable of scavenging oxygen-derived free radicals and sparing other endogenous antioxidants from consumption (316). Vitamin C has been shown to improve endothelial vasomotor function in patients with essential hypertension (317), type 2 diabetes mellitus (318, 319), coronary artery disease (320, 321) hypercholesterolaemia (322), and smokers (296, 297, 323). Vitamin C was also found to be beneficial in patients with both type 2 diabetes and coronary artery disease (324). Although a variety of anti-atherogenic effects have been demonstrated for vitamin C, these have not translated into the clinical setting, since cardiovascular endpoint trials have shown no beneficial effect for vitamin C supplementation alone (325, 326) or in combination with other antioxidant supplements (307). This lack of effect of vitamin C on reducing cardiovascular events could be partially explained by the fact that low dose vitamin C may be pro-oxidant (311). Further cardiovascular outcome trials are needed to establish whether treatment with vitamin C can translate into reductions in cardiovascular risk.

1.3.3.3 Coenzyme Q₁₀

Coenzyme Q₁₀ (CoQ₁₀) is a 1,4-benzoquinone with a long (50- carbon) isoprenoid side chain (Figure 1.6), and was first isolated from beef heart mitochondria by Frederick Crane of Wisconsin, USA in 1957 (327). Various CoQ homologues, containing different numbers of isoprenoid units in the side chain, exist and both CoQ₉ and CoQ₁₀ are present in human plasma. The latter is the dominant homologue (328). CoQ₁₀ is present in the body in both the reduced (ubiquinol, CoQ₁₀H₂) and oxidised (ubiquinone, CoQ₁₀) forms. Oxidised CoQ₁₀ is reduced to CoQ₁₀H₂ in the mitochondria by flavoenzymes, including mitochondrial succinate dehydrogenase and nicotinamide adenine dinucleotide (NADH) dehydrogenase (329). CoQ₁₀ is distributed in all membranes through the cell (330), and transported by lipoproteins in the circulation (193). The percentage of CoQ₁₀H₂ in the plasma of healthy individuals is approximately 96% (331). In human liver, pancreas and intestine, almost all CoQ₁₀ is present in the reduced form, whereas CoQ₁₀ exists mostly in the oxidised state in the brain and lung (80%), which appears to be a reflection of increased oxidative stress in these tissues (332). CoQ₁₀ is present in the highest concentrations in tissues with high energy requirements or metabolic activity, such as the heart, kidney, liver and muscle (332, 333). At a subcellular level, the largest portion of CoQ₁₀ (40-50%) is localised in the inner mitochondrial membrane, reflecting its important role in mitochondrial function (334).

1.3.3.3.1 Roles of CoQ₁₀

1.3.3.3.1.1 Mitochondrial function and cellular bioenergetics

CoQ₁₀ has a fundamental role in mitochondrial function and cellular bioenergetics. CoQ₁₀ is an essential cofactor in mitochondrial oxidative phosphorylation, and is necessary for adenosine triphosphate (ATP) production (Figure 1.7) (335). In this role, CoQ₁₀ functions as a mobile electron carrier, transferring electrons from complex I (NADH coenzyme Q reductase) to complex III (cytochrome bc₁ complex) or from complex II (succinate dehydrogenase) to complex III (335). CoQ₁₀ is also present in protein-bound form in complex III, where it is necessary for the function of the “Q-Cycle” of electron transfer, linked to proton translocation inside the complex. This cycle involves the Rieske iron-sulfur centre, cytochromes b₅₆₆ and b₅₆₀, cytochrome c₁ and ubiquinones (336).

The redox functions of CoQ₁₀ extend beyond its role in the mitochondria (337). There is evidence for a role in proton gradient formation in endomembranes and at the plasma

membrane (335). CoQ₁₀ is native constituent of a lysosomal electron transport chain, which promotes proton translocation across the lysosomal membrane (338), and thereby regulates the luminal pH (339). CoQ₁₀ is necessary for proton transport in the Golgi membranes for acidification of vesicles (340). CoQ₁₀ is also involved in proton movement out of cells, through activation of the plasma membrane NA⁺/H⁺ antiporter (341).

CoQ₁₀ is also an inhibitor of the mitochondrial permeability transition pore (MPTP) in the inner mitochondrial membrane (342-344); considered to be an important cardioprotective function of CoQ₁₀ (345). In this role, CoQ₁₀ preserves sodium potassium-ATPase, activity which necessary for ATP synthesis and stabilises calcium-dependent ion channels, thereby decreasing calcium overload and preventing apoptosis (346-350).

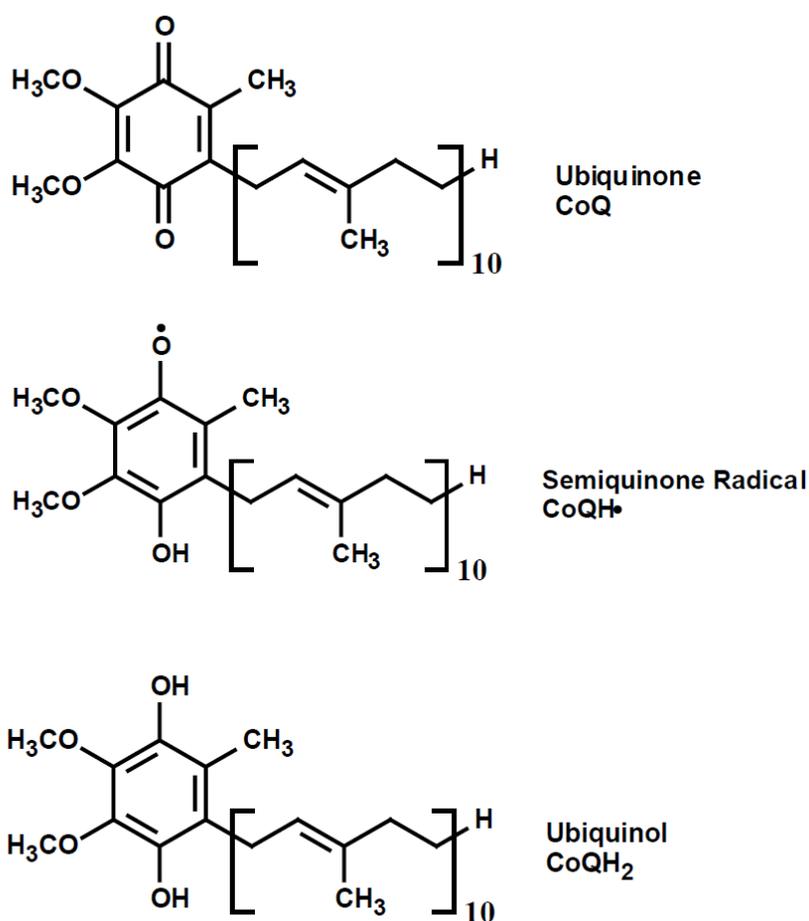


Figure 1.6 CoQ₁₀ and its redox states

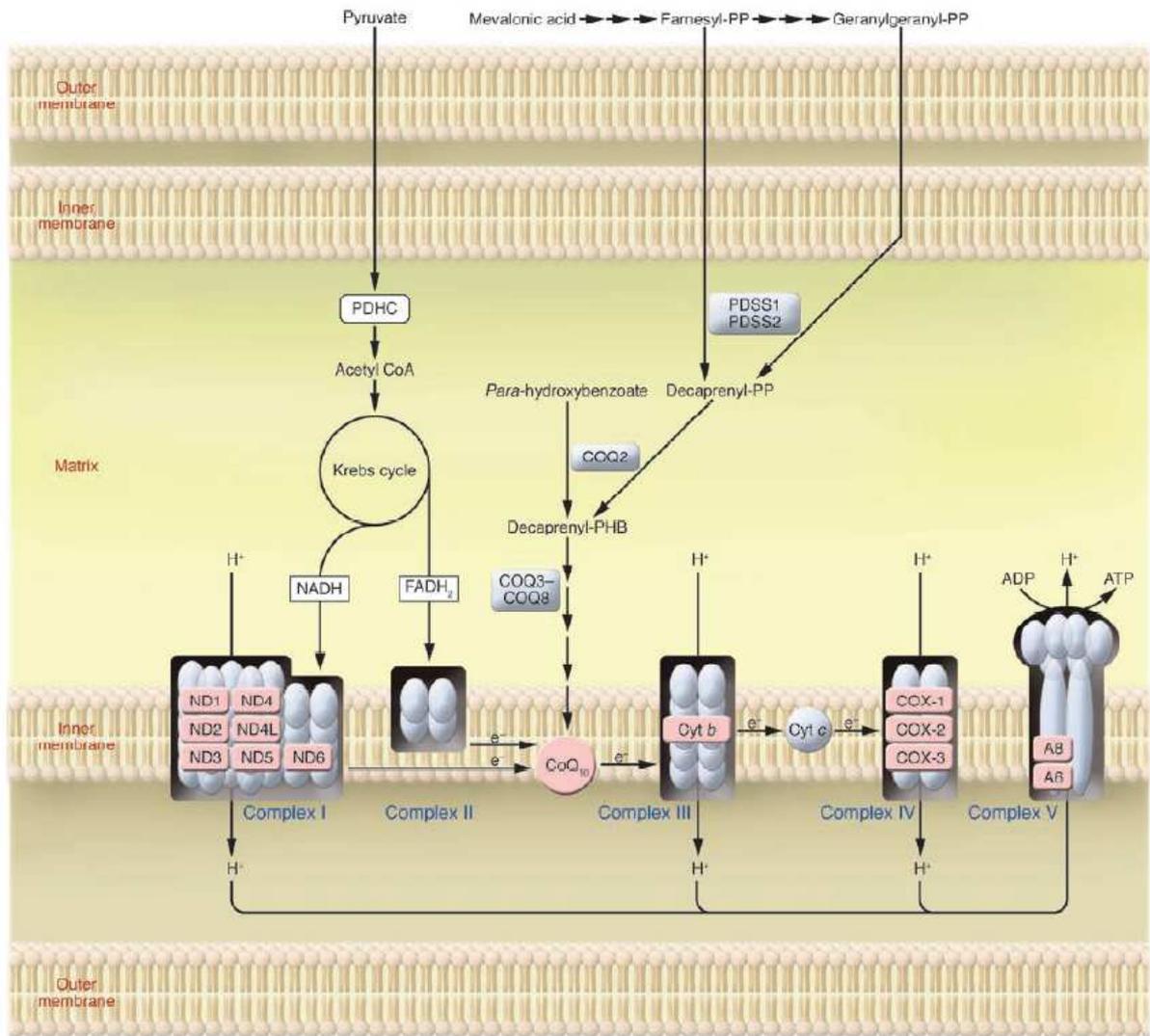


Figure 1.7 CoQ₁₀ biosynthesis and function in mitochondrial metabolism. CoQ₁₀ shuttles electrons (e⁻) from complexes I and II to complex III. Decaprenyl diphosphate is synthesized by COQ1 (PDSSI and PDSS2) from the mevalonate pathway via farnesyl diphosphate and geranylgeranyl diphosphate and is attached to para-hydroxybenzoate by COQ2. At least 6 more COQ enzymes (COQ3-COQ8) catalyse methylation, decarboxylation, and hydroxylation reactions to synthesize CoQ₁₀. A8; ATP synthase subunit 8; Cyt *b*, cytochrome *b*; FADH₂, flavin adenine dinucleotide; ND1-6, subunits 1-6 of NADH dehydrogenase; PP diphosphate; PDHC, pyruvate dehydrogenase complex. Reprinted by permission from American Society for Clinical Investigation: *J Clin Invest.* 2007 March 1; 117(3): 587–589. DiMauro, S *et al.* © (2007)

1.3.3.3.1.2 Antioxidant activity

The reduced form of CoQ₁₀ is now recognised as a major cellular antioxidant, protecting biological membranes and lipoproteins in the circulation (335, 351). CoQ₁₀ is the only endogenously synthesised lipid soluble antioxidant, present in all membranes (335, 351). The earliest observations of an antioxidant function of CoQ₁₀H₂ date back to the sixties, and show that this substance could, like alpha-tocopherol, protect against the light-catalysed peroxidation of mitochondrial lipids (352). These observations have been extensively confirmed during the last two decades.

CoQ₁₀H₂ is present in the membranes and LDL particles, where it is well positioned in close proximity to radical production to act as a primary scavenger of free radicals (335). Since much of the CoQ₁₀ in cell membranes and plasma is present in the quinol form (331, 332), it can be a very effective antioxidant (351). The total content of CoQ₁₀ in many membranes is 3 to 30 times the tocopherol content (353). CoQ₁₀H₂ has an important role in preventing the initiation and propagation of lipid peroxidation in biological membranes, including mitochondria (354), and plasma lipoproteins present in the circulation (351). CoQ₁₀H₂ has been shown to be preferentially utilised and more efficient in preventing LDL-oxidation than the other lipid soluble antioxidants in the blood, lycopene, b-carotene and alpha-tocopherol (355). CoQ₁₀H₂ can also protect against oxidative damage to other important cellular macromolecules such as proteins (356) and DNA (357). CoQ₁₀H₂ is also capable of recycling and regenerating other antioxidants such as alpha-tocopherol and ascorbate to their active form (333, 335, 358).

An important property contributing to the antioxidant efficiency of CoQ₁₀ is the large reducing capacity of the cell which is able to regenerate CoQ₁₀ by reduction at all locations of the cell (351). In mitochondria, the ubisemiquinone radical is formed during respiration which is effectively reduced to CoQ₁₀H₂ by the mitochondrial Q cycle (336). In the plasma and endomembranes, NADH or NADPH reductase enzymes reduce any CoQ₁₀ quinone radical generated by reaction with lipid or oxygen radicals (335).

Supplementation with exogenous CoQ₁₀ has been shown to lead to an increase in the CoQ₁₀H₂ content in plasma and in all of its lipoproteins, and a decrease in the peroxidisability of LDL (359, 360). Raitakari *et al.* (2000) also observed a decrease in the *ex-vivo* LDL oxidisability following CoQ₁₀ administration in patients with hypercholesterolaemia (361).

Conversely, Kaikonen *et al.* (1997) (48) reported that two months of supplementation CoQ₁₀, failed to increase the oxidative resistance of the VLDL + LDL-cholesterol fractions, suggesting the measurement techniques used to determine oxidative resistance of the VLDL + LDL-cholesterol fraction may have lacked sensitivity (362).

CoQ₁₀ may improve endothelial function and cardiovascular risk by reducing oxidative stress through recoupling eNOS or oxidative phosphorylation (12, 363) (Figure 1.8). *In vitro* and animal studies have provided some mechanistic insights into the role of CoQ₁₀ on ameliorating endothelial function, through preventing and attenuating oxidative stress (364, 365). Yokoyama *et al.* (1996) showed that via a direct antioxidant effect, CoQ₁₀ preserved endothelium-dependent vasorelaxation by improving tolerance to ischaemia reperfusion injury in a rat model (366). CoQ₁₀ has been shown to have a direct anti-atherogenic effect, which has been demonstrated in apolipoprotein E-deficient mice fed with a high fat diet (367). In this model, CoQ₁₀ supplementation decreased the absolute concentration of lipid hydroperoxides in atherosclerotic lesions and minimised the size of atherosclerotic lesions in the whole aorta (367).

There is however, conflicting data on the effects of CoQ₁₀ supplementation on oxidative stress from clinical studies. Tiano *et al.* (2007) reported an improvement in the activity of endothelium-bound extracellular superoxide dismutase (ecSOD) in patients with coronary artery disease, which correlated with the improvement in FMD, indicating that the endothelium-ameliorating effects of CoQ₁₀ are related to reductions in local vascular oxidative stress (368). Other studies however, have failed to show any significant changes in markers of oxidative stress, including F2-isoprostane (369-371) and 24-hr urinary 20-hydroxyeicosatetraenoic acid (HETE) levels (370, 371), or serum superoxide dismutase (370) with CoQ₁₀ therapy, despite significant enhancement of endothelial function. It is possible that CoQ₁₀ may have indirect antioxidant effects on NO regulation by reducing other vasoactive mediators, such as endothelin-1 (372), and the eNOS inhibitor, ADMA (373), and further investigation is required.

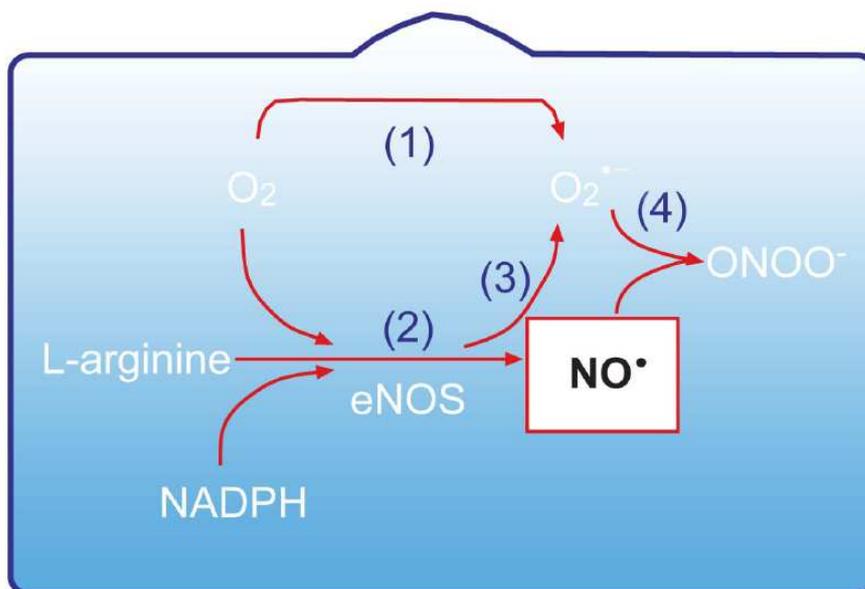


Figure 1.8 Potential antioxidant actions of CoQ₁₀ on the vasculature. CoQ₁₀ may: (1) inhibit mitochondrial production of superoxide (O₂^{•-}); (2) re-couple eNOS activity by maintaining cellular NADPH concentrations; (3) further decrease O₂^{•-} production and; (4) decrease the oxidative catabolism of NO• to peroxynitrite (ONOO⁻). The net consequence would be maintenance of endothelial production of NO• and enhancement bioactivity that could result in improved endothelial dysfunction and blood pressure. Adapted from Hodgson *et al.* (2003) (363)

1.3.3.3.1.3 Gene regulation and expression

Recent studies have revealed that CoQ₁₀ may have a prominent role in modulating the expression of multiple genes involved in cell signalling, intermediary metabolism, transport and transcription control, and inflammation (374-378). Groneberg *et al.* (2005) demonstrated the ability of CoQ₁₀ to target the expression of many genes, particularly in relation to cell signalling, metabolism and nutrient transport (374). Schmelzer and colleagues showed that CoQ₁₀ exerts anti-inflammatory properties via alteration of gene expression (375, 376). Lianne *et al.* (2002) observed significant changes in the expression of 115 genes, including those involved in cell signalling and other metabolic pathways following 25-30 days of CoQ₁₀ supplementation in patients prior to hip surgery (378). Gene regulation and control of metabolic influx may explain the pleiotropic effects of CoQ₁₀ (379, 380). With respect to cardiovascular disease, ongoing gene expression studies will allow elucidation of specific mechanisms by which CoQ₁₀ elicits its beneficial actions on the vasculature.

1.3.3.3.2 Biosynthesis of CoQ₁₀

CoQ₁₀ is synthesised *de novo* by every cell in the body via stepwise addition of isoprenoid units to generate a decaprenyl unit, which is then attached to a quinoid moiety (4-hydroxybenzoate) that has been derived from tyrosine or phenylalanine (Figure 1.7). Decaprenyl diphosphate is synthesised by COQ1 (PDSSI and PDSS2) from the mevalonate pathway via farnesyl diphosphate and geranylgeranyl diphosphate and is attached to para-hydroxybenzoate by COQ2. After condensation, at least six more enzymes (COQ3-COQ8) catalyse methylation, decarboxylation, and hydroxylation reactions to synthesise CoQ₁₀ (381, 382). More recently, another enzyme, COQ9, was identified that is required for the biosynthesis of CoQ₁₀ (383).

1.3.3.3.3 CoQ₁₀: dietary intake, absorption and metabolism

CoQ₁₀ is also obtained from the diet; with meat products being the largest dietary source (384). The dietary contribution is, however, minimal with an average daily CoQ₁₀ intake estimated to be 3–5 mg/day, which has only a marginal effect on plasma CoQ₁₀ concentrations (385). The pathway of dietary CoQ₁₀ absorption, transport and elimination is shown in Figure 1.9. Being lipophilic, the absorption of CoQ₁₀ is thought to follow the same process as that of lipids and vitamin E in the gastrointestinal tract (337), which is thought to be a complex process dependent on active transport mechanisms (386). Absorption of dietary CoQ₁₀ is enhanced in the presence of lipids (337). CoQ₁₀ is absorbed at a constant (zero-order) rate in the gastrointestinal tract with an absorption time of 6.2 hours per 100 mg (387). Following absorption, CoQ₁₀ is incorporated into chylomicrons and transported via the lymphatics to the circulation (388). Chylomicrons are converted to chylomicron remnants in the circulation by lipoprotein lipase and then taken up rapidly by the liver, where CoQ₁₀ is repackaged mostly into VLDL/LDL particles and released into the circulation (387, 389-391). Data indicate that CoQ₁₀ is reduced to CoQ₁₀H₂ either during or following absorption in the intestine (392). A study by Laaksonen *et al.*, (1995) (393) reported that the LDL fraction was the major vehicle for endogenous or supplemented CoQ₁₀H₂. In that study, CoQ₁₀ was not detected in lipoprotein-deficient serum either before or during the supplementation periods, indicating that all endogenous and exogenous CoQ₁₀ is carried in circulating lipoproteins (193). CoQ₁₀ is metabolised in all cells in the body, and the major route of elimination is via biliary and faecal excretion (394), with small amounts excreted in the urine (395).

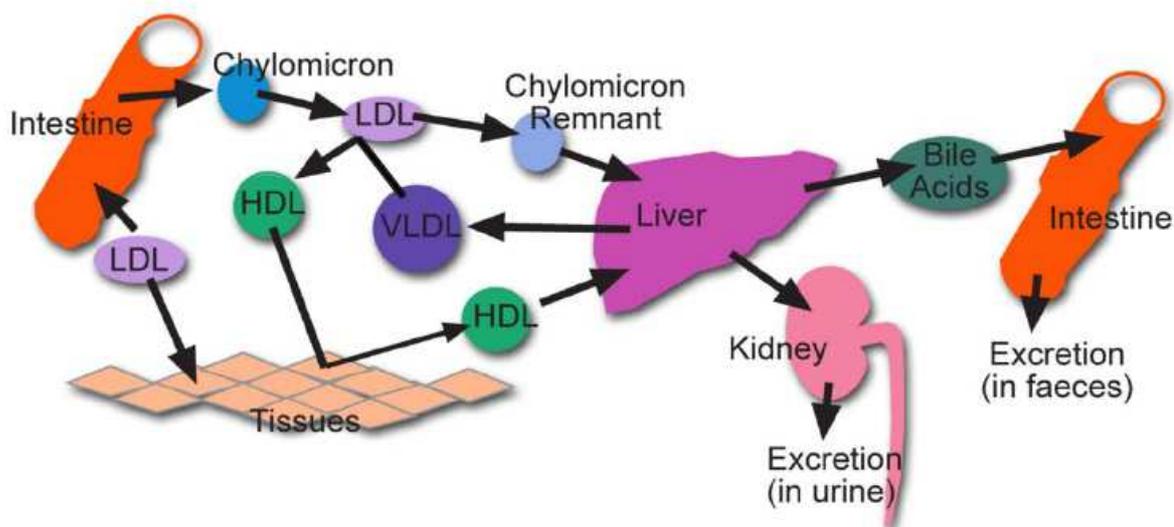


Figure 1.9. CoQ₁₀: dietary absorption, transport and elimination. VLDL; very low-density lipoproteins, LDL; low density lipoproteins, HDL; high-density lipoprotein
Adapted from Molyneux *et al.* 2006 (396)

1.3.3.3.4 Exogenous CoQ₁₀ supplementation

CoQ₁₀ is available as an over-the-counter supplement, and is indicated for the treatment of clinical disorders in some countries, including Japan where it is approved for use in CHF (397). Although CoQ₁₀ plasma concentrations between 0.22–2.31 $\mu\text{mol/L}$ have been reported in healthy people (331) (385, 398-402), therapeutic CoQ₁₀ concentrations in cardiovascular disease are considered to be 2-4 fold higher than normal levels (350).

1.3.3.3.4.1 Pharmacokinetics

An increase in plasma concentrations of CoQ₁₀ after oral supplementation has been well documented, with the extent of absorption depending on the dosage, duration and type of formulation (337). The time (T_{max}) of peak plasma concentration usually occurs six to eight hours after oral administration (387, 403-405). The T_{max} indicates that intestinal absorption of CoQ₁₀ is relatively slow, attributable to its high molecular weight and hydrophobicity (337, 404). The distribution phase occurs during the 6-12 hour period following C_{max} (406). A second peak CoQ₁₀ plasma has also been described at approximately 24 hours after oral administration (387, 403, 404, 407), which may reflect both enterohepatic recycling and redistribution from the liver to circulation (337, 406). Tomono *et al.* (1986) used deuterium-labelled crystalline CoQ₁₀ to investigate pharmacokinetics in humans and established that CoQ₁₀ has a prolonged elimination half life of approximately 33 hours (387). Similar results

have been reported from other single dose studies, where approximately five to six days were required for plasma CoQ₁₀ levels to return to baseline (404, 407). A four-week multiple-dose study demonstrated that two weeks after discontinuation of CoQ₁₀ at 90mg/day or 300mg/day, plasma levels were still 30% and 74%, respectively, above baseline levels (405). This is an important consideration when designing “washout” periods between therapeutic interventions in clinical studies (406). Tomonon *et al.* (1986) provided evidence for zero order absorption of CoQ₁₀ in healthy volunteers (387). Further evidence of non-linear CoQ₁₀ absorption has also been demonstrated by dose-ranging studies (405, 408) and multiple-dose studies (409, 410), where CoQ₁₀ concentrations are proportionally less as the dosage is increased. Upon supplementation, a dose-dependent increase in plasma CoQ₁₀ level has been observed up to a daily dose of 200 mg, which resulted in a 6.1-fold increase in plasma CoQ₁₀ levels (399). Steady-state concentrations are generally achieved by approximately two to four weeks after the initiation of CoQ₁₀ administration (405, 411).

1.3.3.3.4.2 Tissue uptake

The effects of exogenous CoQ₁₀ supplementation on tissue uptake have been mainly studied in animal models. Supplementation increases CoQ₁₀ concentrations in the tissues of both mice and rats, including spleen (412), liver (334, 413) and kidney (413). Increased concentrations in the brain seem to be dose-dependent (413, 414). More importantly, studies in animal models have reported increased CoQ₁₀ concentrations in the mitochondria of heart (414) and skeletal muscle (413, 414), liver (412, 414), spleen (412), and kidney (413). However, many of these studies using animal models are difficult to extrapolate to humans since impractically high doses of CoQ₁₀ are often administered. It has been suggested that uptake of CoQ₁₀ by tissues such as heart, skeletal muscle and brain is low or negligible unless the endogenous levels fall below a crucial physiological threshold (334, 394, 415).

1.3.3.3.4.3 Bioavailability

Intestinal absorption of orally administered CoQ₁₀ is relatively poor in humans due to its high molecular weight and lipophilic nature (404), and is enhanced if taken with a meal (407). Because of its insolubility in water a variety of CoQ₁₀ preparations have been developed to solubilise CoQ₁₀ and enhance its absorption. CoQ₁₀ formulations differ widely, with respect to the form of CoQ₁₀ (reduced or oxidised), the excipients, the type of encapsulation, and the presence of surfactants and emulsifiers, such as lecithin and polysorbate 80 to promote absorption. There is a significant difference in bioavailability of the various brands and

formulations of CoQ₁₀ supplements (362, 404, 407, 411, 416-418). Evidence suggests that CoQ₁₀ is better absorbed from nanoparticulated and solubilised preparations of CoQ₁₀ than oil emulsions of CoQ₁₀ or powder filled preparations (404, 411, 416, 419-421), and the reduced form is more bioavailable than the oxidised form, since it is more hydrophilic (404, 405, 417). The majority of bioavailability studies have utilised delivery systems that target plasma CoQ₁₀ levels rather than the mitochondrion, however new analogues have been developed with a view to enhanced mitochondrial uptake, including mitoquinone-Q (Mito-Q) (422). Moreover, there is large inter-individual variability in absorption of CoQ₁₀ from supplements, and this variability has been reported for many different formulations of CoQ₁₀ (362, 407, 416-418, 423). This highlights the need to measure plasma CoQ₁₀ concentrations during supplementation in order to monitor efficacy.

1.3.3.3.4.4 Tolerability and safety

CoQ₁₀ is well tolerated and has been shown to have a good safety profile (409, 410, 424-433). On average, mild gastrointestinal discomfort has been reported in less than 1% of patients in clinical trials (434). Most trials have not described significant adverse effects that necessitated cessation of therapy, and to date, no serious adverse side effects have been reported (433). CoQ₁₀ dosage guidelines were recently recommended for adults (up to 1200 mg/day) (432), and for children (up to 10mg/kg/day) (435). Toxicity is not usually observed with high doses of CoQ₁₀. In a recent study, a daily dosage up to 3600 mg was found to be tolerated by healthy as well as unhealthy individuals (436). Potential interactions with antiplatelet drugs, including aspirin or clopidogrel may lead to an increase in the risk of bleeding (437) and with warfarin may potentiate its anticoagulant effect (438). The long-term effects of CoQ₁₀ on tolerability and safety require elucidation.

1.3.3.3.5 Measurement of CoQ₁₀

High performance liquid chromatography (HPLC) assays for the measurement of plasma (331, 439) and tissue CoQ₁₀ concentrations (440) have been developed and validated. The relationship between plasma and tissue CoQ₁₀ levels, however is not yet established, and plasma levels should only be regarded as a surrogate for tissue (441), and particularly mitochondrial levels, where any therapeutic effect of CoQ₁₀ may be expected to be most important. A major limitation of measuring tissue concentrations is access to tissue samples, given the invasive nature of sampling. The adult reference interval for plasma or serum CoQ₁₀ has been well established (0.5–1.7 µmol/L) (399, 400, 442, 443). Since plasma CoQ₁₀ and

lipid concentrations correlate strongly, it has been proposed that lipids should be considered when measuring plasma CoQ₁₀ (444, 445), and the ratio of CoQ₁₀ to total- or LDL-cholesterol reported. There is limited data on reference intervals for CoQ₁₀ concentrations in skeletal muscle (446).

1.3.3.3.6 Factors affecting CoQ₁₀ concentrations

1.3.3.3.6.1 Genetic factors

CoQ₁₀ deficiency is associated with four major clinical phenotypes: 1) an encephalomyopathic form, initially reported by Ogasahara in 1989, characterised by mitochondrial myopathy, recurrent myoglobinuria and central nervous system signs, associated with decrease of complex I + III and II + III activity and CoQ₁₀ in muscle (447-449); 2) a pure myopathic form, with lipid storage myopathy and respiratory chain dysfunction (450, 451); 3) a cerebellar form, with cerebellar ataxia and atrophy associated with other manifestations that can include neuropathy, seizures, mental retardation and muscle weakness (452, 453); and 4) a multi-systemic infantile form (454, 455). In most of these phenotypes, family history has indicated an autosomal recessive mode of inheritance (381). Primary CoQ₁₀ deficiencies, due to the presence of mutations in the CoQ₁₀ biosynthetic genes, have been documented in patients with infantile-onset diseases (456) and cerebellar ataxia (455, 457-460). A recent study by Quinzii *et al.* (2008) showed that respiratory chain dysfunction and oxidative stress correlate with the severity of primary CoQ₁₀ deficiency (461). Exogenous CoQ₁₀ supplementation has contributed to significant improvements in the clinical status of some patients with primary and secondary CoQ₁₀ deficiencies (447, 448, 454, 455, 460, 462-464).

1.3.3.3.6.2 Age

Plasma CoQ₁₀ levels have been reported to decline with age, although findings have been inconsistent. Niklowitz *et al.* (2004) (360) found a negative association between the year of life of an infant and plasma CoQ₁₀ concentration after adjustment for lipids. Miles *et al.* (2004) (465) reported significant age-related decreases in the CoQ₁₀ redox ratio after 18 years of age that may be related to the early effects of oxidative stress. In contrast, some studies have demonstrated a positive correlation between CoQ₁₀ and age, which disappears when total cholesterol is included in multivariate analysis (442, 466), or conversely a lack of correlation between CoQ₁₀ and age (465, 467). Reductions in CoQ₁₀ levels with advancing age have been

demonstrated in human tissues, including the brain at 90 years of age (468). Decreasing tissue CoQ₁₀ concentrations with aging may reflect reduced CoQ₁₀ synthesis and/or age-dependent increases in lipid peroxidation (469). It is also possible that reductions in CoQ₁₀ levels with aging may represent a general adaptation to a decreased metabolic rate in older subjects (470).

1.3.3.3.6.3 Gender

Whether a gender difference exists in CoQ₁₀ levels is controversial, with variable findings. Significant differences in CoQ₁₀ with respect to gender have been documented in adults (385, 442, 467), with males having approximately 22.9% higher total CoQ₁₀ levels, or 13.3% higher cholesterol-adjusted CoQ₁₀ levels compared to females (385). Conversely, other studies have reported a lack of difference between genders with regard to plasma CoQ₁₀ concentrations (395, 400, 418). Although Molyneux *et al.* (2005) observed a significant gender difference in both total CoQ₁₀ and the CoQ₁₀ to total cholesterol ratio, the authors found no basis for stratification of the total CoQ₁₀ reference interval for gender according to the Harris and Boyd criteria (442, 471).

1.3.3.3.6.4 Body mass index and plasma lipids

Total CoQ₁₀ levels have been positively correlated with body mass index (BMI) (385, 423), but this correlation is abolished when levels are adjusted for cholesterol (466), and likely reflects the positive association between BMI and total cholesterol (472). Plasma CoQ₁₀ is positively associated with plasma total cholesterol (385, 444, 445, 466, 473), and plasma triglycerides (385, 466, 474), which is anticipated due to the hydrophobicity of the CoQ₁₀ molecule. The correlation between plasma CoQ₁₀ and triglycerides is however, eliminated after adjustment of CoQ₁₀ concentration for total cholesterol (472).

1.3.3.3.6.5 Statin-induced CoQ₁₀ depletion

There is substantial evidence that plasma CoQ₁₀ concentrations are reduced by HMG-CoA reductase inhibitors. This is not surprising, since both CoQ₁₀ and cholesterol are synthesised by the mevalonate pathway (Figure 1.4). Numerous studies have demonstrated reductions of up to 54% in plasma or serum CoQ₁₀ concentrations following statin therapy (186-203). The magnitude of the statin-induced depletion of CoQ₁₀ levels has been shown to be dose related (188, 196), and is reversible on cessation of statin treatment (191). It is possible the reduction in circulating CoQ₁₀ may reflect decreased LDL-cholesterol concentrations. However, several studies have reported a lower CoQ₁₀ to LDL-cholesterol

ratio after statin treatment (188, 194, 197), suggesting CoQ₁₀ depletion may not only be due to a reduction in LDL-cholesterol carriers, although this has not been consistently shown in all trials (191-193).

Circulating concentrations of CoQ₁₀ do not necessarily reflect tissue CoQ₁₀ concentrations, and clinical studies evaluating the effect of statin treatment on skeletal muscle CoQ₁₀ levels are contradictory. In an early trial, four weeks of simvastatin (20 mg/day) produced a 47% increase in muscle CoQ₁₀ concentrations (193), and six months of treatment with 20 mg/day of simvastatin gave a similar result (192). One trial comparing the effect of eight weeks of treatment with simvastatin 80 mg/day, atorvastatin 40 mg/day, or placebo on muscle CoQ₁₀ levels, reported a 34% reduction in those treated with simvastatin (475). However, in a more recent study there was no significant difference in the mean intramuscular CoQ₁₀ concentration in patients with statin-related myopathy compared to statin-treated controls (476). Supplementation with oral CoQ₁₀ can restore plasma CoQ₁₀ levels in patients receiving statin therapy (186, 477, 478). The long-term effect of statin-induced plasma CoQ₁₀ decreases, especially considering the increasingly popular intensive lipid lowering via statins, is not yet clear, and should be monitored.

1.3.3.3.6.6 Disease

CoQ₁₀ deficiency has been implicated in a number of clinical disorders, including but not limited to type 2 diabetes (479), hypertension (480), ischaemic heart disease (481), and CHF (441), and the potential role for CoQ₁₀ supplementation in these cardiovascular disease states is reviewed in section 1.3.3.3.7

1.3.3.3.7 Clinical aspects of CoQ₁₀ in cardiovascular disease

The rationale for the use of CoQ₁₀ as a therapeutic agent in cardiovascular related disorders and statin-induced myopathy is based upon its fundamental role in mitochondrial function and cellular bioenergetics and/or its antioxidant properties. The following sections describe the available data to support the therapeutic value of CoQ₁₀ as an adjunct to standard clinical therapy in patients with metabolic dysregulation and cardiovascular disease.

1.3.3.3.7.1 CoQ₁₀ and type 2 diabetes and the metabolic syndrome

A growing body of evidence indicates that oxidative stress plays a critical role in the pathogenesis of type 2 diabetes mellitus and its complications (479). CoQ₁₀ deficiency in type 2 diabetes results from impaired mitochondrial substrate metabolism (482), and increased oxidative stress (479). In diabetes, CoQ₁₀ deficiency is thought to contribute to endothelial dysfunction, and may also be linked to impaired beta-cell function and the development of insulin resistance (12). Low plasma CoQ₁₀ concentrations have been negatively correlated with poor glycaemic control and diabetic complications (483). Given the important function of CoQ₁₀ in mitochondrial bioenergetics, and its antioxidant effects, oral supplementation represents a potentially attractive therapy in type 2 diabetes mellitus and the metabolic syndrome. Tsuneki *et al.* (2007) (365) demonstrated that CoQ₁₀ can prevent oxidative stress, apoptotic cell death and alteration of cell-adhesion molecules in cultured endothelial cells under high glucose concentrations. The administration of CoQ₁₀ significantly attenuated the increase of oxidative and nitrative stress markers and inflammatory markers in a dose-dependent manner in a rat model of the metabolic syndrome (364). CoQ₁₀ was also associated with improved endothelial function in the mesenteric arteries, in addition to prevention of elevations in serum insulin levels, and reductions in blood pressure, suggesting that the antioxidant properties of CoQ₁₀ may be effective for ameliorating cardiovascular risk in patients with metabolic syndrome (364). A number of clinical trials have shown that CoQ₁₀ can improve glycaemic control (484, 485), and lower plasma insulin (484); although these findings are inconsistent with other studies. In addition, several trials have demonstrated a significant blood pressure lowering effect of CoQ₁₀ in patients with type 2 diabetes mellitus (484-486). Furthermore, improvement in endothelial function of conduit arteries following 12 weeks of oral CoQ₁₀ therapy in patients with type 2 diabetes has been reported (369, 371). Conversely, two further trials in type 2 diabetic patients failed to show any improvement in microcirculatory function with CoQ₁₀ monotherapy (487, 488). Playford *et al.* (2003) (487) did, however, observe a significant increase in endothelium-dependent microcirculatory vasodilation in type 2 diabetes with combined CoQ₁₀ and fenofibrate therapy. CoQ₁₀ supplementation may also enhance the ability of other anti-atherogenic agents such as statins (12). Further studies, including clinical outcome trials are required to confirm whether there is a role for CoQ₁₀ as an adjunct to treatment for patients with type 2 diabetes mellitus and the metabolic syndrome.

1.3.3.3.7.2 CoQ₁₀ and hypertension

CoQ₁₀ deficiency has been demonstrated in patients with essential hypertension (480). A number of clinical trials have provided clinical evidence of a potential antihypertensive effect of CoQ₁₀ in hypertensive patients (480, 484, 489-498). A meta-analysis by Rosenfeldt *et al.* (2007) investigated the efficacy of CoQ₁₀ in the treatment of hypertension (12 clinical trials, 362 patients) and concluded that, in hypertensive patients, CoQ₁₀ has the potential to lower systolic and diastolic blood pressure, without significant side effects (425). A blood pressure lowering effect of CoQ₁₀ was found across three types of studies including randomised controlled, cross-over, and open label, with reductions in systolic blood pressure from 11 to 17 mmHg and in diastolic blood pressure from 8 to 10 mmHg (425). The mechanism for the hypotensive action of CoQ₁₀ is inconclusive, but may be attributed to an ability to induce vasodilation, via a direct effect on the endothelium and underlying smooth muscle (363, 379, 425). More recently, a Cochrane review addressed the evidence for CoQ₁₀ efficacy in the treatment of essential hypertension in three randomised double-blind placebo-controlled studies with a total of 96 participants (499). Although this systematic review found that CoQ₁₀ lowered blood pressure by 11/7 mmHg, the reviewers concluded that larger properly conducted randomised controlled trials are required (499).

1.3.3.3.7.3 CoQ₁₀ and statin myopathy

The underlying pathophysiology of statin-induced myopathy is unknown, but one postulated mechanism is mitochondrial dysfunction through depletion of CoQ₁₀ (119), since CoQ₁₀ is an essential cofactor in the mitochondrial electron transport chain (335) and required for normal skeletal muscle function (500, 501). Post-marketing studies have indicated up to 15% of statin treated patients experience some degree of myopathy (128-130), and as targets for cholesterol reduction become progressively lower, necessitating higher statin doses, the risk of side effects, particularly myopathies, has increased (72, 116, 117). A small number of studies have provided some evidence of impaired mitochondrial function in statin-induced myopathy (151, 194, 204). Philips *et al.* (2002) reported findings consistent with mitochondrial dysfunction in muscle biopsies of four patients with statin-related myopathy but without creatine kinase elevations (204). These abnormalities resolved following discontinuation of statin therapy in the three patients who had repeat biopsies (204). There is however, limited evidence linking mitochondrial dysfunction to reduced muscle CoQ₁₀ concentrations in patients with statin-induced myopathy (475, 476). With regard to clinical

trials, Caso *et al.* (2007) reported a 40% reduction in myopathic pain ($P < 0.001$) after 30 days of 100 mg/day of CoQ₁₀ supplementation compared with no change following 400 IU/day of vitamin E in patients with statin-related myopathy on concurrent statin treatment (502). This trial however, lacked a placebo-control design and patients were not on a standardised dose or type of statin. Further studies are required to confirm whether CoQ₁₀ supplementation can improve tolerance to statin therapy.

1.3.3.3.7.4 CoQ₁₀ and chronic heart failure

Given the importance of CoQ₁₀ in mitochondrial electron transport and ATP synthesis, its depletion has been postulated to compromise myocardial energy generation and lead to “energy starvation” of the myocardium, considered to be a pathogenic mechanism of CHF (503). Myocardial depletion of CoQ₁₀ has been demonstrated in CHF and the severity of the deficiency has been found to correlate with the severity of symptoms, with patients in NYHA class IV having significantly lower CoQ₁₀ in endomyocardial biopsy samples than those in NYHA class I (441). This myocardial CoQ₁₀ deficiency in patients with cardiomyopathy was also reversed by CoQ₁₀ therapy (441). Molyneux *et al.* (2008) (504) showed that CoQ₁₀ levels were an independent predictor of total mortality in an observational study of 236 subjects with heart failure for whom the mean follow-up time was 2.7 years. The strength of the association between CoQ₁₀ and mortality was greater than observed for N-terminus pro-B type natriuretic peptide (BNP) (504). More recently, a pre-specified substudy of CORONA (Controlled Rosuvastatin Multinational Study in Heart Failure) examined plasma CoQ₁₀ levels in 1,1,91 patients with ischaemic systolic heart failure in relation to clinical outcomes (505). Although lower CoQ₁₀ was associated with a higher risk of death in unadjusted analyses, CoQ₁₀ was not an independent predictor of mortality in multivariate analysis. Compared to the study by Molyneux and colleagues (504), the CORONA study included many more patients and events, and thus had the ability to adjust for more covariates and provide more precise estimates of the independent relationship between baseline variables and outcomes (506). The findings from the CORONA study suggest that while low CoQ₁₀ appears to be a marker of more advanced disease, it is unlikely to be a clinically important prognostic biomarker in heart failure (505).

Meta-analyses of CoQ₁₀ supplementation in CHF have been undertaken (507, 508). Soja *et al.* (1997) reviewed eight double-blind placebo-controlled studies and reported a significant improvement in stroke volume, ejection fraction, cardiac output, cardiac index and end

diastolic volume index, as a consequence of CoQ₁₀ supplementation (509-516). In a more recent meta-analysis, Sander *et al.* (2006) reviewed eleven studies (508), ten that evaluated ejection fraction (509, 511-513, 516-521) and two that evaluated cardiac output (515, 517) with CoQ₁₀ doses ranging from 60-200 mg/day and treatment periods ranging from 1-6 months. Overall, a 3.7% (95%CI 1.59-5.77) net improvement in the ejection fraction was found, and cardiac output was increased on average of 0.28 L/minute (95%CI 0.03-0.53) (508). Recent randomised controlled trials have also shown significant clinical benefits in patients with NYHA classes II and III CHF (522, 523). Dai *et al.* (2011) (370) reported significant improvements in endothelial function in patients with ischaemic left ventricular systolic dysfunction following eight weeks of CoQ₁₀ administration. In this study, improved FMD was positively correlated with a reduction in the lactate/pyruvate ratio, suggesting that CoQ₁₀ ameliorates endothelial function via reversal of mitochondrial dysfunction in patients with ischaemic left ventricular systolic dysfunction (370). An international, randomised, double-blind multi-centre intervention study, “Q-SYMBIO” has been initiated with CoQ₁₀ supplementation in CHF patients and focuses on symptoms, biomarker status (BNP) and long-term outcomes (503). Coupled with the findings of the meta-analyses (507, 508), a positive result in Q-SYMBIO may be expected to increase the acceptance of CoQ₁₀ as an adjunctive therapy in addition to current medical strategies in CHF.

1.3.3.3.7.5 CoQ₁₀ and ischaemic heart disease

A deficiency of plasma CoQ₁₀H₂ and total CoQ₁₀ has been observed in patients with ischaemic heart disease (481, 524, 525). Randomised controlled trials in patients with ischaemic heart disease were initiated in the mid-1980s, with the first study reported by Hiasa *et al.* (1984) (526), in which 18 patients were randomised to either treatment with intravenous CoQ₁₀ or placebo. Protection against myocardial ischaemia was demonstrated by improved exercise tolerance, a reduction in ischaemic changes on ECG, and a reduction in angina, in treated patients compared to placebo (526). Further randomised, placebo-controlled trials in patients with ischaemic heart disease showed similar benefits with oral CoQ₁₀ therapy (527-529). Kumar *et al.* (2005) (530) revealed that adjunctive CoQ₁₀ supplementation in patients with acute coronary syndrome produced symptomatic improvement in angina scores, decreased the development of left ventricular dysfunction, and decreased the need for revascularisation by percutaneous transluminal angioplasty or coronary artery bypass, and reduced mortality at six months follow-up. These beneficial effects may be related to a direct

effect on myocardial metabolism, as has been demonstrated in patients with ischaemic heart disease following treatment with CoQ₁₀ (531).

The role of reactive oxygen species is now well established in ischaemia and reperfusion injury, making CoQ₁₀ an attractive therapy in this setting. There is increasing evidence to suggest that the beneficial effects of CoQ₁₀ supplementation in myocardial ischaemia may also be related to its antioxidant properties. Under conditions of high oxidative stress, such as coronary artery disease, the rate of inactivation of NO to peroxynitrite by superoxide anions may be reduced by CoQ₁₀ (532). Tiano *et al.* (2007) (368) reported an improvement in the activity of endothelium-bound extracellular superoxide dismutase (ecSOD) in subjects with coronary artery disease following four weeks of CoQ₁₀ therapy in a randomised double-blind, placebo controlled study. Patients with lower levels of extracellular superoxide dismutase (ecSOD), a major antioxidant system of the vessel wall, demonstrated greater improvements than patients with normal ecSOD levels (368). Furthermore, the increase in ecSOD activity was significantly correlated with the improvement in FMD of the brachial artery, suggesting that the endothelium-enhancing effects of CoQ₁₀ are related to improvements in local vascular oxidative stress (368). CoQ₁₀ treatment was also associated with enhancement of peak VO₂ in these patients, which is likely to be due to the bioenergetic effect of CoQ₁₀ (368). CoQ₁₀ may also influence vascular function indirectly via inhibition of oxidative damage to LDL (355, 361). With regards to acute myocardial infarction, a trial in 144 patients reported a significant reduction in the total number of cardiovascular events in those receiving CoQ₁₀ compared to placebo (533). In another randomised, double-blind controlled trial of 71 patients, treatment with CoQ₁₀ for 12 months resulted in significant reductions in total cardiovascular events, non fatal cardiovascular events and cardiovascular mortality in the intervention compared to the placebo group (534). Further clinical endpoint trials are needed to confirm the benefits of CoQ₁₀ on cardiovascular risk in patients with ischaemic heart disease.

1.4 Residual cardiovascular risk

Despite the recent advances in therapeutic strategies for controlling risk factors that contribute to coronary artery disease, stroke and hypertension, mortality from cardiovascular disease continues to increase worldwide. The increasing prevalence of obesity, which is contributing to an epidemic of type 2 diabetes mellitus and its subsequent increased risk of

cardiovascular diseases, is a particularly pertinent issue. It is estimated that by 2020, cardiovascular disease will surpass infectious and communicable disease as a cause of loss of productive life years worldwide (535).

Even with current standards of care, including achievement of LDL-cholesterol goals and intensive control of blood pressure and glucose, the risk of cardiovascular events still persists in the majority of patients (536). Reducing LDL-cholesterol with statin therapy has been clearly shown to reduce major cardiovascular events by 25% to 35%; however there remains a considerable residual risk of cardiovascular disease, with approximately two-thirds of all cardiovascular events still occurring on statin treatment (57, 59, 65-68) (Figure 1.10). The 10-year risk in some patients with known CHD has been shown to be over 20% despite statin treatment (65-67). Even in primary prevention trials, individuals have exhibited an 11% to 13% 10-year risk on statin therapy (57, 59). Furthermore, a high level of residual cardiovascular risk has also been observed in patients who were treated with intensive high-dose statin therapy, where LDL-cholesterol levels were lowered beyond current targets (72, 537, 538). Although cardiovascular risk remains in all statin-treated subjects, residual risk is particularly high in patients with type 2 diabetes on statin therapy. Major statin trials have demonstrated that the absolute risk for cardiovascular events is still greater in patients with diabetes (9.6% to 33.4%) than the risk for untreated non-diabetic patients (8.7% to 25.7%) (61, 66, 67, 75, 134, 539). Thus, statins do not eliminate the increased cardiovascular risk associated with type 2 diabetes. Post-hoc analyses have also demonstrated residual risk of coronary artery disease in statin-treated patients with the metabolic syndrome (540-542).

Other modalities for treating dyslipidaemia, such as fibrate therapy are also associated with significant reductions in cardiovascular event rates in both primary (543-545) and secondary prevention trials (544, 546, 547), yet a high residual cardiovascular risk still exists in these populations despite treatment. With respect to intensive glycaemic control, trials have also documented that the majority of cardiovascular events are not prevented. The Action in Diabetes and Vascular: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) trial study showed that although aggressive glycaemic control (HbA1c of 6.5% versus 7.3% with conventional control) lowered the incidence of combined microvascular and major macrovascular events by 10%, there was considerable absolute residual cardiovascular risk (548).

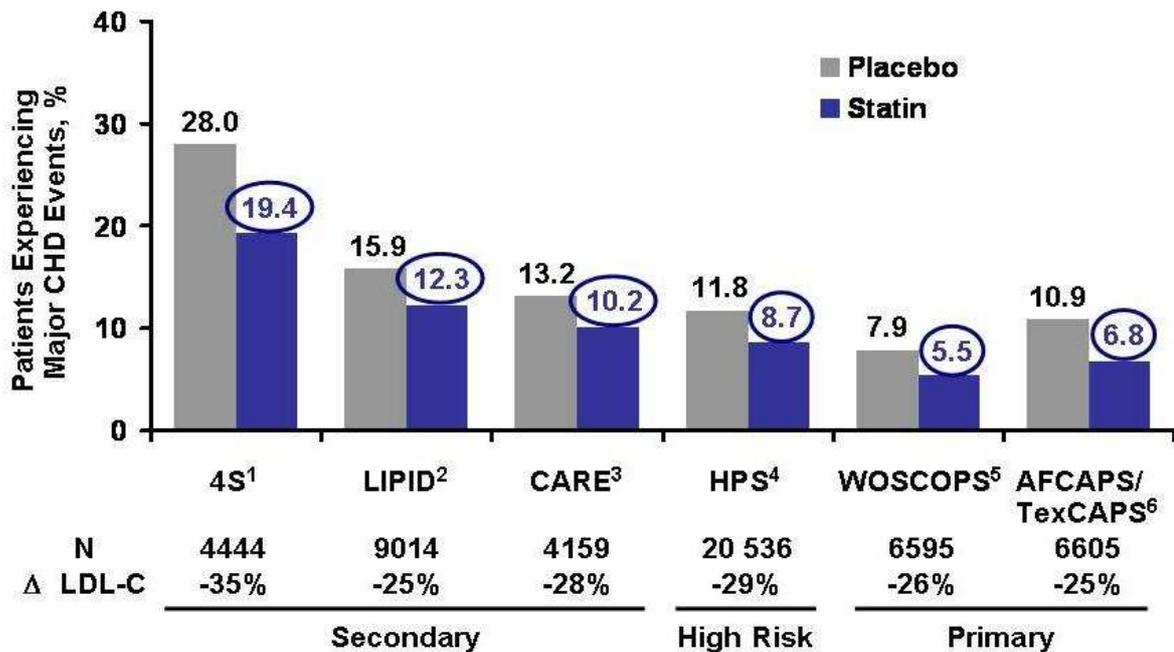


Figure 1.10 Percentage of patients experiencing major coronary events, according to several large statin outcome trials. 4S, Scandinavian Simvastatin Survival Study; HPS, Heart Protection Study; LIPID, Long-term Intervention with Pravastatin in Ischaemic Disease study; WOSCOPS, West of Scotland Coronary Prevention Study; AFCAPS/TexCAPS, Air Force Coronary Atherosclerosis Prevention Study/Texas Coronary Atherosclerosis Prevention Study. ¹4S Group. *Lancet*. 1994;334: 1383-1389. ²LIPID Study Group. *N Engl J Med*. 1998;339: 1349-1357. ³Sacks FM, et al. *N Engl J Med*. 1996;335: 1001-1009. ⁴HPS Collaborative Group. *Lancet*. 2002;360: 7-22. ⁵Shepherd J, et al. *N Engl J Med*. 1995;333: 1301-1307. ⁶Downs, JR, et al. *JAMA*. 1998;279: 1615-1622. From www.heart.org

There is clearly a need for renewed focus on effective interventions that are capable of reducing the residual risk of cardiovascular events in patients receiving optimal therapy in accordance with current standards of care (549). Lifestyle modifications together with new therapeutic strategies are urgently needed to reduce the global burden of cardiovascular disease. The Residual Risk Reduction Initiative was established in 2005, with a focus on multifactorial approaches to treat atherogenic dyslipidaemia in patients with established cardiovascular disease, type 2 diabetes mellitus and the metabolic syndrome (549). Ongoing clinical outcome trials with anti-glycaemic agents (550, 551), and combination therapy with statin and niacin (223), or ezetimibe (230) will help to address the potential of these agents to reduce residual risk of cardiovascular events. Several lipid modifying agents in development

may potentially reduce the residual cardiovascular risk. Such therapies that are currently under investigation include microsomal triglyceride transfer protein (MTP) inhibitors (552), antisense ApoB (553), lipoprotein-associated phospholipase A₂ (Lp-PLA₂ antagonists) (554), PCSK9 antisense and antibodies (555), and cholesterylester transfer protein (CETP) inhibitors (556). Aliskiren, a direct renin inhibitor, and dospirenone, a progestin with aldosterone receptor antagonist activity used in combination with 17beta-estradiol, represent new approaches in the treatment of hypertension, but have yet to be assessed in cardiovascular outcome trials (557, 558). Ongoing and future studies with clinical outcomes will clarify their efficacy in clinical practice.

Dietary supplementation with CoQ₁₀ may represent another therapeutic approach to modify cardiovascular risk, given its potent antioxidant properties and key role in mitochondrial bioenergetics (335). In addition, strategies are also required to improve the tolerability of statin therapy, given its established role in cardiovascular risk reduction, in order to enhance cardiovascular management in affected individuals. In view of the well documented pleiotropic effects of statins (93, 94, 96, 99-103), these agents may also confer cardiovascular benefit in patients with non ischaemic CHF and this requires evaluation.

1.5 Research hypotheses

The work described in the thesis was designed to explore further strategies for modifying endothelial dysfunction and cardiovascular risk, and is based on four main hypotheses:

1. CoQ₁₀ supplementation will improve cardiovascular risk status in high-risk patient populations by enhancing endothelial response, reducing arterial blood pressure and improving simvastatin tolerability.
2. Genetic susceptibility to underlying metabolic myopathies and altered statin pharmacokinetics are determinants of statin intolerance.
3. Patients with genotypic FH on long term statin therapy will have lower plasma CoQ₁₀ concentrations than patients with phenotypic FH and untreated controls, and CoQ₁₀ levels will be inversely correlated with vascular dysfunction.

4. Restoration of endothelial function with atorvastatin therapy in patients with CHF is mediated in part, through reductions in plasma levels of the potent eNOS inhibitor, ADMA.

1.6 Research objectives

As a result of these hypotheses, the primary objectives of the research described in this thesis were:

1. To investigate the effects of CoQ₁₀ supplementation on endothelial function and 24-hr blood pressure in patients with the metabolic syndrome.
2. To establish the effects of CoQ₁₀ on statin intolerance and explore genetic risk factors contributing to statin myopathy.
3. To examine the effects of high dose statins on arterial stiffness and CoQ₁₀ levels in patients with FH.
4. To elucidate potential mechanisms for statin-induced amelioration of endothelial dysfunction in patients with CHF.

Chapter three examines the effects of CoQ₁₀ treatment on endothelial function in simvastatin-treated patients with the metabolic syndrome in a randomised, double-blind, placebo controlled 12-week cross-over study.

Chapter four reports the results of a randomised, double-blind, placebo-controlled 12-week cross-over study designed to investigate the effects of adjunctive CoQ₁₀ therapy on 24-hr ambulatory blood pressure in patients with the metabolic syndrome and inadequately blood pressure control, despite use of conventional antihypertensive agents (559). Patients with the metabolic syndrome were chosen in both of these studies because they are likely to have increased oxidative stress and may therefore be more responsive to dietary CoQ₁₀ supplementation.

Chapter five addresses the effects of CoQ₁₀ on statin tolerance in patients with prior self reported statin-related myalgia in a randomised, double-blind, placebo-controlled 12-week study of CoQ₁₀ supplementation in combination with upward dose titration of simvastatin (560).

Chapter six reports the findings of a case-control study undertaken to confirm whether polymorphisms and mutations in candidate genes associated with underlying metabolic myopathies and alterations in statin pharmacokinetics predict an increased risk of statin intolerance. This study incorporated two independent cohorts of patients with a history of statin-induced myopathy and statin tolerant controls, and pooled data was reviewed in a meta-analysis.

Chapter seven describes a case-control study in statin-treated FH patients designed to establish whether plasma CoQ₁₀ concentrations are lower in LDL receptor (LDLR) mutation positive FH patients than LDLR mutation negative patients on long-term statin therapy, and whether low plasma CoQ₁₀ concentrations are a determinant of increased systemic arterial stiffness (561). Plasma levels of CoQ₁₀ and systemic arterial stiffness were also compared between treated FH subjects and untreated healthy controls with similar lipid levels.

Chapter eight examines the effect of six weeks of atorvastatin therapy on plasma ADMA levels in patients with stable, non-ischaemic CHF to establish whether reductions in eNOS inhibitor concentrations are one of the mechanistic pathways by which atorvastatin augments endothelial function (562).

2 Methodology

2.1 Measurement of endothelial function

There are a number of techniques to assess endothelial function *in vivo* such as iontophoresis and laser Doppler flowmetry (31, 32). This section will review the two most widely used techniques for endothelial function assessment: flow-mediated dilation (FMD) and invasive strain-gauge plethysmography, as these methods were incorporated in the studies presented in this thesis. FMD and strain-gauge plethysmography are the standard research tools to assess endothelial function (27, 28). Two ultrasound FMD methodologies were incorporated in this thesis in Chapters 3 and 8, respectively. The first protocol was setup in the Vascular Ultrasound Unit at Christchurch Hospital as part of the work described in the current thesis and will be described in detail in the sections below. The second protocol, established in conjunction with Dr Chris Strey, formed a significant component of his PhD thesis (563), and will thus only be outlined in brief in the following sections. The invasive strain-gauge plethysmography methodology was also established and validated at Christchurch Hospital as part of Dr Chris Strey's thesis (563) and will therefore be briefly summarised.

2.2 Flow-mediated dilation of the brachial artery

2.2.1 History and underlying principal

Brachial ultrasound is used to assess endothelial function in conduit arteries. In 1970, Rodbard (564) proposed that the endothelium may sense and respond to shear stress generated by flowing blood. In 1980, Furchgott and Zawadski (1980) discovered that acetylcholine-mediated vasodilation requires participation of endothelium (565). Rubanyi and colleagues (566) subsequently showed that in response to blood flow, the endothelium released a substance that possessed the characteristics of Furchgott's endothelium-derived relaxing factor, later identified as nitric oxide (NO) (567). More recent studies have demonstrated that degree of vasodilation is proportional to blood flow induced increases in shear stress, not blood flow per se (7, 568). In 1992, Celermajer developed the FMD technique as a non-

invasive method to measure peripheral conduit artery function following a period of distal limb ischaemia (569). FMD has subsequently become a widely used research tool to test for the presence of impaired endothelium-dependent vasodilation in patients with risk factors for cardiovascular disease or to demonstrate benefits of therapeutic agents that improve endothelial function (570). FMD is typically assessed in the brachial artery, but can also be assessed in other conduit vessels, including radial, posterior, tibial or femoral arteries.

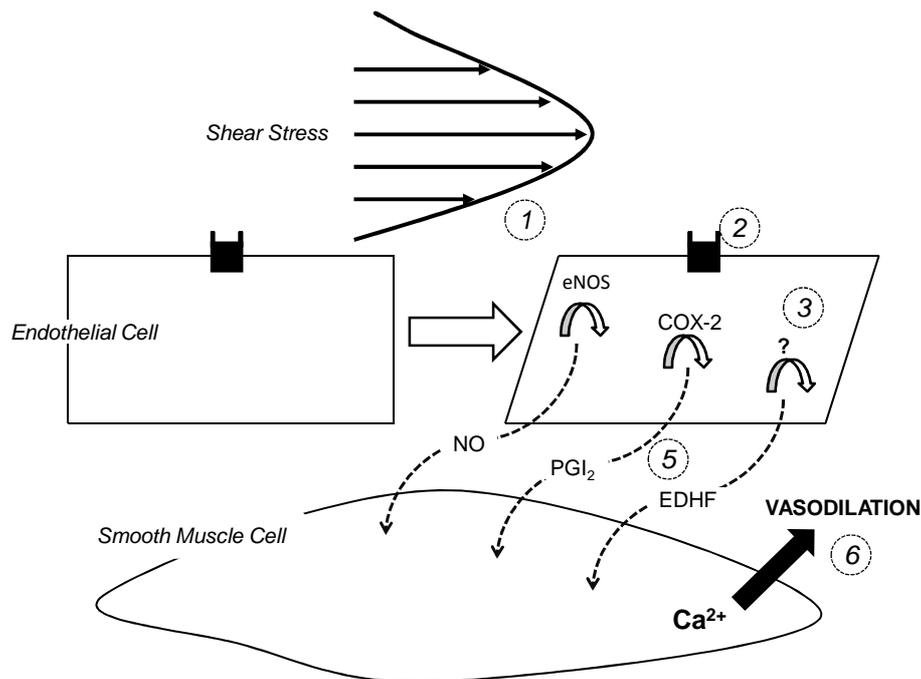


Figure 2.1 Endothelium-dependent dilation. Shear stress results in deformation of the endothelial cells (1), which is detected by mechanoreceptors on the cell membrane (2). In response to mechanotransduced shear stress, a signalling cascade results in the production of nitric oxide (NO), PGI₂ (prostaglandins) and EDHF (endothelial-derived hyperpolarizing factor) (3). The vasodilators diffuse cross the interstitial space (5) and enter the vascular smooth muscle cells, where they initiate a signalling cascade which lowers the calcium concentration and results in vasodilation (6). eNOS, endothelial NO synthase. (Permission obtained from Stoner *et al.* (2011) (10)

The underlying principle of FMD is as follows: vasodilation of an artery following an increase in luminal blood flow derived shear stress (Figure 2.1). Vascular occlusion, typically achieved via inflation of a cuff to suprasystolic levels in the arm, creates a region of ischaemic tissue distal to the point of occlusion (569). Post-ischaemia, a rapid, transient increase in

blood flow is seen; a phenomenon termed reactive hyperaemia. The increased blood flow augments laminar shear stress on endothelial cells lining the artery (571). Blood flow associated shear stress is sensed by deformation of the mechanosensitive ion channels in the cell membrane (572). In response to mechanotransduced shear stress, a signalling cascade results in the endothelial cell release of vasodilators, including NO (573-575), prostaglandins (PGI₂) (576), and a putative endothelial-derived hyperpolarizing factor (EDHF) (577, 578). Vasodilators diffuse from the endothelial cell into the smooth muscle cell, where they trigger a signalling cascade that results in lower calcium concentrations, thereby inducing relaxation of the smooth muscle and subsequently vasodilation. FMD is typically expressed as the percentage increase in the artery diameter above baseline. Endothelium-independent function of conduit arteries can be assessed by measuring the vasodilatory responses to glyceryl trinitrate (GTN), which acts downstream of the endothelium and provides an index of smooth muscle cell function.

While multiple vasodilators (PGI₂, EDHF and NO) may be involved in the FMD process, the standard FMD protocol is thought to serve as a non-invasive bioassay for endothelium-derived NO bioavailability (27). Given the vasoprotective properties of NO, (579, 580), efforts have been directed at identifying specific protocols and stimulus profiles that are able to isolate the NO pathways.

2.2.2 Equipment and set-up for brachial ultrasound

The brachial ultrasound methodology was established in the Vascular Ultrasound Unit at Christchurch Hospital for the purposes of this thesis and formed the basis of the data collected in Chapter 3. The setup is shown in Figure 2.2.

2.2.2.1 Equipment to image the artery and measure blood flow

2.2.2.1.1 Ultrasound device

The ACUSON Antares™ ultrasound machine, Siemens Medical Solutions, USA, was used for imaging of the brachial artery. This machine possessed duplex Doppler functionality, which enabled simultaneous real-time imaging of the brachial artery and measurement of blood velocity. Ultrasound settings were optimised by comparing images of the same brachial artery for clarity and quality of edge detection that were obtained from different image

settings. The image setting that gave the clearest image and which was read most reliably by the analysis software was used as the default set up in all recordings. Ultrasound global (acoustic output, gain, dynamic range, gamma, and rejection) and probe-dependent (zoom factor, edge enhancement, frame averaging, and target frame rate) settings were standardised.

The screen display was set so that 2/3 of the screen was allocated to the B-Mode image of the artery and 1/3 to the Doppler trace. This set up enabled simultaneous capture of blood velocity measurements in addition to the brachial artery diameter.



Figure 2.2 Setup for brachial ultrasound assessment

2.2.2.1.2 Ultrasound transducer

Accurate analysis of flow mediated dilation is highly dependent on the quality of the ultrasound images. To optimise the imaging of superficial, vascular anatomical structures like the brachial artery, a broad-spectrum, 9-4 MHz Linear Array transducer (VFX9-4, Siemens Medical Solutions, USA) was used. When making simultaneous diameter and blood velocity measurements, a compromise has to be made. An optimal B-Mode image is obtained when the ultrasound probe is perpendicular (90 degrees) to the imaged vessel, whereas an optimal

velocity signal is obtained with a beam-vessel angle ≤ 60 degrees, and ideally at 0 degrees. Error associated with incorrect estimation of insonation angle increases exponentially with angles ≥ 60 degrees (581). On this basis, the Doppler insonation angle was set to between 45 – 60 degrees for Doppler curve/blood flow velocity measurements.

2.2.2.2 Equipment to position arm and ultrasound transducer

2.2.2.2.1 Bed

Participants rested on a specialised examination bed with a stiff foam mattress to minimise movement artefacts. The elevation and inclination of the bed could be changed electrically with a foot switch, and the bed could be positioned horizontally.

2.2.2.2.2 Armrest

The non-dominant, test arm was positioned on a specially designed, foam cushioned cradle, fixed onto a metal frame which could be adjusted for height and length. The armrest included a mounting for the stereotactic clamp.



Figure 2.3 Setup of non-dominant arm lying on the armrest with the transducer fixed in position with the stereotactic clamp

2.2.2.2.3 Stereotactic clamp

In order to precisely fixate the ultrasound probe into one position a stereotactic clamp was used (Figure 2.3). To accommodate the VFX9-4 ultrasound probe a steel gripper was

designed at the Bio-Engineering Department of Christchurch Hospital. The clamp was attached to a commercially available fixation device (NF Holder-61003, Noga Engineering LTD, Israel) consisting of three arms connected through three, 360 degrees joints mounted onto a magnetic base. All three joints could be screwed tight with a single bolt. The probe could therefore be moved seamlessly in all directions and then fixated in the desired position, while one hand held the probe in position, the other hand was able to screw the bolt tight. Once fixed in position, fine adjustments could be made to the probe on the vertical and horizontal axis by manipulating screws on the gripper to ensure precise positioning and minimal pressure on the artery.

2.2.2.3 Equipment for data acquisition

2.2.2.3.1 Video capture device

In the vascular laboratory, all ultrasound images were recorded and stored on a laptop equipped with video acquisition hardware. The video files were collected at 25 frames/s in the Moving Picture Experts Group (MPEG 2) format via a USB 2.0 video capture adaptor (XH3371, Dick Smith Electronics, New Zealand).

2.2.2.3.2 Laptop

A standard, commercially available laptop was fitted with a graphics board and software for data capture (Ulead Systems Inc, Taiwan), video decompilation (Blaze Media Pro, version 7.0, Mystik Media) and image analysis (semi-automated edge-detection custom software).

2.2.3 Protocol

The protocol was undertaken in accordance with the Guidelines from the International Brachial Artery Reactivity Task Force 2002 (570) and updated by Thijssen in 2011(27), except that mean diameters were calculated instead of end diastolic diameters. Patients underwent testing at the same time of the morning after a 12-hr overnight fast by an operator trained in the technique of brachial ultrasound. Testing was conducted at the Vascular Laboratory, Department of Radiology, which is a quiet, air-conditioned room with a stable temperature. Subjects abstained from vitamin supplementation prior to and for the duration of the clinical study. Patients were asked to avoid strenuous exercise, caffeine, nicotine and alcohol during the 24-hours prior to the procedure and to fast during the 12 hours before the vascular assessment, since these factors are known to influence FMD. Patients abstained from

medications on the morning of the visits. During all recordings, the patient was instructed to remain still and quiet. The participant rested in a recumbent position for at least 20 minutes prior to commencement of vascular measurements. Endothelium-dependent vasodilation was assessed using the standard flow-mediated dilation (FMD) protocol. Endothelium-independent vasodilation (EID) was assessed using a sublingual glyceryl trinitrate (GTN) challenge.

The non-dominant arm of the participant was positioned in the arm rest in a specially designed cradle at approximately 80 degrees from the torso. A pneumatic cuff (Hokanson, Inc., Seattle, Washington) was placed around the forearm, distal to the insonated artery and a blood pressure cuff was placed on the dominant arm. The brachial artery was imaged in the longitudinal plane 3-7 cm proximal to the antecubital fossa (Figure 2.4). Care was taken to ensure that the vessel clearly extended across the entire (un-zoomed) imaging plane to minimize the likelihood of skewing the vessel walls. Magnification and focal zone settings were adjusted to optimise imaging of the proximal and distal vessel walls. Once the best image had been obtained, the ultrasound transducer was carefully positioned and fixed on the brachial artery using a specialised stereotactic clamp. If feasible, an anatomical landmark was identified and recorded.

The lower third of the image was set to display Doppler velocity of the brachial artery blood flow in real time. The shape of the Doppler curves was optimised by placing the Doppler sample into the centre of the brachial artery. Sonication angle was kept constant at 45 – 60 degrees for all measurements. Pulse repetition frequency was adjusted between stages of testing to prevent aliasing. Blood flow velocities were determined automatically using the vascular package supplied with the ultrasound machine, and were specifically programmed to display the time-averaged maximum velocities.

After all set up procedures were completed, the image of the brachial artery simultaneously with blood velocities were captured using a commercial Laptop equipped with a video capture device for one minute to assess resting brachial artery diameter and blood velocity. The occluding cuff was then rapidly (1-2 seconds) inflated to a pressure of approximately 50 mmHg above the systolic blood pressure for five minutes. Diameter and blood velocity recordings resumed 30 seconds before cuff deflation and continued for 2.5 minutes following cuff release to allow measurement of the post-ischaemic response. After the completion of the FMD test, a 10 minute rest period followed to allow the arterial

diameter to return to baseline, during which time the ultrasound transducer remained fixated by the stereotactic clamp and the participant remained supine. A second baseline recording was made for 1 minute. Maximum EID was then assessed after administration of 400 µg of sublingual GTN (1 spray). Diameter and blood velocity recordings recommenced two minutes after GTN administration and were continued for at least four minutes.

Systolic and diastolic blood pressures were measured in duplicate on the non-imaged arm using a validated oscillometric sphygmomanometer (BpTRU BPM-200, BpTRU Medical Devices, Coquitlam, BC Canada) following recordings obtained at baseline, post-ischæmia, second baseline, and post-GTN.

2.2.4 Analysis

2.2.4.1 Diameter analysis

Ultrasound images were captured using a commercial Laptop (Toshiba) equipped with a video capture device. Video files were collected at 25 frames per second and converted to Joint Photographic Experts Group (JPEG) images by Blaze Media Pro, and subsequently used to make 25 diameter measurements per second. JPEG images provide comparable accuracy for ultrasound image measurements compared to the Digital Image and Communications in Medicine (DICOM) standard (582). Images were measured offline using semi-automated edge-detection software custom written to interface with the LabVIEW data acquisition platform (version 8.1, National Instruments, Austin, Texas) (Figure 2.5) (583, 584). After calibration of the images, the investigator manually inputted a line corresponding with the long axis of the artery and then selected a region of interest (ROI) along the length of the artery. The artery walls were then represented by a line of best fit located via gradient-based detection within the ROI. Arterial diameter was estimated as the distance between the upper and lower wall via a least-squared-error model fit. The software success rate in detecting the arterial wall was maximised by increasing the ROI length along the artery as much as possible and keeping the probe perfectly stable during the entire experiment. Custom written Excel Visual Basic (VB) code was used to fit peaks and troughs to diameter waveforms in order to calculate systolic, diastolic and mean diameters. Mean diameters were used for analysis. A recent study demonstrated that calculating FMD based on mean diameter yields comparable results to calculations based on end-diastolic diameters (585). Data was automatically cleaned by the VB in order to eliminate any outliers. A summary diameter output was then generated

using a “SUMM” function, which graphically displayed the diameter waveforms and the summary of the diameter calculations. Figure 2.6 shows the diameter waveforms in response to five minutes of ischaemia.

Vessel diameter was defined as the distance between the distal and proximal luminal–intimal interface. FMD and EID were expressed as the percentage increase in vessel diameter from the preceding baseline [(peak diameter post-ischaemia or post-GTN administration minus resting diameter) / resting diameter] x 100. In our vascular laboratory, normal FMD and EID values are considered to be >7 % and >15 %, respectively. It is acknowledged that when FMD is expressed as % of change, the initial baseline diameter has the potential to introduce mathematical bias into the FMD assessment, with larger vessels appearing less reactive, and vice versa (580). Thus, in addition to FMD expressed as a percentage it is important to document baseline diameters, the absolute change in diameter, and the hyperaemic shear response. Absolute FMD and EID were expressed as the peak diameter post-ischaemia or GTN administration minus the resting diameter. The time to peak diameter (seconds) was calculated from the point of cuff deflation to the maximum diameter post-ischaemia.

2.2.4.2 Blood velocity analysis

Blood flow velocities were manually recorded offline from the JPEG images. Obvious outliers were removed and missing values were replaced using linear interpolation.

2.2.4.3 Shear rate analysis

The 25 diameter measurements/second were aggregated to 1 per second and synchronised with blood velocities. Shear rates were calculated as:

$$\text{Shear rate (s}^{-1}\text{)} = (8 * \text{mean blood velocity}) / \text{diameter}$$

Time averaged maximum velocities were used to calculate the shear rate. Time averaged maximum velocity is the average of the highest velocities throughout the cardiac cycle. Time averaged blood are more reliable when compared to time averaged mean blood velocities (586).

Shear rates were calculated at: resting baseline, peak post-ischaemia, second resting baseline and maximum GTN. Shear rate has been used as a surrogate measure of shear stress in a number of previous studies (587, 588)

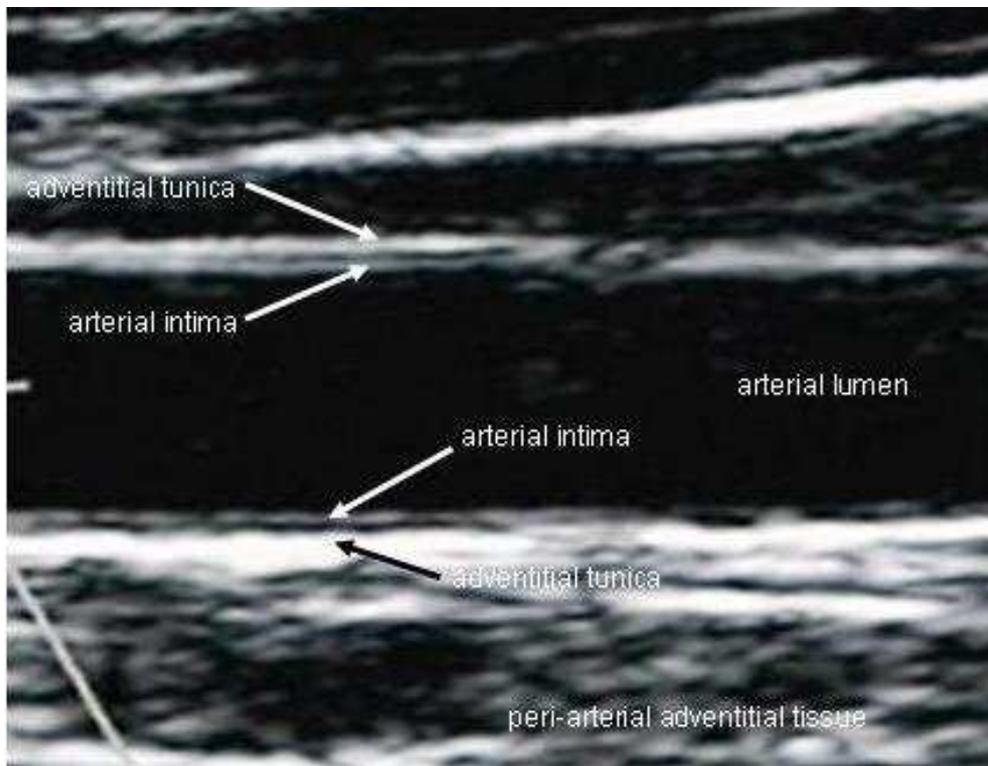
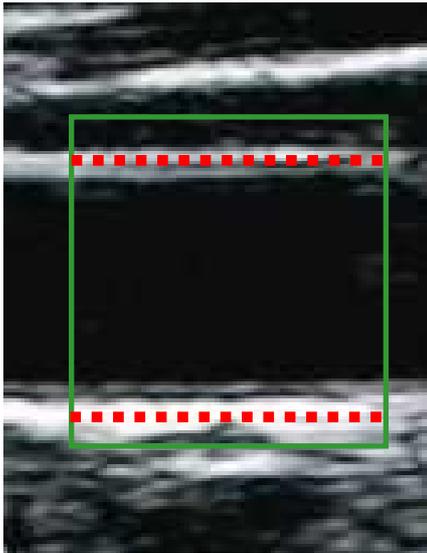
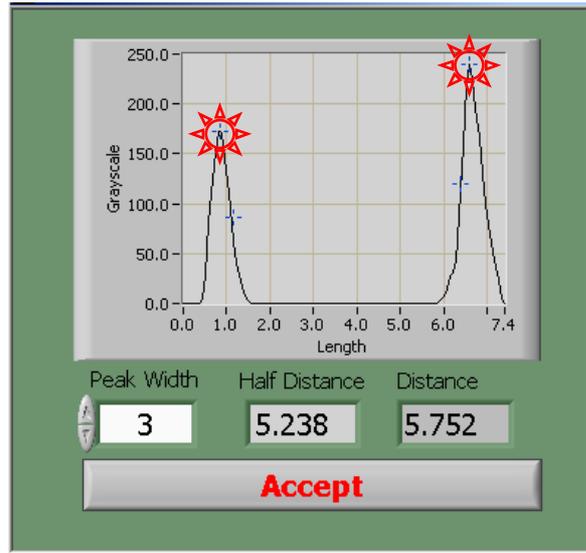


Figure 2.4 **Ultrasound image of the brachial artery**

(a) B-Mode Image



(b) Histogram



(c) Diameter waveforms

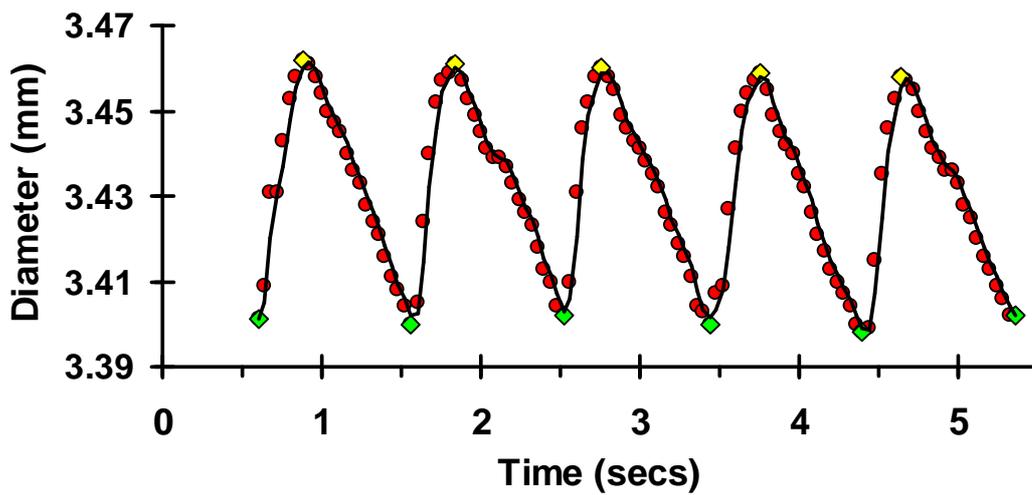


Figure 2.5 Semi-automated diameter analysis. (a) Pixel brightness on the B-mode image corresponds with (b) histogram. The peaks (stars) correspond with the walls. Distance between brightest horizontal segments is recorded. (c) Diameter waveforms from five cardiac cycles. Green markers represent diastole, yellow markers represent systole

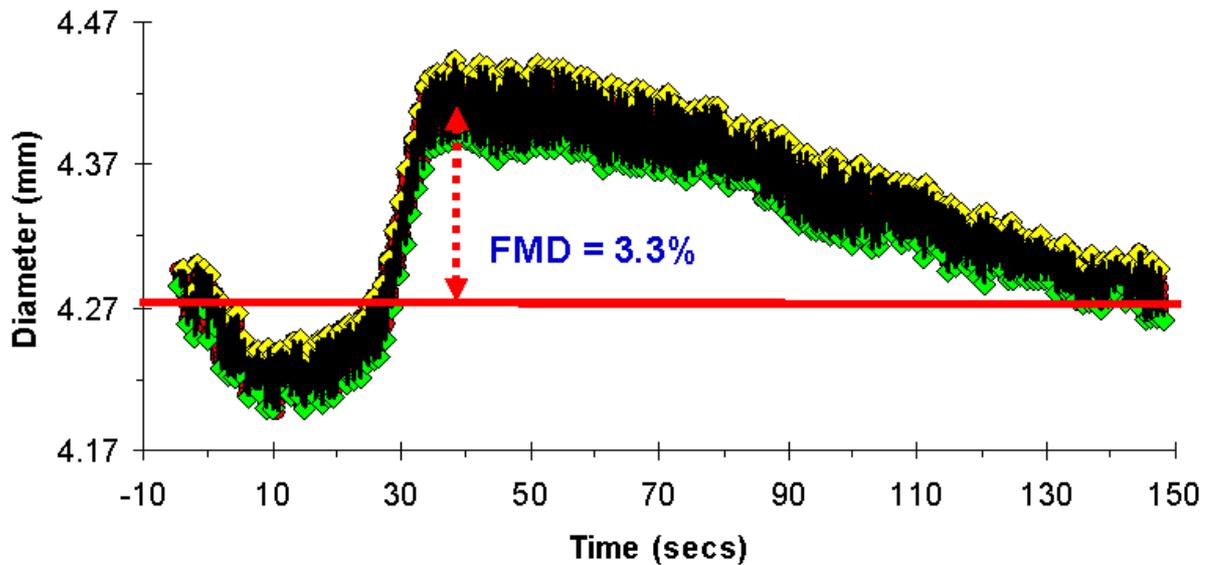


Figure 2.6 Diameter arterial waveforms in response to five minutes of ischaemia. Green markers represent diastole, yellow markers represent systole. The horizontal line represents resting diameter. Flow-mediated dilation (FMD) is the peak percentage increase in diameter above rest. A healthy artery dilates > 7% (589)

2.2.5 FMD reproducibility study

2.2.5.1 Aims

The aims of the present study were: 1) to determine the long-term reproducibility and sources of variation of brachial arterial FMD using high resolution ultrasound in subjects with the metabolic syndrome; and 2) to establish reference change values for the parameters.

2.2.5.2 Methods

2.2.5.2.1 Subjects

Fourteen males, aged 35 – 65 years, with the metabolic syndrome as defined by International Diabetes Federation (IDF) 2005 guidelines (590), and either statin naïve or treated with ≤ 40 mg simvastatin daily or equivalent were enrolled in the study. All subjects had a waist circumference ≥ 94 cm, triglycerides ≥ 1.7 mmol/L, and a systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg or treatment for hypertension. Participants

who were on endothelial influencing therapies were stabilised for at least one month prior to screening.

2.2.5.2.2 Study design

Following a screening visit, eligible participants attended two further visits four weeks apart. Non-invasive assessment of endothelial function using brachial ultrasound was assessed once at each visit by the same operator. The protocol is described in detail in Section 2.2.3. All measurements were performed at the same time of the day and after a 12-hour overnight fast. Participants were asked not to alter their medication or undertake any significant lifestyle changes during the study. The study protocol was approved by the Upper South B Regional Ethics Committee (NZ), and written informed consent was obtained from all participants.

2.2.5.2.3 Biochemistry

All biochemical analyses were performed by Canterbury Health Laboratories, an ISO15189 accredited (human) pathology laboratory. Plasma total cholesterol, triglycerides, and HDL-cholesterol were determined by an enzymatic colorimetric method (Architect c8000 analyser, Abbott Laboratories, Abbott Park, Illinois, U.S.A.). LDL-cholesterol was calculated from the Friedewald equation. Plasma glucose, urea, creatinine and liver function were also measured (Architect c8000 analyser).

2.2.5.2.4 Statistical analysis

All statistical analyses were performed using SPSS Base version 17.0 (SPSS, Inc., Chicago, Illinois) or MedCalc version 11.5.0.0 (MedCalc Software, Broekstraat 52, 9030 Mariakerke, Belgium). The differences between weeks 0 and 4 were tested using paired t-tests. Standard variance decomposition methods (Restricted maximum likelihood (REML)) were used to estimate the variance contributions of measurements from two sources: week, within-subjects four weeks apart, and subjects, variation between subjects. All data are reported as means \pm SD. Statistical significance was inferred when $P < 0.05$ (two tailed).

Reproducibility of parameters was assessed by measuring the intra-class correlation coefficient (ICC) and coefficient of variation (CV). The ICC assesses the reproducibility of measurements by comparing the variability of different measurements within the same subject to the total variation across all measurements and all subjects. In general, ICC values above

0.75 are considered to indicate excellent reproducibility and values between 0.4 and 0.74 fair to good reproducibility (591). The ICC was calculated according to the formula:

$$\text{ICC} = \text{SD}_b^2 / (\text{SD}_b^2 + \text{SD}_w^2)$$

where: SD_b^2 and SD_w^2 are the between and within-subject variance of the measured variable, respectively.

The CV was calculated according to the formula:

$$\text{SD}_w / \bar{x} * 100$$

where SD_w is the standard deviation representing within-subject variation and \bar{x} is the mean of the measured variable.

The reference change value (RCV) is defined as the critical difference in a parameter that must be exceeded between two sequential results in order for a significant (or true) change to occur in an individual (592). Absolute RCV was calculated according to the formula:

$$Z * \sqrt{2 * \text{SD}_w^2}$$

where: Z is 1.96 for $P < 0.05$ (two-tailed) and SD_w is the variation between weeks.

2.2.5.3 Results

Baseline characteristics of subjects are summarised in Table 2.1.

2.2.5.3.1 Reproducibility of brachial ultrasound measurements

The mean baseline diameter was 4.6 ± 0.6 mm. There were no significant differences in measurements of arterial diameters, blood velocities or time to peak post-ischaemic diameter between weeks 0 and 4, as shown in Table 2.2. Likewise the FMD and EID responses did not differ significantly between weeks 0 and 4 (Table 2.2).

2.2.5.3.1.1 *Intra-class correlation coefficients*

Resting, peak post-ischaemic, and maximum EID diameters were highly reproducible when measured at four weekly intervals, providing ICC values ≥ 0.88 (Table 2.3). Repeated measurements of absolute EID and percentage EID also demonstrated excellent reproducibility with ICC values ≥ 0.78 , whereas absolute FMD and percentage FMD showed lower reproducibility, with ICC values of 0.50 and 0.37, respectively. ICC values also indicated that resting and peak blood velocities and time to post-ischaemic diameters were less reproducible when measured at four weekly intervals.

2.2.5.3.1.2 *Coefficients of variation*

CVs were $\leq 4.7\%$ for resting, peak post-ischaemic, and maximum EID diameters measurements recorded at four-weekly intervals indicating good reproducibility (Table 2.3). CVs for repeated measurements, both absolute and percentage EID were $\leq 13.5\%$, and substantially higher for absolute and percentage FMD at $\leq 43.9\%$. CVs for resting and peak blood velocities and time to peak post-ischaemic diameter ranged from 13.8 to 27.6%.

2.2.5.3.1.3 *Sources of variation*

Between-subject variation accounted for 87.7 to 91.7% of the variability in resting, peak post-ischaemic, and maximum GTN diameter measurements (Table 2.3). Similarly, 78.0 to 83.8% of the variability in absolute EID and percentage EID was explained by between-subject variation. In contrast, between-subject variation explained only 37.4 to 50.0% of the total variation in absolute FMD and FMD measurements. Similarly, between-subject variation in resting and peak blood velocities and time to peak post-ischaemic diameter accounted for 2.4 to 45.2% of the total variation, indicating high variability in the measurements taken at four-weekly intervals.

2.2.5.3.2 *Absolute reference change values*

Table 2.4 shows the changes in FMD and EID responses required to show significant differences at the 95% confidence level (two-tailed) in an individual studied on two occasions. The table also incorporates the number of recordings taken at any given visit (i.e. the average of 1, 2 or 3 recordings). For example, a subject who is studied once before and after an intervention would need to show a greater than 4.6% absolute increase in FMD to be confident that the change is due to a genuine effect and not merely a consequence of

variability. If the number of recordings increases to three per visit, the absolute RCV drops to 2.7%.

Table 2.1 Baseline characteristics

Parameter	(n=14)
Age, y	57 ± 8
Systolic blood pressure, mmHg	134 ± 13
Diastolic blood pressure, mmHg	84 ± 8
Heart rate, bpm	66 ± 9
Waist circumference, cm	102.7 ± 5.4
Weight, kg	94.0 ± 11.6
Body mass index, kg/m ²	29.5 ± 3.0
Body fat, %	29.4 ± 5.3
Ex-smokers, n (%)	4 (28.6)
Alcohol intake, units per week	4.3 ± 5.9
Plasma glucose, mmol/L	5.5 ± 0.5
Total cholesterol, mmol/L	4.6 ± 1.3
LDL-cholesterol, mmol/L	2.7 ± 1.2
Triglycerides, mmol/L	1.8 ± 0.5
HDL-cholesterol, mmol/L	1.07 ± 0.21
Statin therapy, n (%)	12 (85.7)
Antihypertensive therapy, n (%)	12 (85.7)
Aspirin, n (%)	8 (57.1)
Coronary heart disease, n (%)	2 (14.3)
Type 2 diabetes mellitus, n (%)	4 (28.6)

Values are mean ± SD or number (percentage).

Table 2.2 Repeated brachial ultrasound parameters

	Week 0	Week 4	<i>P</i> -value†
Resting characteristics			
Diameter, mm	4.7 ± 0.6	4.5 ± 0.6	0.17
Blood velocity, cm/sec	26.6 ± 5.8	30.8 ± 9.5	0.19
Flow-mediated dilation			
Peak diameter, mm	4.8 ± 0.6	4.7 ± 0.7	0.30
Absolute FMD, mm	0.16 ± 0.06	0.19 ± 0.13	0.37
FMD, %	3.5 ± 1.2	4.1 ± 2.8	0.34
Peak velocity, cm/sec	94.3 ± 23.1	91.7 ± 13.7	0.71
Time to peak FMD, s	47.6 ± 11.9	44.6 ± 9.7	0.36
EID-mediated dilation			
Resting diameter, mm	4.7 ± 0.6	4.6 ± 0.7	0.41
Maximum diameter, mm	5.3 ± 0.7	5.2 ± 0.8	0.22
Absolute EID, mm	0.61 ± 0.19	0.57 ± 0.20	0.23
EID, %	12.9 ± 3.4	12.3 ± 3.8	0.38

Values are mean ± SD.

†Paired t-test for between-week comparisons.

EID, endothelium independent vasodilation.

Table 2.3 Long-term reproducibility and sources of variation of brachial ultrasound parameters

	Reproducibility			Sources of variation	
	Mean	ICC	CV	Weeks	Subjects
Resting characteristics					
Diameter, mm	4.6	0.88	4.7	4.6 (12.3)	32.8 (87.7)
Blood velocity, cm/sec	28.7	0.02	27.6	63.0 (97.6)	1.5 (2.4)
Flow-mediated dilation					
Peak diameter, mm	4.8	0.89	4.5	4.7 (11.3)	36.9 (88.7)
Absolute FMD, mm	0.18	0.50	40.2	0.5 (50.0)	0.5 (50.0)
FMD, %	3.8	0.37	43.9	2.8 (62.6)	1.7 (37.4)
Peak velocity, cm/sec	93.9	0.43	13.8	167.1 (56.9)	126.8 (43.1)
Time to peak FMD, s	46.1	0.45	17.4	64.4 (54.8)	53.1 (45.2)
EID-mediated dilation					
Resting diameter, mm	4.6	0.90	4.4	4.1 (10.2)	36.0 (89.8)
Peak diameter, mm	5.2	0.92	4.1	4.6 (8.3)	51.1 (91.7)
Absolute EID, mm	0.59	0.84	13.2	0.6 (16.2)	3.1 (83.8)
EID, %	12.6	0.78	13.5	2.9 (22.0)	10.3 (78.0)

Values are mean, ICC, intra-class correlation coefficient; CV, coefficients of variation; and the variance (percentage of total variance) associated with each source.

EID, endothelium-independent vasodilation.

Table 2.4 Absolute reference change values for FMD and EID according to the number of recordings per visit

	Number of recordings per visit		
	1	2	3
Absolute FMD, mm	0.2	0.1	0.1
FMD, %	4.6	3.3	2.7
Absolute EID, mm	0.2	0.2	0.1
EID, %	4.7	3.3	2.7

FMD, flow-mediated dilation; EID, endothelium-independent dilation.

2.2.5.4 Discussion

It is important to assess reproducibility in patients groups, as well as healthy controls, since FMD is a widely used method, yet studies are typically powered on the basis of reproducibility studies which have used healthy populations. The present study demonstrated good between-week reproducibility for brachial artery diameters at baseline, peak diameters following reactive hyperaemia (FMD), and peak diameters following GTN (EID) in patients with the metabolic syndrome. Peak hyperaemic mean blood velocity also demonstrated good reproducibility in this patient group. However, reproducibility of FMD was lower in these patients than reported in studies of healthy subjects (593-599). The EID response showed good reproducibility, as did the peak blood velocity.

A large number of studies have assessed the reproducibility of brachial artery diameter measurements, both at baseline and following reactive hyperaemia. Baseline and peak diameters have been shown to have relatively small variability, with CV ranges of 1– 13% (593, 594, 600-609), and 1 – 11% (597, 601, 603, 604, 606, 607), respectively. The present results were consistent with these reproducibility studies. The present study revealed high ICC values (≥ 0.88) for baseline and peak hyperaemic and GTN diameters, indicating excellent between-weeks reproducibility for these variables.

In comparison to absolute diameter measurements, the FMD response has been associated with a much greater range of variability, with CV's ranging from 1–84% (593-599, 603-607, 610). The best reproducibility of FMD (overall CV of 1.8%) was reported by Sorensen *et al.* (1995) (596), although the method of their variability calculation was not

exactly described. The current results for repeated measurements of FMD showed higher CVs than reported in previous studies of healthy subjects, but lower CVs than observed in some other studies (603-607, 610). The CV is considered an inappropriate index of variability when values of the measured parameter, i.e. FMD range close to zero, particularly in subjects with the metabolic syndrome. In such cases, high CV values that have been observed may not necessarily indicate poor reproducibility. The ICC provides an appropriate statistical method for measuring consistency and conformity of repeated measurements. In the present study, however, high variability of FMD measurements was demonstrated by ICC values of 0.50 and 0.37 for absolute FMD change and percentage FMD change, respectively. Previous studies assessing the short-term reproducibility of brachial artery FMD have reported ICC values of between 0.10 and 0.92 (601, 610-612). Malik *et al.* (2004) (610) showed poor reproducibility of FMD, with an ICC of 0.10. In contrast, Welsch *et al.* (2002) (611) reported an ICC of 0.92 comparing between-day FMD values. Peretz *et al.* (2007) revealed an ICC value of 0.60 for FMD (601).

In a previous study, Craiem *et al.* (2007) (613) showed that the reproducibility of FMD measurements is lower in subjects with cardiovascular risk factors compared to healthy controls. For the most part, the poor reproducibility is likely attributable to the low FMD values of the present patient group, i.e., any small change in FMD will equate to a large degree of variance when the FMD reference values are so low. The population tested in the current study had a baseline diameter of 4.6mm and a FMD of 3%, equating to a 0.138mm change in diameter - close to resolution capabilities of the ultrasound machine. Indeed, Stoner *et al.* (2011) (614) found the SEM for the LA39 probe to be 0.130mm.

With regard to the EID response, again the best reproducibility for repeated measurements has been demonstrated by Sorensen *et al.* (1995) (596). Good between-week reproducibility was observed for the EID response in the present study, comparable to that obtained by Hijmering *et al.* (2001) (594). ICC values for the EID response, expressed as an absolute and percentage, were 0.84 and 0.78 respectively, indicating good between-week reproducibility.

The reproducibility of blood flow velocities has been less frequently documented. Between-day measurements of resting blood velocities have been reported to be lower than observed for between-week measurements in the present study (602, 615). However, the peak hyperaemic mean blood velocity showed good reproducibility in the present study, consistent

with that reported by Malik *et al.* (2004) (610) and West *et al.* (2004) (615). This is important because blood velocity can be used to calculate shear stress, the primary stimulus for FMD (587, 616). Given the high variability of FMD in the current study, accounting for shear stress may help to improve the reliability of the test in patients with the metabolic syndrome. However, it remains to be determined how much this method would increase the reliability of the FMD measurement in this population,

Absolute RCV's were established for the FMD and EID responses to determine the treatment effects required in individuals that would ensure individual responses are due to a real effect and not due to biological variation. Absolute RCV's for the FMD response were comparable to that obtained by Sorensen *et al.* (1995) (596). Taken together, these studies show that by performing several assessments before and after therapeutic intervention, the chances of showing a real benefit are increased. The magnitude of clinically important changes in FMD is 1% (38, 39), which is small compared to the absolute RCV, even when measured in triplicate on two occasions. In order to be confident of a clinically significant change in the FMD an individual would require multiple replications that would be impractical.

2.2.6 Brachial ultrasound (2nd method)

The second protocol was established in conjunction with Dr Chris Strey during his PhD. A more detailed description of the methodology is provided in his thesis (563). The setup is shown in Figure 2.7. This protocol was employed in the study presented in Chapter 8. The methodology differs from the procedure presented above in the following aspects: ultrasound machine, stereotactic clamp, data capture, and image analysis. These differences will be outlined below.



Figure 2.7 Setup for brachial ultrasound measurement (2nd method)

2.2.6.1 Equipment and set up (2nd method)

2.2.6.1.1 Ultrasound device

The Logiq 700 Expert Series ultrasound machine (GE Medical Systems) was used for imaging of the brachial artery. This machine also possessed duplex Doppler functionality, which enabled simultaneous real-time imaging and measurement of blood velocity in the brachial artery. Ultrasound settings were optimised and standardised for all assessments.

2.2.6.1.2 Ultrasound transducer

A broad-spectrum, 6-13 MHz linear array transducer (LA39, GE Medical Systems) was used.

2.2.6.1.3 Stereotactic clamp

A stereotactic clamp was developed in order to precisely fixate the ultrasound probe into one position. This clamp was the same as described above, except for the gripper. A Plexiglas gripper was designed and molded at the Bio-Engineering Department, Christchurch Hospital to accommodate the LA39 ultrasound probe.

2.2.6.1.4 Videocassette recorder

Ultrasound images were recorded and stored on S-VHS videotapes with a Sony SVO-9500MDP S-VHS videocassette recorder. For later analysis the videocassettes were played in a second S-VHS videocassette recorder (Model number: NVHS 870, Panasonic, Japan) which was linked to a personal computer.

2.2.6.1.5 Personal computer

A standard, commercially available personal computer was fitted with a data acquisition board and software (DV500 Plus, Pinnacle Systems®), so that the signals from the S-VHS videocassettes could be digitised and displayed on screen.

2.2.6.2 Protocol (2nd method)

The protocol was very similar to the one described above, with several exceptions:

1) Simultaneously recorded brachial artery images and blood flow at the first resting baseline, post-ischæmia, second resting baseline, and post-GTN were recorded on an S-VHS video tape. The other protocol captured images directly onto a laptop via a video capture device.

2) Recordings were recommenced ten seconds prior to cuff release and continued for two minutes to assess the post-ischæmic response. In the other protocol, recordings were recommenced 30 seconds prior to cuff release and continued for 2.5 minutes to ensure a normal FMD response was obtained.

3) Patients were administered 800 micrograms of GTN (2 sprays), and after four minutes, video sequences were recorded for two minutes to measure the response to GTN. In the other protocol, a GTN dose of 400 micrograms was administered to reduce the risk of side

effects, and recordings were taken two minutes post-GTN administration and were continued for a further four minutes.

2.2.6.3 Analysis (2nd method)

Data was recorded on S-VHS tapes, digitised using a data acquisition board and analysed with in-house software (Sinclair Bennett, Christchurch Hospital) as described in detail in Dr Chris Strey's PhD (563). Image analysis differed from the method described above in that only end diastolic diameters were analysed. Custom developed semi-automated edge detection software recognised the proximal and distal arterial-lumen interfaces for diameter calculation.

Analysis of the ultrasound images involved five steps: i) acquisition of the video signal; ii) conversion of the video sequence into frames; iii) diastolic frame extraction; iv) placement of tracer lines; and v) measurement of diameter. Ultrasound video sequences were digitised using Pinnacle Systems interface box and converted from Audio Video Interleave (AVI) files into single Graphics Interchange Format (GIF) frames with Adobe Premier. Simultaneously recorded and displayed Doppler signal was used to extract only those images which were taken prior to the systolic peak flow. Electronic indicators placed on diastolic "snapshots", each taken exactly at the same time prior to systole were then combined to create a video clip of diastolic frames.

A ROI box was used to identify the lumen-intima interfaces in the first diastolic frame, where two tracer lines detected the brightness of the image. The software then recognised lumen-intima interfaces in the ROI boxes in all diastolic frames and measured the arterial distance from left to right by "slicing" the ROI into one-pixel wide cross-sections. A summary output was then displayed of arterial diameters-averages calculated from each frame in sequence along the time axis. In order to eliminate any outliers, two horizontal lines could be adjusted from above and below the summary output to exclude all diastolic frames outside these lines. In averaging mode, the mean resting arterial diameters for the first and second baseline and the maximum GTN diameter were calculated. A horizontal line was lowered onto the peak section of the summary output to determine the post-ischaemic diameter.

2.2.6.4 Reproducibility (2nd method)

The reproducibility of this method was established by two operators in eighteen healthy volunteers who were not taking any medication, as detailed in Chris Strey's thesis (563). The study was approved by the Canterbury Ethics Committee and all participants completed written informed consent prior to the initiation of the study. Brachial artery ultrasound measurements were recorded during two identical study visits one week apart by the same investigator. At each study visit, FMD was measured twice, i.e. before and after a 15 minute break (617), and the GTN response was measured once.

CVs for repeated measurements on the same day were: 2% for the resting diameter, 2% for the peak diameter following reactive hyperemia, and 21% for FMD. Within-day CVs for resting velocity measurements and peak velocity measurements were 8% and 11%, respectively. Between-week CVs were 3% for the first resting diameter, 3% for the peak diameter following reactive hyperemia, 23% for the FMD response, 2% for the second resting diameter, 2% for the peak diameter following GTN, and 10% for the EID response. Between-week CVs for resting velocity measurements and peak velocity measurements post-ischaemia were 7% and 10%, respectively.

2.2.7 Advantages and clinical utility of brachial ultrasound

FMD has become a well used research tool for the assessment of endothelial function, given its non-invasive nature and the relatively short duration required for measurement – a typical session can be completed within 30 minutes. The FMD technique is associated with only a few side effects, including the inability to endure the discomfort caused by the interruption of arterial blood flow for five minutes. Although the initial investment for an ultrasound machine is considerable, the actual brachial ultrasound measurements have minimal costs. The FMD test represents an important tool to improve physiological insight and understanding of mechanisms that alter endothelial function (27). Pre-clinical detection of endothelial dysfunction will enable interventions to be targeted and monitored at an early stage in the disease process (569).

There is now a good evidence base for the prognostic implications of impaired FMD in the brachial artery (38, 39). FMD has the capacity to predict cardiovascular events in patients with established cardiovascular disease and in asymptomatic subjects (38), and FMD is at least as predictive as traditional risk factors (38). In a recent meta-analysis, Inaba *et al.* (2010)

(38) assessed the prognostic strength of brachial artery FMD in fourteen studies, involving 5,547 participants. After adjusting for confounding risk factors, a 1% increase in brachial artery FMD was associated with a relative risk of 0.87, which is a 13% decrease in the risk of future cardiovascular events (38). Furthermore, FMD has been shown to correlate with invasively assessed endothelial function in the coronary arteries (618, 619), although not always consistently (620).

Although some FMD protocols are less NO-dependent, such as proximal cuff placement or the use of steady state shear stress (575), a recent re-analysis of the meta-analysis by Inaba *et al.* (2010), demonstrated that studies conducting FMD using proximal cuff placement provided a better prognosis for CVD risk than distal cuff placement (17% versus 9% decrease in CV risk for every 1% increase in FMD, respectively) (39). These findings need to be confirmed, but suggest that all endothelial-based mechanisms are significant for vascular disease (39). However, the distinction of the dilatory pathways is important when establishing interventions designed to improve endothelial dysfunction, particularly when the interventions target the eNOS-dependent pathway, and a systematic investigation of all potential pathways involved in the FMD response and their potential links to vascular disease need to be established.

While the potential benefits of a non-invasive test that allows early risk identification cannot be ignored, the clinical utility of the methodology of brachial artery FMD testing is currently limited by the variability of FMD responses between research centres, primarily as a result of different methodological approaches (detailed in section 2.2.8). Efforts to standardise the FMD technique have been underway since 1992 (570), however there are still some aspects of the FMD protocol that are lacking expert consensus. Presently, FMD remains a valuable research tool for study populations rather than individuals. Despite the widespread use of this tool in the research environment and its established prognostic value, a robust protocol would need to be established before ultrasound FMD could be integrated into the clinical environment. That would require national and international standardisation, however, not only to define abnormal cut-off points, but also to ensure quality control and comparability of results between institutions (563). Furthermore, the additive predictive value of brachial ultrasound FMD beyond established traditional risk factors is yet to be determined (38). Ongoing studies are continuing to address the standardisation of FMD.

2.2.8 Limitations of brachial ultrasound

The principle disadvantage of FMD of the brachial artery is at present, its limited clinical utility. FMD is technically demanding and subject to biological and methodological variability, which can critically impact the nature and magnitude of the FMD response (575, 621, 622). As a consequence, poor reliability has been reported (623). For example, a meta-analysis was conducted on 250 studies that used FMD and revealed that technical aspects of the measurement (i.e. occlusion, location and duration) may explain the differences in FMD observed among studies (623). Standardised protocols for the performance and interpretation of FMD are therefore essential for implementation into the clinical setting. At present, the lack of a scientific consensus on a standardised protocol for measuring FMD precludes the accurate comparison of data between research centres (624). In an effort to standardise the FMD technique, initial guidelines for the assessment of ultrasound FMD of the brachial artery were published by an International Task Force (570), but failed to agree on the position of the cuff in relation to the ultrasound probe. Despite considerable advancements in the application of the FMD technique and comprehensive updates to the guidelines on the FMD methodology and analysis (27, 580, 625, 626), recommendations are not currently available for all aspects of the FMD protocol due to the lack of an evidence base.

2.2.8.1 Influence of cuff position and duration of ischaemia

For example, to date there is no consensus regarding the placement of the occlusion cuff relative to the site of measurement. However, there is growing support and recommendation for distal placement relative to the ultrasound probe, since this method is thought to yield a predominantly NO-endothelium-dependent vasodilation (27, 575, 627). Placement of the cuff proximal to the ultrasound probe elicits a greater hyperaemic response and subsequent FMD, attributable to vasoactive mechanisms in addition to NO (575, 601, 608, 627, 628). The duration of ischaemia is still a matter of some debate (579, 629), although more recent evidence supports the use of a five minute occlusion period, since longer durations of occlusion are thought to be more NO-independent (630, 631).

2.2.8.2 Contribution of NO to FMD responses

The conduit artery being studied may also influence the relative contribution of NO, since the eNOS content is not uniform throughout the arterial tree (632), and thus further work is needed to establish the NO dependence of FMD responses in conduit vessels (27). In

addition, the relative importance of NO to FMD may be down-regulated when testing patients with diseased states, such as chronic heart failure (633) or hypertension (634), and this should be taken into consideration when making comparisons between patient groups. With respect to the transient nature of the hyperaemic response, there is debate as to the optimal time resolution necessary to accurately determine peak diameter (621).

2.2.8.3 Lack of normalisation of FMD to shear stress

FMD has been called into question due to lack of normalisation to its primary stimulus, i.e. shear stress. The magnitude of the stimulus may be influenced by the presence of cardiovascular risk factors rather than impaired local conduit artery response (576). Therefore, in order to efficaciously compare groups the stimulus should at least be considered. For instance, Mitchel *et al.* (2004) found that variations in baseline and evoked hyperaemic shear stress were closely related to cardiovascular risk factors, supporting the idea that many risk factors impair brachial artery FMD by reducing the hyperaemic stimulus for dilation rather than by impairing brachial artery endothelial function (576). Shear rate is used as a surrogate measure for shear stress, and is typically estimated using a simplified mathematical model based on Poiseuille's law (587, 616). However, estimations of shear stress using Poiseuille's Law still involves considerable and unavoidable error (635). For instance, the most appropriate quantification of shear stress remains to be determined – should the shear stimulus be predicted as the peak response or some integral. The integral of shear rate over time (i.e. area under the curve) until peak dilation is generally considered the optimal method to quantify accumulated shear that contributes to the FMD response (636). However, a recent study in healthy, young males found that the addition of peak shear rate to time-integrated shear rate significantly explained further variation for change in diameter, indicating that peak shear rate may be an additional important predictor of FMD (637). Despite the well established importance of quantifying shear stress during the FMD response (580, 588, 636), there is currently no consensus for the appropriate normalisation of FMD to the shear rate (27).

A number of studies have attempted to account for the effect of the shear stimulus on FMD by dividing FMD by shear stress/rate (588, 636). However, the following assumptions must hold true for the use of ratios to normalise FMD to shear: 1) there must be at least a moderate correlation between the two variables (i.e. shear and FMD), 2) the relationship between shear and diameter must be linear, 3) the intercept for the regression slope must be

zero, 4) variance must be similar between groups, and 5) data must be normally distributed (638). A recent study found that all assumptions for the reliable use of shear-diameter ratios were violated (639). The use of analysis of covariance (ANCOVA), with shear rate as a cofactor is also not currently accepted (640), since the covariate is related to the independent variable, and the relationship between shear and FMD is variable and unstable. Another alternative approach is to normalise the FMD response to shear using hierarchical linear modelling. The disadvantage of this method is that multiple stimuli are required to generate a reliable shear-diameter relationship (641). Further study is required to establish the most appropriate normalisation of FMD to the shear stimulus.

2.2.8.4 Influence of secondary flow characteristics

In addition to time-averaged shear stress, secondary flow phenomenon including flow turbulence (642) and velocity acceleration (643, 644)– the large rate of change at the onset of flow (10) - can influence the regulation of endothelial cells and thereby affect FMD responses. The rate of velocity acceleration can effect progression of atherosclerosis (645), endothelial function (645) and vascular tone (646). In a recent study of healthy individuals, a 14% increase in velocity acceleration significantly attenuated FMD by 11% (10). This finding suggests that mean velocity may not adequately characterise the shear stimulus, and the importance of secondary flow phenomena to estimations of shear rate need to be clarified, particularly when comparing groups with secondary flow abnormalities (641).

2.2.8.5 Transient versus sustained shear stress

Since the peak post-hyperaemic response is transient in nature and thus hard to capture, endothelial function can be evaluated by using sustained increases in shear stress, for example through local hand warming and low intensity hand grip exercise (587, 602, 617). Whilst the relationship between shear rate and vasodilation is comparable when shear rate is increased transiently (ischaemia-induced) or in a sustained manner (local hand warming and hand-grip induced exercise), when FMD responses are normalised for shear stress (637), the response to sustained shear stress is likely to be less NO dependent (575). Nevertheless, the endothelium is still thought to primarily govern vasodilation under steady-state shear rate conditions (641).

2.2.8.6 Shear-rate-diameter dose response curves

It has been proposed that a more accurate assessment of endothelial function may be achieved by estimating shear-rate-diameter dose response curves (584, 602, 647, 648). The slope (change in diameter per one unit change in shear rate) would most accurately reflect endothelial function, and the maximum response reflects the degree of arterial stiffness (583, 584). This approach has the advantages of: 1) the shear stimulus is directly accounted for in a manner that does not violate statistical assumptions, 2) improved sensitivity i.e. the slope can be clearly identified, 3) improved reliability i.e. the dose response slope is more resistant to measurement error compared to a single measurement, and 4) more information is provided. However, further studies are required to determine whether shear-rate dose response curves offer greater statistical power and provide greater prognostic strength (641, 649).

2.3 Forearm strain gauge plethysmography

2.3.1 History and underlying principal

“Plethysmography” derives from the Greek words “plethysmo”, meaning “to increase”, and “grapho”, meaning “to write”, which translated literally means “the measurement of increases”. Currently, plethysmography is the term used to describe a method for recording changes in volume. Forearm venous occlusion plethysmography is the most widely used technique to assess endothelial function of resistance vessels by quantifying local blood flow response to reactive hyperaemia or to intra-arterial drug administration (650). In the forearm about 70% of the resting forearm blood flow (FBF) is through skeletal muscle, with skin blood flow accounting for most of the remainder (651). Since the blood supply of skeletal muscles is regulated by resistance vessels, the FBF measured by venous occlusion plethysmography primarily reflects resistance vessel function.

The underlying principle of forearm venous occlusion plethysmography is as follows: by inflating a brachial cuff to supra-venous blood pressure levels, but below arterial pressure during diastole, the venous outflow is briefly interrupted and the forearm proximal to the interruption starts to swell due to the continuing arterial inflow (652). Changes in forearm volume are measured by a plethysmograph using a mercury-filled strain gauge and result in a corresponding change in arm circumference and therefore strain gauge length, which is

detected as an alteration in electrical resistance of the gauge (653). By calculating the slope of the increase in the circumference over time, the volume change can be estimated. The arterial FBF rate between the brachial cuff and the wrist cuff at the moment of venous occlusion can be determined from the volume change, if calculated as percent per minute (654). FBF is usually expressed as ml/dl of forearm tissue/min. This concept and formula are shown in Figure 2.8 (655, 656).

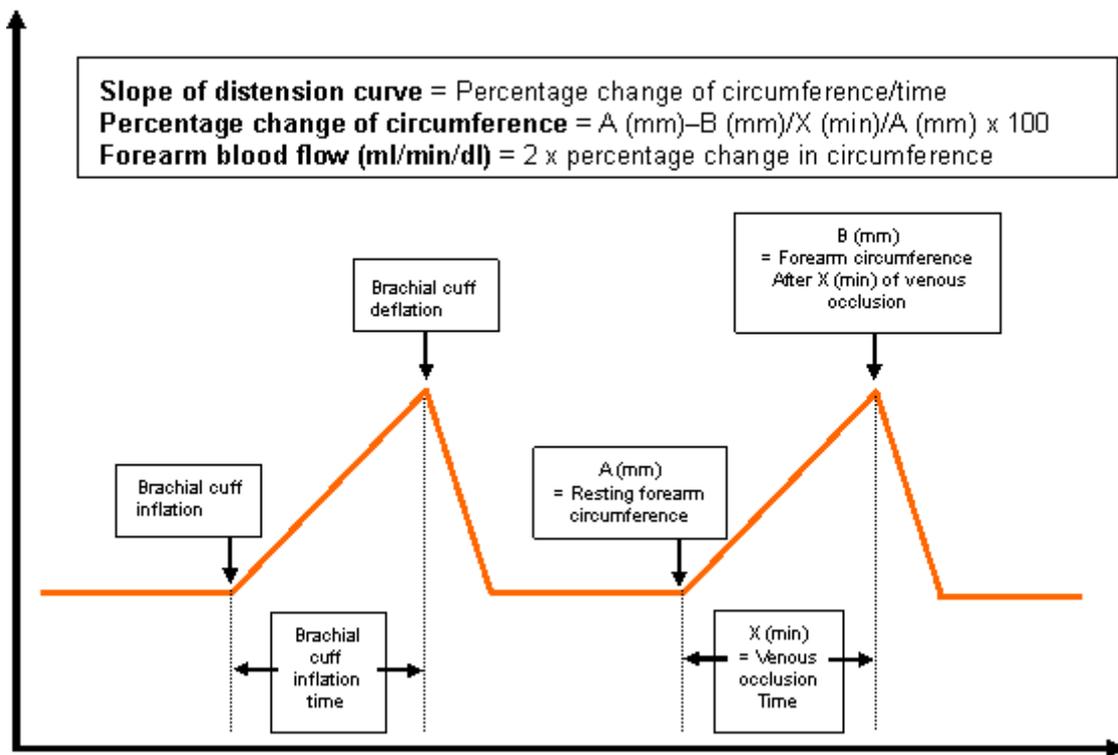


Figure 2.8 Schematic of forearm blood flow calculation from the slope of forearm distension curves (Adapted from Strey *et al.* (2007) (563))

2.3.1.1 Stimulation of endothelial cells through reactive hyperaemia

The FBF response during reactive hyperaemia following five minutes of ischaemia of the distal forearm is used as a non-invasive indirect assessment of endothelial function in the resistance vessels (657). The hyperaemic response can be calculated by measuring the slope of the peak flow curve, the percentage change of flow from baseline to the peak flow, or the entire time-flow curve during reactive hyperaemia until blood flow has returned to basal levels (297). Endothelial dysfunction is associated with a small time-flow curve and a rapid recovery, reflecting impaired flow mediated dilation (654). The major disadvantage of this technique is that the post-ischaemic response is caused by multiple factors and is thus relatively non-specific to the endothelium. Studies have demonstrated that endothelium-

derived NO plays a minor role in the peak FBF response to reactive hyperaemia, and mainly contributes to the mid-to-late phase of the FBF response after peak vasodilation (658-661). On the other hand, this technique offers the advantages of being non-invasive and straightforward, and does not require highly trained operators.

2.3.1.2 Stimulation of endothelial cells through infusion of vasoactive substances

FBF can be measured during the intra-arterial infusion of endothelium modifying vasoactive compounds that can stimulate or block endothelium-dependent functions. The resulting change in FBF is thus largely endothelium-dependent. The infusion of varying concentrations of the same vasoactive compound allows the calculation of dose response curves, and the infusion of selective blockers can give insights into the precise endothelial mechanisms. Vasoactive substances that act directly on the smooth muscle can also be infused to assess endothelium-independent vasodilation. Three vasoactive compounds were used in the plethysmography study described in Chapter 8, namely, acetylcholine chloride, N^G-monomethyl-L-arginine (L-NMMA) and sodium nitroprusside. A major advantage of invasive plethysmography is its specificity for endothelial function, whereas the inherent invasiveness of this method is a key limitation.

2.3.1.2.1 Acetylcholine Chloride

Acetylcholine (Miochol®, CIBA Vision GmbH, Switzerland) is a parasympathomimetic neurotransmitter. Acetylcholine acts on the muscarinic receptors located on the surface of endothelial cells and subsequently activates NOS. NO is produced by L-arginine in the presence of eNOS. The released NO diffuses into the blood stream and stimulates cytosolic guanylate cyclase, and thereby increases cyclic guanosine monophosphate (cGMP) content in vascular smooth muscle cells, resulting in relaxation of vascular tone (662). Acetylcholine therefore induces endothelium-dependent vasodilation.

2.3.1.2.2 NG-Monomethyl-L-Arginine Acetate

L-NMMA (Clinalfa, Weidenmattweg, Switzerland) is a substance exclusively used in research. It is an arginine analogue, which inhibits the generation of NO by selectively inhibiting inducible NOS (663). Basal release of NO can be assessed in the forearm with L-NMMA infusion into the brachial artery (663). It only has local effects as it is rapidly degraded in the microvasculature of the forearm. Co-infusion of L-NMMA with acetylcholine

can be used to assess the contribution of NO to acetylcholine-induced vasodilation, thereby demonstrating endothelium-dependent function (664).

2.3.1.2.3 Sodium nitroprusside

Sodium nitroprusside (FauldingDBL, Leamington SPA, UK) is used clinically as a vasodilating drug to reduce cardiac pre- and afterload in heart failure, angina pectoris and hypertensive crises (665). Infused into the forearm, sodium nitroprusside causes the release of NO from erythrocytes, which acts directly on the smooth muscle cells in the vascular wall, resulting in endothelium-independent vasodilation.

2.3.2 Equipment and set up

2.3.2.1 Equipment to distribute and regulate pressure

A standard hospital oxygen supply port was used as a pressurised air source and connected to the rapid cuff inflator with polyethylene tubing. The rapid cuff inflator (E20, D.E. Hokanson, Inc., Bellevue, WA, USA) regulates the pressurised air and allows rapid inflation and deflation of two venous occlusion cuffs, which are connected with the device by one polyethylene tube dividing into two separate tubes for each cuff at a T-junction. The rapid inflation venous occlusion cuffs (D.E. Hokanson, Inc., Bellevue, WA, USA) are placed around each upper arm just above the elbow crease. Arterial occlusion cuffs (D.E. Hokanson, Inc., Bellevue, WA, USA) are inflated independently from the timed inflation of the brachial cuffs using a sphygmomanometer and a medical oxygen bottle as a pressure source. The inflation pressure for the wrist cuffs is supra-arterial to exclude the circulation in the hand from the forearm circulation for the duration of each set of measurements. One standard blood pressure cuff can be applied to the dominant arm over the rapid inflation cuff to measure the post-ischaemic response.

2.3.2.2 Equipment to measure and record volume changes

Mercury filled silica strain gauges were used, which are based on the principle of piezoresistance. An increase in the length of the mercury column results in an increase of resistance, which can be quantified by applying an electrical current. Strain gauges are designed to be slightly stretched at rest, and the appropriate length is chosen by adding 2-3 cm to the forearm length. Two plethysmographs (EC4, D.E. Hokanson, Inc., Bellevue, WA,

USA), one for each arm, are used to create a current through the strain gauge and back to the plethysmograph. Any change in resistance of the mercury is recorded by the plethysmograph as a voltage change, thereby allowing an exact measurement of stretch changes caused by volume changes in the forearm. Voltage changes are visualised on an analogue display and recorded over time as curves either with an external chart recorder through an analogue output or digitally on a computer through a RS232 data output port. A specially designed arm rest allowed positioning of the arm and elbow at the appropriate height to ensure a reproducible setup between measurements.

2.3.2.3 Equipment for data acquisition

A standard computer fitted with a commercially available data acquisition board was used to display and store data from the plethysmograph. In collaboration with Dr Sinclair Bennett (Christchurch Hospital), a software package was designed to transform data recorded by the plethysmograph into curves on the computer screen. Infusion of vasoactive substances into the brachial artery required access to the arterial lumen. 27G needles (Cooper Needleworks, Birmingham, United Kingdom) were attached to a 18G epidural catheter (Portex Limited, UK) fitted with a standard Luer lock connector, so the needle-catheter unit could be connected to a standard extension set (Codon US corporation, USA). The needle-tubing units were sterilised with ethylene oxide for up to 48 hours. Two constant rate infusers (Terumo® perfusion syringe pump, model STC-523, Terumo Medical Corporation, USA) were used to continuously infuse various vasoactive substances or normal saline through the administration set and intra-arterial needle into the brachial artery.

2.3.2.4 Drug preparation

Terumo® syringe pump compatible, 50ml syringes fitted with a standard Luer lock connector were used for infusion. Christchurch Hospital Pharmacy delivered all active substances for intra-arterial infusion in pre-prepared syringes within 30 minutes prior to the commencement of the blood flow studies. The 50ml syringes were connected to mini-bore, polyethylene extension sets fitted with slide clamps and Luer lock connectors (Codon US corporation, USA) and connected to the needle-tubing unit via a three-way-tap (Connecta TM Plus 3, Becton Dickinson, Sweden).

2.3.3 Protocol

All blood flow measurements were undertaken in a specially designed, air-conditioned temperature-controlled environment. FBF measurements commenced at the same time of the morning after a 12-hr overnight fast. Patients were asked to avoid strenuous exercise, caffeine, nicotine and alcohol during the 24-hours prior to the procedure and to fast during the 12 hours before the procedure and to refrain from taking medications, smoking and undertaking strenuous exercise on the morning of the visits. During all recordings, the patient was instructed to remain still, and relaxing music was played via headphones. The standardised setup is shown in Figure 2.9.

Infusion sets were prepared at the Christchurch Hospital Pharmacy and used immediately that morning. The application set including the 27G needle was primed and checked for patency, and the brachial artery in the non-dominant arm was cannulated. The appropriately sized strain gauges were determined and placed at the widest circumference of each forearm. Both arms were positioned onto the arm rests, followed by application of the rapid cuff inflation cuffs to the upper arms, a blood pressure cuff to the dominant upper arm over the rapid inflation cuff, and wrist cuffs to both wrists. After 10 minutes of supine rest, blood pressure was measured using the automated, digital sphygmomanometer Omron HEM-705-CP (Omron Corporation, Japan). Blood pressure measurements were repeated during breaks between intra-arterial infusions and at the end of the study visit. Recording commenced 15 min after supine rest, beginning with a strain gauge sensitivity demonstration. Blood flow curves were balanced and calibrated. Wrist cuffs were inflated to 250 mmHg during each set of different vasoactive infusions and deflated during the breaks.

A Terumo® syringe pump driver delivered intra-arterial infusions of 0.9 % saline for 30 minutes (baseline), acetylcholine at 7.5, 15 and 30 µg/minute each for three minutes, sodium nitroprusside at 1.5, 3, 10 µg/minute each for three minutes and L-NMMA at 4.0 units/minute alone and co-infused with acetylcholine at the above concentrations for three minutes each. There was a break of 15 minutes between the different vasoactive infusions during which the saline infusion continued. Control blood flow measurements were made in the dominant (non-infused) arm. Forearm distension was achieved by inflating and deflating brachial cuffs to supra-venous pressure levels (45 mmHg) with a rapid cuff inflator for five and ten seconds, respectively. Ten inflation/deflation cycles were recorded for each stage of the protocol and averaged.



Figure 2.9 Standardised setup for forearm plethysmography measurement

2.3.4 Analysis

Semi-automated software for the analysis of plethysmography data was specifically designed for the research project in Chapter 8 (reviewed in Dr Chris Strey's PhD thesis (563)). A multifunctional Windows program was created using the rapid application development product Borland Delphi 4. The program allowed visualisation, optimisation, analysis and storage of plethysmography data. Signals from the plethysmograph were digitised with a standard data acquisition board and then visualised as curves on the computer screen. The depicted curves represent changes in resistance along a time axis and are the direct reflection of volume changes recorded with the mercury in silica strain gauges placed across the forearm (Figure 2.10). In order to calculate the arterial FBF the brachial venous occlusion cuffs are inflated rapidly with the rapid cuff inflator. Continuing arterial inflow, but obstructed venous outflow, results in swelling of the forearm proportional to arterial inflow. The stretch of the strain gauge results in a slowly increasing resistance signal that is measured by the plethysmograph. Consequently the blood flow curve deflects in an upward direction, due to the superimposed resistance fluctuations caused by the arterial pulse, and the slope of this tracing forms the basis for the FBF calculation. The slope can be calculated in absolute

figures or expressed as the area under the curve (AUC) according to the trapezoid rule. FBF was also expressed as the ratio between ml/min/dl in each arm, and the ratio between AUC in the infused and the non-infused arm, to allow correction for any systemic changes, such as fluctuations in blood pressure or sympathetic activity that may impact on blood flow (655).

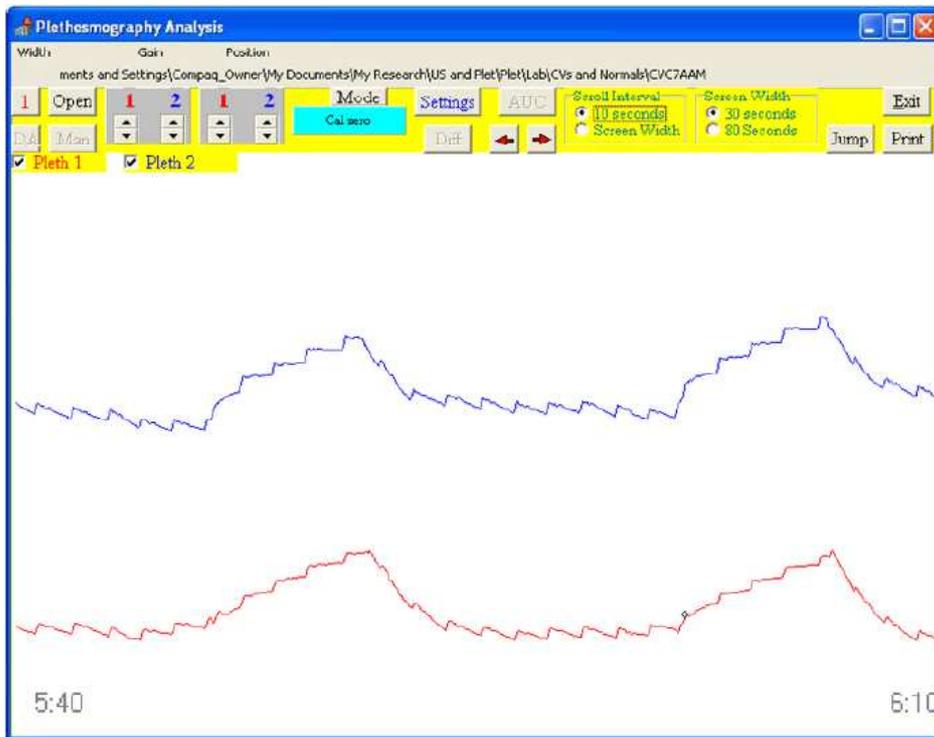


Figure 2.10 Screen display of blood flow curves using semi-automated plethysmography software. The white area is the curve display field and the area above the display is the control panel

Automated analysis was achieved by exaggerating changes within the blood flow curves through calculation of a differential curve. Adjustable parameters were accessed through pop-up menus allowing alterations of pre-defined parameters, for atypical blood flow curves. AUC analysis modes were also integrated into the program. A set of interpretation rules was employed to ensure good reproducibility of blood flow curve analysis.

2.3.5 Reproducibility

The reproducibility of this method was established by two operators, in nine healthy volunteers who were not taking any medication, as detailed in Dr Chris Strey's thesis (563). The study was approved by the Canterbury Ethics Committee and written, informed consent

was obtained from all participants prior to the initiation of the study. FBF at rest and post-ischaemia was recorded in each participant four times during two study visits one week apart by the same investigator.

The intra-observer (between repeats) CV of FBF measurements were 18% for right resting FBF, 21% for left resting FBF, 11% for peak post-ischaemic FBF, and 21% for the ratio of post-ischaemic FBF in dominant arm/non-dominant arm. The intra-observer (between repeats) CV of AUC measurements were 14% for right resting AUC, 16% for left resting AUC, 14% for peak post-ischaemic AUC, 19% for the ratio of post-ischaemic AUC in dominant arm/non-dominant arm. The intra-observer (between weeks) CV of FBF measurements were 28% for right resting FBF, 29% for left resting FBF, 14% for peak post-ischaemic FBF, 33% for the ratio of post-ischaemic FBF dominant arm/non dominant arm. The intra-observer (between weeks) CV of AUC measurements were 22% for right resting AUC, 19% for left resting AUC, 17% for peak post-ischaemic AUC, and 27% for the ratio of post-ischaemic AUC dominant arm/non dominant arm.

The CVs for resting FBF are comparable with similar reproducibility studies, with lower variation observed between repeated measurements on the same day (666, 667) than week-to-week measurements (668-670). The CV for post-ischaemic response was similar to that of Altenkirch *et al.* (1989) (667). Studies have reported inconsistent results for the CV when using ratios for expression of blood flow data (670-672).

2.3.6 Advantages and clinical utility of forearm strain gauge plethysmography

Forearm strain-gauge plethysmography is a relatively easy to method to learn and apply once the basic functions and the principle of this technique are understood. When the equipment has been obtained the running costs for non-invasive plethysmography studies are inexpensive, apart from the vasoactive drugs. A key benefit of forearm strain-gauge plethysmography is the ability to examine specific functional pathways of the endothelium when vasoactive drugs are infused intra-arterially. This allows an insight into the pathogenic mechanisms of endothelial dysfunction and can allow investigation of endothelium modulating therapies. Although peripheral endothelial dysfunction of the resistance vessels assessed by forearm strain-gauge plethysmography is predictive of future cardiovascular

events (40-42) and is closely correlated with coronary endothelial dysfunction (618), this technique will likely remain a research tool only for the foreseeable future. As mentioned, non-invasive forearm strain-gauge plethysmography is not sufficiently specific for the assessment of endothelial function; therefore vasoactive substances have to be infused intra-arterially. The invasive nature of forearm strain-gauge plethysmography limits subject acceptability since this technique requires arterial cannulation for the infusion of vasoactive mediators (28). Currently, the high variance of blood flow data precludes this technique from becoming a useful clinical tool. Whilst this method can be utilised in a research setting to assess endothelial function in a group of patients, the lack of sensitivity of this technique means it cannot be utilised to assess cardiovascular risk status or response to interventions in individual patients. Furthermore, plethysmography is unlikely to be suitable as a screening tool, as it is too time-consuming and sensitivity data is lacking. Furthermore, there is currently no universally standardised protocol for forearm strain gauge plethysmography, making it difficult to compare results from different research groups.

2.3.7 Limitations of forearm strain gauge plethysmography

The principle disadvantage of forearm strain-gauge plethysmography is its limited clinical utility, particularly since invasive assessment with vasoactive agents requires arterial cannulation. Data acquisition has to be performed in a designated area and thus requires considerable space as it is unpractical to dismantle the testing equipment. Forearm strain-gauge plethysmography is time consuming, and typically only one participant can be studied in a single day with protocol often lasting between 5 to 6 hours. Using strain gauge plethysmography, endothelial NO production can be estimated as slopes of voltage changes; however these are estimated on the basis of a cascade of conversion steps, each of which is potentially prone to error. Rigorous matching of the groups to be compared is therefore essential; for example, the amount of subcutaneous tissue or the percentage of forearm muscle mass can have a spurious influence on blood flow measurements.

2.4 Measurement of central pressure waveforms

Direct invasive measurement of aortic blood pressures can be performed by fluid filled catheters or high fidelity catheter micro-tip pressure transducers; the later is considered the gold standard and most accurate invasive method for blood pressure measurement (673). These methods are however, limited by their invasive nature and expense and in some circumstances reduced accuracy that may relate to the unavoidable distortion resulting from the fluid-filled catheter and transducer characteristics (674). Non-invasive aortic pressures can be attained by using statistical methods relating brachial to central blood pressure, or by estimating indirectly from the radial artery waveform using transfer functions or directly by pressure wave estimation at the common carotid artery. There are several techniques available including echo-tracking, plethysmography and applanation tonometry, but this section will focus on radial artery applanation tonometry and pulse wave analysis using the commercially available SphygmoCor system (AtCor Medical, Sydney, Australia). The protocol was based on previously published studies by Wilkinson and colleagues (675, 676), and was setup and validated in the Vascular Ultrasound Laboratory, at Christchurch Hospital for the purposes of the research described in this thesis.

2.5 Applanation tonometry and pulse wave analysis

2.5.1 History and underlying principle

Applanation tonometry and pulse wave analysis is a non-invasive method for determining central arterial pressures and waveform characteristics. “Tonometry” means “measuring of pressure” and applanation means “to flatten”. Modern recording of the pulse waveform became possible through the invention of the sphygmograph by Etienne Jules Marey in 1860 (677). The first arterial tonometer was built by G.L. Pressman and P.M. Newgard in 1963, inspired by ocular tonometry used for diagnosis of eye disease (678). The underlying principle of applanation tonometry is flattening of a peripheral artery between the sensor and the underlying structure that enables transmission of the intra-arterial pressure pulse from the vessel to the sensor, where it is recorded digitally (Figure 2.11). An averaged aortic pressure waveform is derived from peripheral brachial blood pressure and concomitant recording of a pulse pressure wave with radial tonometry via a generalised transfer function

(679). Pulse wave analysis permits examination of the characteristics of the aortic pressure waveform. The peak and trough of the radial pulse wave correspond respectively to systolic and diastolic blood pressure measured conventionally on the brachial artery, since blood pressure is practically identical in brachial and radial arteries (680). Mean blood pressure is determined by integration of the radial wave with brachial blood pressures, from which the derived aortic pulse waveform is calibrated and central blood pressures are derived. The shape of the aortic pressure waveform provides a number of other key measures in addition to central blood pressure values, including pressure–time indices and aortic wave reflection indices. Carotid applanation tonometry can also be performed by directly recording waveforms at the carotid artery with proper calibration. However radial tonometry has several advantages over carotid tonometry, since 1) carotid tonometry may activate baroreceptors, which can result in the dislodgement of carotid plaques; 2) the bony structure underlying the radial artery allows easier more optimal measurement; and 3) carotid arterial tonometry can be associated with some discomfort, which induces gagging or coughing.

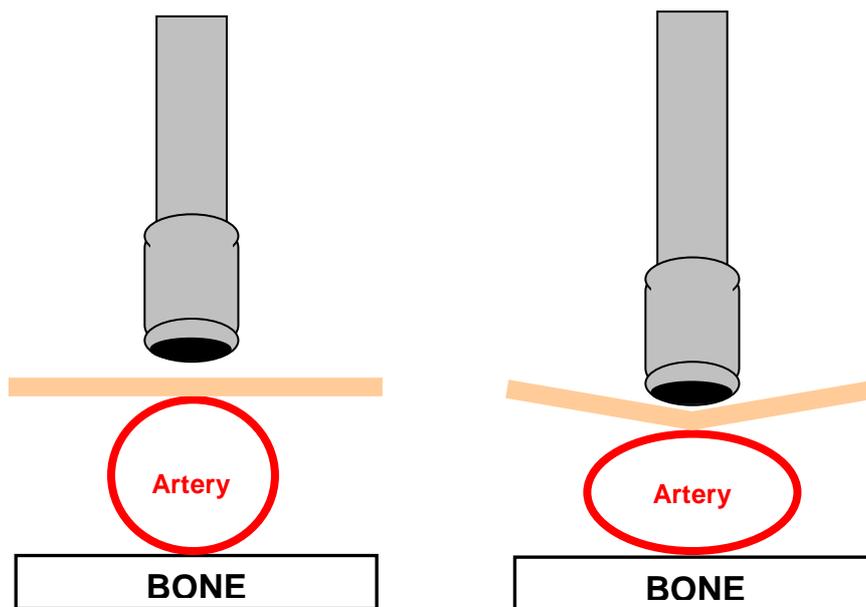


Figure 2.11 Schematic of an applanated artery

2.5.2 Features of the aortic pulse pressure waveform

Figure 2.12 shows features of the aortic pulse pressure waveform. Central systolic, diastolic and pulse pressure can be determined by measuring the peak and trough of the derived aortic pressure waveform. Ejection duration is calculated by defining the distance from the onset of the pulse wave to the dicrotic notch on the descending side of the waveform. The subendocardial viability ratio (SEVR) or Buckberg ratio gives an estimate of myocardial perfusion relative to cardiac workload (681). This is determined by $SEVR = DPTI / TTI \times 100\%$, where DPTI is the diastolic pressure time index, a determinant of myocardial blood supply, and TTI is the tension time index, a determinant of left ventricular load and thus myocardial demand.

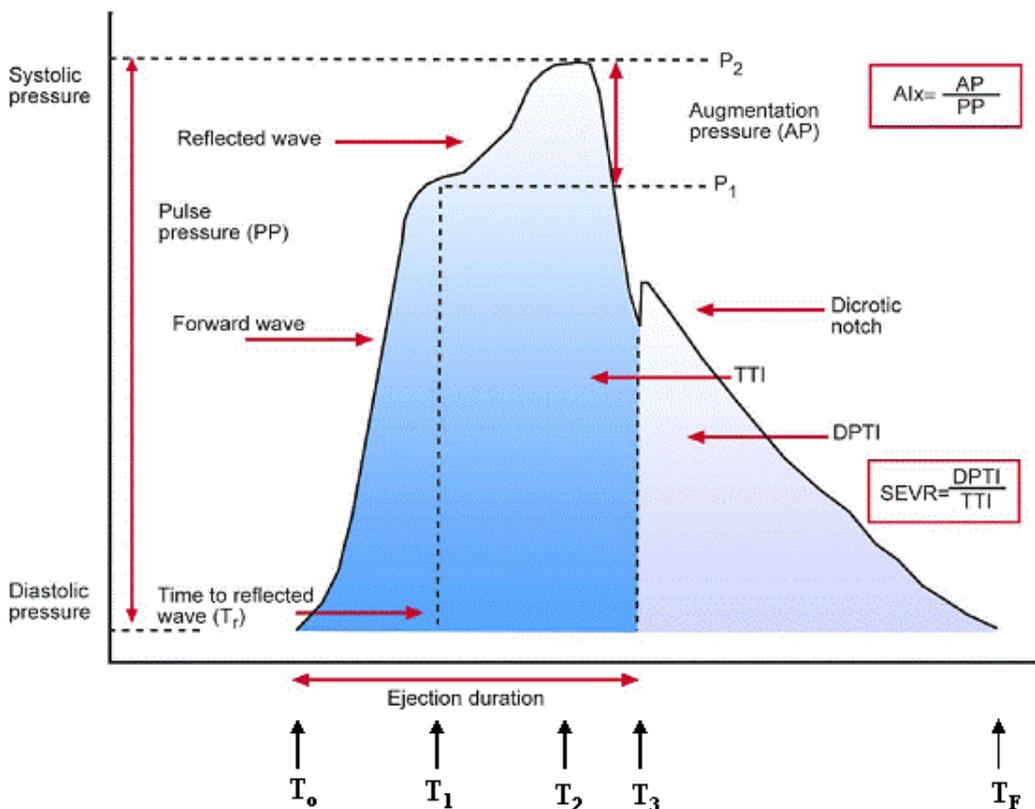


Figure 2.12 Features of the aortic pulse waveform. AIX, augmentation index; T_0 , time at the onset of ejection; T_1 , time to the peak of the primary left ventricular ejection pressure (i.e. time to reflection (T_r)); T_2 , time to peak of the reflected wave; T_3 , time at the end of ejection, associated with closure of aortic valve; T_F , time at the end of the aortic waveform. P_1 , pressure at first peak; P_2 , pressure at second peak, SEVR, subendocardial viability ratio, TTI, tension time index; DPTI, diastolic pressure time index

Pressure wave reflections are generated within the arterial tree at every cardiac cycle due to discontinuity in the geometrical and/or mechanical properties of arteries (682). Increases in pulse wave velocity and the amplitude of the reflected wave contribute to increased central wave reflection, such that the reflected wave arrives earlier, and augments central systolic blood pressure (683, 684). The amplitude and timing of reflected pressure waves are determined primarily by vascular elasticity, peripheral vascular resistance, heart rate and left ventricle function (685). Three major indices characterise the properties of wave reflections: the augmentation pressure (AP), augmentation index (AIx), and arrival time of reflected waves at the central aorta (T_r). The AP is the pressure added to the incident wave by the reflected wave from the periphery, and represents the increased afterload on the left ventricle (686). The AP is expressed as the difference between the first and second systolic peaks of the central arterial waveform. The AIx is defined as the difference between the first and second systolic peaks of the central arterial waveform (AP), expressed as a percentage of the pulse pressure, and is thus a composite measure of the magnitude of wave reflection and systemic arterial stiffness (683, 686, 687). The AIx is influenced by heart rate (688), and therefore an index normalised for a heart rate at 75 beats per minute (AIx@HR75) is derived. T_r is the time from the onset of the ejected pulse waveform to the onset of the reflected wave (i.e. the arrival time of reflected waves at the central aorta), and related directly to pulse wave velocity (689, 690). Increased aortic wave reflections have well established adverse effects on ventricular afterload and coronary perfusion, and their pathological role has been demonstrated in cardiovascular related disease states (691-694).

2.5.3 Stimulation of endothelial cells through administration of NO agonists

Pulse wave analysis using the SphygmoCor system (AtCor Medical, Sydney, Australia) combined with provocative administration of salbutamol and GTN (GTN) has been shown to provide a non-invasive assessment of endothelium-dependent and -independent function (33, 695, 696). This protocol allows determination of the salbutamol and GTN-mediated reductions in the arterial wave reflections (AIx). The technique has been clinically validated in patients with hypercholesterolaemia, coronary heart disease, and peripheral arterial disease, where significantly blunted responses to inhaled salbutamol were demonstrated compared to healthy controls, but similar responses to sublingual GTN (33, 695, 697).

2.5.3.1 Salbutamol sulphate

Salbutamol (GlaxoSmithKline, Auckland, New Zealand) is a β_2 -agonist, which in part reduces wave reflections by activation of the L-arginine-NO pathway (33, 698). L-NMMA attenuates the effect of salbutamol on wave reflections (33, 698). Salbutamol is thus, as an endothelium-dependent, NO-mediated stimulus. Furthermore, salbutamol-mediated reductions in AIx have been correlated with the effect of acetylcholine on FBF (33). Inhaled salbutamol is used clinically for the relief of bronchospasm in asthma, bronchitis and emphysema. In this protocol, a 100 μg dose of salbutamol was administered four times via a spacer, providing a total dose of 400 μg at each visit. Salbutamol was dispensed by the Christchurch Hospital pharmacy.

2.5.3.2 Glyceryl trinitrate

Glyceryl trinitrate (GTN) (Healthcare Logistics, Auckland, New Zealand) is an organic nitrate vasodilator that reduces wave reflections by direct activation of the cGMP pathway (699), and is therefore an endothelium-independent stimulus. Sublingual GTN is used therapeutically in the treatment of acute angina and chronic heart failure. In this protocol, GTN was administered sublingually at a dose of 150 μg ($\frac{1}{4}$ tablet). GTN tablets were quartered and dispensed by the Christchurch Hospital pharmacy.

2.5.4 Equipment and set up

2.5.4.1 Equipment to measure arterial pulse pressure waveform

2.5.4.1.1 Tonometer

A high-fidelity Millar SPT-301 tonometer (Millar Instruments Inc., Houston, USA) was used to record the radial artery pressure waveforms by applying gentle pressure over the non-dominant radial artery (Figure 2.13). This is a hand-held device with a single strain gauge sensor placed at the tip of the tonometer.

2.5.4.1.2 Resting aid

The non-dominant, test arm was positioned on a specially designed, foam cushioned cradle fixed onto a metal frame which could be adjusted for height and length. This allowed the patient's wrist to be extended, to enable optimal pulse waveform capture.



Figure 2.13 Applanation tonometry is performed by placing a pressure sensor over the radial artery (arm rest not shown) (SphygmoCor device, AtCor Medical, Sydney, Australia)

2.5.4.1.3 Electronics module

The electronic module (MM3, AtCor Medical, Sydney, Australia) was connected to the laptop, as well as the tonometer via a 12-way panel plug inside the tray, thereby allowing the digital capture of the pulse waveform recordings (Figure 2.14).

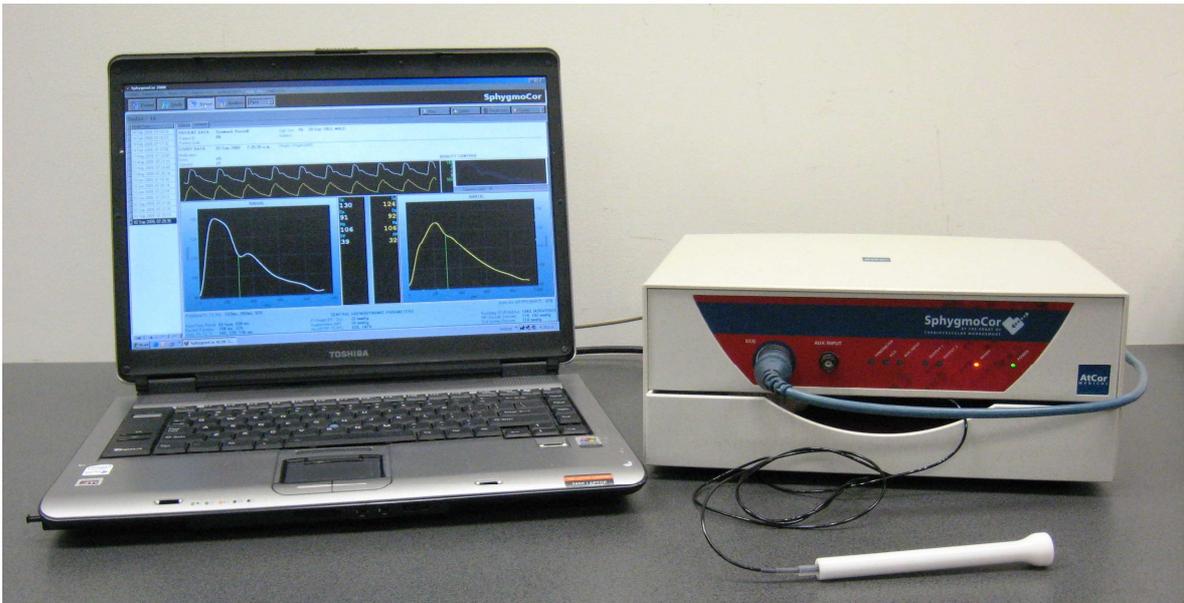


Figure 2.14 Electronics module (SphygmoCor device, AtCor Medical, Sydney, Australia)

2.5.4.2 Equipment for data acquisition

2.5.4.2.1 Computer

A laptop installed with SCOR-Px version 7.1 software (AtCor Medical, Sydney, Australia) was used to display and store data from the electronics module.

2.5.4.2.2 Data capture and analysis software

Integral software (SCOR-Px, software version 7.1, AtCor Medical, Sydney, Australia) was used for the capture, storage and analysis of peripheral artery waveforms from radial artery tonometry. A new database was created for a new clinical study, and the subsequent data for each patient was stored within a dedicated folder. At the beginning of each patient session, brachial blood pressure was measured in the non-dominant arm and the recordings were entered electronically to allow calibration of the central pulse pressure waveforms. After 20 sequential waveforms were acquired, an averaged peripheral waveform was generated, and a corresponding central waveform was then derived from a validated transfer function by the integral software. The validity of derived aortic parameters has been confirmed by simultaneous direct central aortic measurements (679). Parameters are derived from five-time relative points of the averaged aortic waveform; the onset of ejection (T_0), the peak of the primary left ventricular ejection pressure or first shoulder (T_1) (i.e. time to reflection (T_r), the

peak of the arterial reflection wave or second shoulder (T_2), the end of ejection (T_3), and the end of diastole (T_F) (Figure 2.12).

Figure 2.15 shows an example of an output from a radial artery applanation tonometry recording. Quality control data is included in the output display so that only waveforms of sufficiently high quality are included in the analysis. The quality control area allows the operator to ensure that the measurement was within the quality controls limits (pulse height ≥ 100 units; pulse height variation $\leq 5\%$; and diastolic variation $\leq 5\%$). The overlaid radial waveforms allow visual inspection to ensure there is as little variability in the waveforms as possible. The operator index, gives an indicator of overall quality of the captured signal, with readings $\geq 85\%$ considered acceptable.

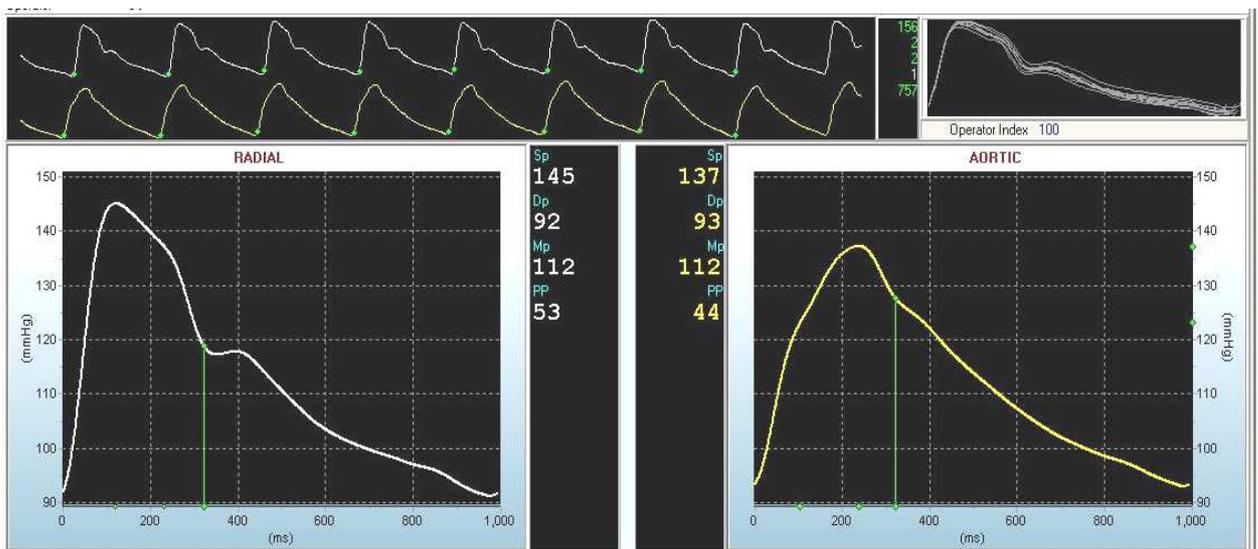


Figure 2.15 Radial artery applanation tonometry recording. The upper long panel shows the radial pressure waveform above the derived central pressure waveform. The upper right panel shows the overlaid radial waveforms, including the operator index, and the middle panel shows the quality control indices. The bottom left panel demonstrates an averaged radial arterial waveform. Systolic and diastolic pressures are 145/92 mm Hg. The bottom right panel provides an averaged derived central pressure waveform. Central pressure is 137/93 mm Hg

2.5.5 Protocol

The protocol was undertaken in accordance with the Expert Consensus Document on Arterial Stiffness: Methodological Issues and Clinical Applications (2006) (700), using the SphygmoCor PWA system (AtCor Medical, Sydney, Australia). Visits were conducted in a quiet, temperature-controlled environment by an operator who was adequately trained in the technique of pulse wave analysis. Patients were asked to avoid strenuous exercise, caffeine, nicotine and alcohol during the 24-hours prior to the procedure and to fast during the 12 hours before the procedure, since these factors can influence vascular reactivity, heart rate and blood pressure. Patients abstained from medications on the morning of the visits. During all recordings, the patient was instructed to remain still with legs uncrossed and to not talk.

2.5.5.1 Pulse wave analysis

After the patient was lying for at least five minutes in the recumbent position, systolic and diastolic blood pressure was measured twice in duplicate in the non-dominant arm using a validated automated oscillometric sphygmomanometer (HEM-705CP; Omron Corporation, Japan) or (BpTRU BPM-200, BpTRU Medical Devices, Coquitlam, BC Canada), depending on the clinical study. The mean of the two blood pressure measurements was entered into the appropriate fields within the SphygmoCor Study Screen to allow calibration with the pulse wave analysis measurements. If consecutive blood pressure readings differed by more than ~5 mmHg, a third reading was obtained and mean of the closest two readings was taken. After two minutes, radial artery waveforms were recorded in duplicate. A high-fidelity tonometer was used to obtain radial artery waveforms by applying gentle pressure over the non-dominant radial artery and repositioning the device until the largest pulse was detected. Optimal recordings are obtained when the wrist was bent outward in the “dorsiflex” position. Data was collected directly into a microcomputer and recordings were assessed visually to ensure that the best possible recording was obtained with minimisation of movement related artefacts. After 20 sequential waveforms were acquired, an averaged peripheral waveform was generated and a corresponding aortic waveform was derived. The quality of the waveforms captured was checked both visually and numerically, and only high quality readings, defined as a quality index $\geq 85\%$ were considered acceptable (described in section 2.5.4.2.2). When consecutive $AIx@HR75$ readings differed by more than ~4%, a third reading was obtained and the mean of the closest two readings was taken. This procedure equates to one set of readings. Blood pressure and pulse wave analysis measurements were

repeated in duplicate after a further two minute interval, and mean values were calculated for analysis.

2.5.5.2 Endothelial function assessment

Endothelium-independent and -dependent vasodilation was assessed by measuring the maximum changes in aortic wave reflection parameters using pulse wave analysis coupled with the administration of GTN as a direct nitrovasodilator, and the administration of the β_2 -agonist, salbutamol, as an endothelium-dependent, NO-mediated stimulus, according to an established protocol (33). The final set of haemodynamic and vascular recordings (described in section 2.5.5.1), served as the baseline for the GTN response. 150 μ g of GTN ($\frac{1}{4}$ tablet) was administered sublingually, while the patient remained in the recumbent position. Blood pressure was measured in duplicate at 1, 7, 13, 18, 23 and 28 minutes after GTN administration. Radial waveforms were recorded in duplicate at 3, 5, 10, 15, 20, 25 and 30 minutes after GTN administration. Since there was usually insufficient time to measure blood pressure between the 3 and 5 minute time points, the blood pressure reading at 7 minutes was used for both the 5 and 10 minute radial artery recordings. If baseline levels were not achieved within 30 minutes after GTN administration, recordings were continued at five minute intervals until baseline values were obtained. The final set of recordings following GTN administration served as the baseline for the salbutamol test. Salbutamol was administered via a spacer with the patient in the upright position. The spacer was depressed once (100 μ g) by the operator and the patient was asked to breathe out completely, and then breathe in deeply and slowly through the spacer and hold their breath for 10 seconds. This entire procedure was then repeated three times, providing the patient with a total dose of salbutamol of 400 μ g. The patient was instructed to lie in the supine position for the rest of the procedure. Blood pressure was measured in duplicate at 3, 8, 13 and 18 minutes after salbutamol administration. Radial waveforms were recorded at 5, 10, 15 and 20 minutes after salbutamol administration. If baseline levels were not achieved within 20 minutes after salbutamol administration, the measurement period was extended until baseline recordings were obtained. All haemodynamic and vascular assessments were made in duplicate and performed by the same investigator at each visit.

2.5.6 Analysis

The peripheral and aortic pulse pressure waveform data was exported from the SphygmoCor study database and saved as a Microsoft excel file. For each subject, the duplicate peripheral and aortic pulse pressure waveform recordings were averaged. For endothelial function assessment, the maximum changes in AIX, AIX@HR75, and T_r from baseline following GTN and salbutamol were calculated. Summary pulse pressure waveform data was exported into SPSS Base version 17.0 (SPSS, Inc., Chicago, Illinois) for statistical analysis.

2.5.7 Reproducibility study

2.5.7.1 Aims

The aims of the present study were: 1) to determine the reproducibility and sources of variation of: a) aortic wave reflections by pulse wave analysis and b) endothelial function assessment by pulse wave analysis and pharmacological challenge in healthy controls and subjects with the metabolic syndrome; 2) to establish clinically relevant changes, between two consecutive readings, in an individual, and 3) to establish the sample size requirements for intervention trials using pulse wave analysis alone and combined with pharmacological testing.

2.5.7.2 Methods

2.5.7.2.1 Subjects

Eighteen subjects were recruited; nine healthy male subjects, aged 40 – 65 years and nine age-matched male subjects with the metabolic syndrome as defined by the IDF 2005 guidelines (590). Control subjects were not taking medications that may influence endothelial function and did not have any clinical evidence of cardiovascular disease. None of the metabolic subjects had type 1 diabetes mellitus, type 2 diabetes mellitus using oral anti-glycemic agents or insulin, treated hypertension or existing vascular disease. Medication use among the subjects with the metabolic syndrome included statins (33%). All subjects were treated with aspirin (300mg/day) prior to the study in order to reduce the contribution of prostacyclin by inhibiting the release of endothelial cyclo-oxygenase (701). None of the study

subjects were a current smoker, had significantly deranged liver function (alanine aminotransferase >3 x upper level of normal), significant renal impairment (creatinine >150 µmol/L), or was receiving vitamin supplementation. The study was approved by the Upper South B Regional Ethics committee (NZ), and written informed consent was obtained from all participants.

2.5.7.2.2 Study design

Following a screening visit, eligible subjects attended two study visits one week apart. Subjects were asked not to alter their medication or undertake any significant life-style changes during the study and to refrain from taking medications on the morning of the visits. At each visit, subjects were assessed in the morning after a 12-hour overnight fast. Studies were conducted in a quiet, temperature controlled room. Subjects lay in the recumbent position for 10 minutes before assessments were performed. Brachial blood pressure and aortic wave reflection parameters were assessed by two different operators in a random order, twice at each visit. Endothelium-independent and -dependent vasodilation using pulse wave analysis combined with the administration of 150µg sublingual GTN and 400µg of inhaled salbutamol was assessed once at each visit, by the same operator. The protocol is described in detail in section 2.5.5. All haemodynamic and vascular assessments were made in duplicate and mean values were calculated for analysis.

2.5.7.2.3 Biochemistry

All biochemical analyses were performed by Canterbury Health Laboratories, an ISO15189 accredited (human) pathology laboratory. Plasma total cholesterol, triglycerides, and HDL-cholesterol were determined by an enzymatic colorimetric method (Aeroset analyser Model LN, Abbott Laboratories, Illinois IL, USA). LDL-cholesterol was calculated from the Friedewald equation. Plasma glucose, urea, creatinine and liver function were also measured (Aeroset analyser Model LN).

2.5.7.2.4 Statistical analysis

All statistical analyses were performed using SPSS Base version 17.0 (SPSS, Inc., Chicago, Illinois) or MedCalc version 11.5.0.0 (MedCalc Software, Broekstraat 52, 9030 Mariakerke, Belgium). Differences in variables between metabolic subjects and controls were tested using independent t-tests or Chi-square or the Fishers exact test, as appropriate. Absolute changes in variables after pharmacological challenge were assessed by paired t-tests

and compared between groups by independent t-tests. Standard variance decomposition methods (REML) were used to estimate the variance contributions to: 1) aortic stiffness measurements from four sources: repeats, intra-observer variation within-subjects for the same observer on the same day; week, intra-observer variation within subjects one week apart; observer, variation within-subjects on the same day; and subjects, variation between subjects; and 2) endothelial function from two sources; week: within subjects one week apart; and subjects, variation between subjects. All data are reported as means \pm SD. Statistical significance was inferred when $P < 0.05$ (two tailed).

Reproducibility of parameters was assessed by measuring the ICC and absolute RCVs were calculated as described in section 2.2.5.2.4.

Bland-Altman analysis was used to provide an indication of systematic bias and random error when comparing measurements taken from two different observers (702). The 95% limits of agreement were calculated using the equation:

$$d \pm 1.96 * SD$$

where: d = the sample bias (mean difference) and SD is the standard deviation of the differences.

Power calculations were undertaken to determine the sample sizes required to detect specified differences between groups in aortic wave reflection parameters for parallel and cross-over study designs, based on an 80% power at a significant level of $\alpha = 0.05$ (two-tailed). The calculations integrated all relevant sources of variation to determine the sample size.

2.5.7.3 Results

2.5.7.3.1 Study population characteristics

Baseline characteristics and haemodynamic parameters of the groups are summarised in Table 2.5. Mean $AIx@HR75$, AP and $AP@HR75$ were significantly higher in metabolic subjects ($p < 0.05$), and there was a trend towards higher AIx and T_r values in this group.

2.5.7.3.2 Aortic wave reflection parameters

2.5.7.3.2.1 Reproducibility of aortic wave reflection parameters

There were no significant differences in AIx, AIx@HR75, AP, AP@HR75 and T_r between repeated measurements on the same day for controls or metabolic subjects ($P>0.05$) (data not shown). Likewise AIx, AIx@HR75, AP, AP@HR75 and T_r also did not differ significantly between weeks 1 and 2 or between observers ($P>0.05$).

2.5.7.3.2.1.1 Intra-class correlation coefficients

Total ICC values for measurements of AIx, AIx@HR75, AP and AP@HR75 were 0.84, 0.86, 0.81, and 0.82, respectively, indicating excellent reproducibility, whereas the total ICC value for measurement of T_r was 0.62 (Table 2.6). In general, ICC values showed comparable reproducibility between subjects with and without the metabolic syndrome. The ICC values for repeated measurements of AIx and AIx@HR75 were ≥ 0.90 , indicating excellent reproducibility in both controls and subjects with the metabolic syndrome. Repeated measurements of AP and AP@HR75 also demonstrated excellent reproducibility with ICC values ≥ 0.84 , whereas T_r showed good reproducibility in both controls and subjects with the metabolic syndrome. AIx, AIx@HR75, AP and AP@HR75 were highly reproducible when measured at weekly intervals, providing ICC ≥ 0.85 , while T_r showed less reproducibility for healthy controls (ICC=0.75) than metabolic subjects (ICC=0.99). Between-observer measurements of AIx, AIx@HR75, AP, AP@HR75 and T_r demonstrated excellent reproducibility, with ICC values of 1.00 for all parameters.

2.5.7.3.2.1.2 Sources of variation

Between-subject variation accounted for 78.0 to 85.5% of the variability in AIx and AIx@HR75 measurements (Table 2.7). Similarly, 75.9 to 77.2% of the variability in AP and AP@HR75 measurements was explained by between-subject variation. In contrast, between-subject variation explained only 51.9 to 62.5% of the total variation in T_r measurements. The within and between-subject variation in each of the aortic wave reflection parameters was comparable between both groups, with the exception of T_r , where the week-to-week differences were substantially higher in healthy controls than metabolic subjects.

2.5.7.3.2.1.3 Bland Altman analysis

Bland-Altman analysis demonstrated good between-observer reproducibility for AP and AP@HR75, but higher between-observer variability in repeated measurements of T_r (Table 2.6). There was no evidence of systematic bias or a remarkable trend for the reproducibility of these parameters to vary with the underlying mean. Overall, the SD of the mean differences between repeated aortic wave reflection measurements did not differ between controls and metabolic subjects.

2.5.7.3.2.2 *Absolute reference changes values for aortic wave reflection parameters*

Table 2.8 shows the changes required to show significant differences in aortic wave reflection at the 95% confidence level (two-tailed) in an individual studied on two occasions, e.g. before and after a medical intervention. The table also shows the influence of additional repeat recordings taken at any given visit. For example, a subject who is studied once before and after an intervention would need to show a greater than 8.4% absolute reduction in AIx@HR75 to be confident that the change is due to a genuine effect and not merely a consequence of random effect variability. If the number of recordings increases to four per visit, the absolute RCV drops to 4.2%.

2.5.7.3.2.3 *Sample size calculations for clinical trials using aortic wave reflection parameters*

Appendix 1 shows the number of participants required in parallel and cross-over design studies to give an 80% power of detecting absolute differences in aortic wave reflection parameters between groups, at a significance level of $\alpha=0.05$ (two tailed). The sample size estimations also incorporate the number of recordings taken at any given visit (i.e. the average of 1, 2 or 3 recordings). For AIx@HR75; to detect a small difference (4%) 66 subjects are required for a parallel design, and 9 subjects for cross-over design. These sample sizes assume only one recording per visit; if the number of recordings increases to three, sample sizes drop to 63 and 6, respectively.

Table 2.5 Characteristics of study populations

	Healthy controls (n=9)	Metabolic Syndrome (n=9)	<i>P</i> -value*
Baseline characteristics			
Age, yr	50 ± 7	50 ± 8	0.86
Systolic blood pressure, mmHg	124.3 ± 10.2	128.4 ± 6.7	0.33
Diastolic blood pressure, mmHg	76.3 ± 4.6	80.6 ± 5.1	0.09
Height, m	1.8 ± 0.1	1.8 ± 0.1	0.47
Waist circumference, cm	89.9 ± 4.3	102.6 ± 2.8	<0.001
Body mass index, kg/m ²	24.9 ± 2.4	30.8 ± 2.8	<0.001
Glucose, mmol/L	4.7 ± 0.4	5.2 ± 0.6	0.06
Triglycerides, mmol/L	1.3 ± 0.3	2.3 ± 1.0	0.01
HDL-cholesterol, mmol/L	1.38 ± 0.23	1.12 ± 0.19	0.02
Central haemodynamic parameters			
Systolic blood pressure, mmHg	110.3 ± 4.5	117.9 ± 5.2	0.004
Diastolic blood pressure, mmHg	76.5 ± 3.6	80.5 ± 4.9	0.07
Mean arterial pressure, mmHg	91.2 ± 3.6	96.6 ± 4.5	0.01
SEVR	182.8 ± 23.9	170.3 ± 17.9	0.23
Heart rate, bpm	55.7 ± 5.3	58.1 ± 6.5	0.41
Wave reflection parameters			
AIx, %	18.2 ± 7.8	24.6 ± 7.0	0.08
AIx@HR75, %	9.0 ± 7.3	16.6 ± 6.2	0.03
AP, mmHg	6.1 ± 2.5	9.5 ± 3.7	0.04
AP@HR75, mmHg	2.7 ± 2.1	5.7 ± 2.4	0.01
T _r , msec	158.7 ± 9.9	149.5 ± 11.2	0.08

Values are mean ± SD or number (percentage). SEVR indicates subendocardial viability ratio; AP@HR75, augmentation pressure corrected for a heart rate of 75 bpm; AIx, augmentation index; AIx@HR75, augmentation index corrected for a heart rate of 75 bpm; T_r, time to reflection. *Between-group comparisons using independent t-tests.

Table 2.6 Reproducibility of pulse wave analysis measurements

Variable	Group	Total	Within-observer (1) Repeat 1 – Repeat 2	Within-observer (2) Week 1 – Week 2	Between-observer Operator 1 – Operator 2	
		ICC	ICC	ICC	ICC	Mean difference (SD)
AIx, %	Pooled	0.84	0.92	0.91	1.00	0.56 (7.26)
	Healthy	0.85	0.91	0.94	1.00	-0.17 (4.62)
	Metabolic	0.78	0.90	0.86	1.00	1.28 (9.10)
AIx@HR75, %	Pooled	0.86	0.93	0.92	1.00	0.46 (6.48)
	Healthy	0.84	0.91	0.92	1.00	-0.09 (5.82)
	Metabolic	0.81	0.91	0.88	1.00	1.01 (7.06)
AP, mmHg	Pooled	0.81	0.89	0.89	1.00	0.25 (3.32)
	Healthy	0.77	0.84	0.90	1.00	-0.28 (3.06)
	Metabolic	0.77	0.88	0.86	1.00	0.28 (2.36)
AP@HR75, mmHg	Pooled	0.82	0.90	0.90	1.00	0.00 (2.74)
	Healthy	0.76	0.85	0.88	1.00	-0.28 (3.06)
	Metabolic	0.76	0.88	0.85	1.00	0.28 (2.36)
T _r , msec	Pooled	0.62	0.67	0.89	1.00	0.36 (16.00)
	Healthy	0.52	0.63	0.75	1.00	-0.28 (14.00)
	Metabolic	0.63	0.63	0.99	1.00	1.00 (18.12)

Values are intra-class correlation coefficient (ICC), mean difference \pm SD of repeated measurements.

AIx, augmentation index; AIx@HR75, augmentation index corrected for a heart rate of 75 bpm; AP, augmentation pressure; AP@HR75 augmentation pressure corrected for a heart rate of 75 bpm; T_r, time to reflection.

Table 2.7 Sources of variation for pulse wave analysis measurements

	Group	Mean	Repeats	Week	Observer	Subjects
AIx, %	Pooled	21.4	5.5 (7.7)	5.8 (8.1)	0.0 (0.0)	59.8 (84.2)
	Healthy	18.2	5.7 (8.4)	3.9 (5.7)	0.3 (0.4)	58.0 (85.5)
	Metabolic	24.6	5.3 (8.8)	7.7 (12.9)	0.2 (0.3)	46.4 (78.0)
AIx@HR75, %	Pooled	12.8	4.2 (6.4)	4.9 (7.4)	0.0 (0.0)	56.9 (86.2)
	Healthy	9.0	5.0 (8.1)	4.6 (7.4)	0.0 (0.0)	52.0 (84.5)
	Metabolic	16.6	3.5 (7.7)	5.2 (11.5)	0.1 (0.3)	36.7 (80.6)
AP, mmHg	Pooled	7.8	1.4 (9.6)	1.4 (9.5)	0.0 (0.0)	12.1 (80.9)
	Healthy	6.1	1.1 (14.3)	0.7 (8.5)	0.0 (0.0)	5.9 (77.2)
	Metabolic	9.5	1.8 (10.1)	2.2 (12.7)	0.0 (0.0)	13.5 (77.2)
AP@HR75, mmHg	Pooled	4.2	0.8 (9.0)	0.9 (9.3)	0.0 (0.0)	7.5 (81.7)
	Healthy	2.7	0.8 (13.7)	0.6 (10.3)	0.0 (0.0)	4.7 (76.0)
	Metabolic	5.7	0.8 (10.3)	1.1 (13.9)	0.0 (0.0)	6.0 (75.9)
T _r , msec	Pooled	154.1	58.2 (30.9)	14.2 (7.5)	0.0 (0.0)	116.0 (61.6)
	Healthy	158.7	48.9 (30.4)	28.4 (17.7)	0.0 (0.0)	83.4 (51.9)
	Metabolic	149.5	67.5 (36.7)	1.5 (0.8)	0.0 (0.0)	115.1 (62.5)

Values are mean and the variance (percentage of total variance) associated with each source.

AIx, augmentation index; AIx@HR75, augmentation index corrected for a heart rate of 75 bpm; AP, augmentation pressure; AP@HR75, augmentation pressure corrected for a heart rate of 75 bpm; T_r, time to reflection.

Table 2.8 Absolute reference change values for aortic wave reflection parameters according to the number of recordings per visit

	Number of recordings per visit			
	1	2	3	4
AIx, %	9.3	6.6	5.4	4.6
AIx@HR75, %	8.4	5.9	4.8	4.2
AP, mmHg	4.7	3.3	2.7	2.3
AP@HR75, mmHg	3.6	2.5	2.1	1.8
T _r , sec	23.6	16.7	13.6	11.8

2.5.7.3.3 Endothelial function assessment

2.5.7.3.3.1 Mean haemodynamic changes relative to baseline after pharmacological challenge

Overall, there were significant mean reductions in AIx@HR75 and AIx with GTN in both groups ($P < 0.01$). The maximum changes in AIx@HR75 and AIx after GTN did not differ significantly between metabolic subjects and controls. Mean AIx@HR75 and AIx fell after administration of salbutamol in the two groups ($P < 0.001$), and there was a trend towards a blunted response in patients with the metabolic syndrome compared to controls for AP@HR75 ($P = 0.17$). Mean T_r was not altered significantly after GTN or salbutamol in either group ($P > 0.05$). GTN, but not salbutamol mediated reductions in mean MAP in both metabolic and control subjects ($P < 0.01$). Mean heart rate was increased following both GTN and salbutamol administration in the two groups ($P < 0.05$).

2.5.7.3.3.2 Reproducibility of haemodynamic parameters before and after pharmacological challenge

Table 2.9 shows the week-to-week repeated measurements of haemodynamic parameters following pharmacological challenge. There were no significant differences in baseline values between the two visits for controls or metabolic subjects (data not shown). The response to GTN and salbutamol did not differ between visits for controls. There was some evidence of systemic bias within the metabolic subjects; GTN-mediated decreases in AIx@HR75 ($P = 0.03$) and T_r ($P = 0.04$), and salbutamol-mediated increases in heart rate ($P = 0.04$) were significantly lower at the second visit in metabolic subjects.

2.5.7.3.3.2.1 Intra-class correlation coefficients

The ICC values for baseline AIX@HR75 and AIX were above 0.85 in the control group, indicating excellent reproducibility, and values were above 0.60 in the metabolic group, demonstrating good reproducibility (Table 2.10). Similarly, the GTN-mediated changes in AIX@HR75 and AIX were above 0.75, indicating very good reproducibility in both groups. The salbutamol-mediated response to AIX@HR75 also showed good reproducibility, with a higher ICC value in the metabolic subjects versus controls. In contrast, the ICC values for salbutamol-mediated changes in AIX indicated fair to moderate reproducibility in controls and metabolic subjects, respectively. ICC values for baseline T_r showed moderate to good reproducibility in controls and metabolic subjects, respectively, whereas ICC values for GTN and salbutamol-mediated changes in T_r were substantially lower in both groups.

2.5.7.3.3.2.2 Sources of variation

Between-week variation accounted for 8.3 – 40.1% of the variability in baseline AIX@HR75 and AIX measurements, and 7.5 – 24.1% of the variability in GTN-induced changes in AIX@HR75 and AIX measurements, with higher variability observed in the metabolic subjects (Table 2.11). In contrast, there was higher between-week variability for salbutamol-induced changes in AIX@HR75 and AIX measurements (24.6 – 50.9%), with more variation in the control subjects. Between-week variation explained 19.4 % and 56.2% of the total variation in T_r measurements in metabolic subjects and controls, respectively. Furthermore, between-week variability was substantially higher for GTN and salbutamol-induced changes in T_r , explaining 55.6 – 100% of the total variation in these measurements.

2.5.7.3.3.3 *Absolute reference changes values following pharmacological challenge with glyceryl trinitrate and salbutamol*

Table 2.12 shows the changes required to show significant differences in GTN- and salbutamol-mediated AIX@HR75 responses at the 95% confidence level (two-tailed) in an individual studied on two occasions, e.g. before and after a medical intervention. The table also shows the influence of additional repeat recordings taken at any given visit (i.e. the average of 1, 2, 3 or 4 recordings). For example, a subject who is studied once before and after an intervention would need to show a greater than 8.2% absolute reduction in the response of AIX@HR75 to salbutamol to be confident that the change is due to a genuine effect and not merely a consequence of random effect variability. If the number of recordings increases to four per visit, the absolute RCV drops to 4.1%.

Table 2.9 Repeated haemodynamic measurements following pharmacological challenge with glyceryl trinitrate and salbutamol

	Controls (<i>n</i> =9)			Metabolic syndrome (<i>n</i> =9)		
	First week	Second week	<i>P</i> -value	First week	Second week	<i>P</i> -value
GTN-mediated						
AIx@HR75 (%)	-15.3 ± 5.4***	-16.2 ± 5.4***	0.25	-17.6 ± 4.7***	-16.0 ± 3.6***	0.03
AIx (%)	-16.3 ± 5.8***	-17.3 ± 6.0***	0.35	-20.3 ± 5.9***	-18.0 ± 4.4***	0.06
T _r (ms)	5.7 ± 17.5	8.7 ± 12.8	0.63	6.8 ± 10.2	-1.2 ± 12.2	0.04
MAP (mmHg)	-5.9 ± 4.1**	-6.5 ± 3.8**	0.78	-6.7 ± 6.7*	-4.3 ± 3.2**	0.34
HR (bpm)	1.9 ± 3.4	2.0 ± 4.1	0.94	5.4 ± 5.2*	4.2 ± 4.1*	0.34
Salbutamol-mediated						
AIx@HR75 (%)	-8.2 ± 4.9**	-8.3 ± 4.0***	0.91	-5.3 ± 3.2**	-5.0 ± 2.5***	0.68
AIx (%)	-9.0 ± 5.8**	-10.3 ± 5.2***	0.58	-8.1 ± 5.0**	-5.5 ± 3.8**	0.08
T _r (ms)	-1.6 ± 4.9	-1.6 ± 9.8	0.99	7.0 ± 16.6	1.0 ± 15.5	0.57
MAP (mmHg)	-2.1 ± 7.7	-3.3 ± 4.9	0.69	-1.9 ± 7.0	-0.8 ± 4.4	0.73
HR (bpm)	1.7 ± 4.0	4.3 ± 4.3*	0.19	6.2 ± 5.0**	1.1 ± 3.2	0.04

Values are mean ± SD.

* *P*<0.05, ** *P*<0.01, *** *P*<0.001 for comparison of changes from baseline using paired t-tests.

GTN, glyceryl trinitrate; AIx@HR75, augmentation index corrected for a heart rate of 75 bpm; AIx, augmentation index; T_r, time to reflection; MAP, mean arterial pressure; HR, heart rate.

Table 2.10 Reproducibility of baseline haemodynamic measurements and changes following pharmacological challenge with glyceryl trinitrate and salbutamol

	Pooled (n=18)	Controls (n=9)	Metabolic syndrome (n=9)
AIx@HR75 (%)			
Baseline	0.87	0.92	0.60
Δ GTN	0.89	0.93	0.85
Δ Salbutamol	0.66	0.56	0.75
AIx (%)			
Baseline	0.83	0.91	0.62
Δ GTN	0.82	0.86	0.76
Δ Salbutamol	0.41	0.30	0.49
T_r (ms)			
Baseline	0.63	0.44	0.81
Δ GTN	0.36	0.32	0.44
Δ Salbutamol	0.00	0.00	0.00

Values are intra-class correlation coefficient (ICC).

AIx@HR75, augmentation index corrected for a heart rate of 75 bpm; AIx, augmentation index; T_r, time to reflection; Δ GTN, glyceryl trinitrate-mediated change; Δ Salbutamol; salbutamol-mediated change.

Table 2.11 Sources of variation for baseline haemodynamic measurements and changes following pharmacological challenge with glyceryl trinitrate and salbutamol

		Group	Mean	Days	Subjects
AIx@HR75, %	Baseline	Pooled	12.7	9.9 (13.3)	64.3 (86.7)
		Healthy	7.9	6.0 (8.3)	66.3 (91.7)
		Metabolic	17.4	13.8 (40.1)	20.6 (59.9)
	GTN	Pooled	-16.3	2.4 (10.9)	20.0 (89.1)
		Healthy	-15.8	2.2 (7.5)	27.0 (92.5)
		Metabolic	-16.8	2.7 (15.3)	15.0 (84.7)
	SAL	Pooled	-6.8	5.3 (34.1)	10.4 (65.9)
		Healthy	-8.3	8.7 (44.4)	10.9 (55.6)
		Metabolic	-5.2	2.0 (24.6)	6.2 (75.4)
AIx, %	Baseline	Pooled	21.5	13.4 (17.0)	65.2 (83.0)
		Healthy	17.4	7.1 (9.2)	70.1 (90.8)
		Metabolic	25.5	19.7 (37.8)	32.4 (62.2)
	GTN	Pooled	-18.0	5.8 (18.5)	25.5 (81.5)
		Healthy	-16.8	4.8 (13.7)	30.1 (86.3)
		Metabolic	-19.1	6.7 (24.1)	21.3 (75.9)
	SAL	Pooled	-8.3	15.6 (40.7)	10.7 (59.3)
		Healthy	-9.7	20.6 (69.9)	8.9 (30.2)
		Metabolic	-6.8	10.7 (50.9)	10.3 (49.1)
T _r , msec	Baseline	Pooled	157.3	75.8 (36.5)	131.6 (63.5)
		Healthy	160.2	108.3 (56.2)	84.4 (43.8)
		Metabolic	154.3	43.8 (19.4)	180.5 (80.6)
	GTN	Pooled	5.0	116.6 (64.2)	64.9 (35.8)
		Healthy	7.2	155.3 (68.2)	72.4 (31.8)
		Metabolic	2.8	77.8 (55.6)	62.1 (44.4)
	SAL	Pooled	1.2	243.1 (100.0)	0.0 (0.0)
		Healthy	-1.6	58.2 (100.0)	0.0 (0.0)
		Metabolic	4.0	428.1 (100.0)	0.0 (0.0)

Values are mean and the variance (percentage of total variance) associated with each source. AIx@HR75, augmentation index corrected for a heart rate of 75 bpm; AIx, augmentation index; T_r, time to reflection; ΔGTN, glyceryl trinitrate-mediated change; ΔSalbutamol; salbutamol-mediated change.

Table 2.12 Absolute reference change values following pharmacological challenge with glyceryl trinitrate and salbutamol according to the number of recordings per visit

	Number of recordings per visit			
	1	2	3	4
AIx@HR75 Δ GTN (%)	4.1	2.9	2.4	2.0
AIx@HR75 Δ Salbutamol (%)	8.2	5.8	4.7	4.1

2.5.7.3.3.4 Sample size calculations for clinical trials for endothelial function assessment

Sample size estimates are presented in Appendix 2. Analysis of the combined cohorts in our study indicates that in a parallel design, 16 participants per group will provide an 80% power for detecting an absolute difference in salbutamol-induced changes in AIx@HR75 of 4%, at a significant level of $\alpha=0.05$ (two tailed). In a cross-over design, 5 participants are needed to give an 80% power of detecting an absolute difference in salbutamol-induced changes in AIx@HR75 of 4%, at a significance level of $\alpha=0.05$ (two tailed).

2.5.7.4 Discussion

2.5.7.4.1 Pulse wave analysis

The present study demonstrated high within- and between-observer reproducibility of aortic wave reflection parameters: AIx, AI@HR75, AP and AP@HR75 derived from radial tonometry in subjects with the metabolic syndrome and healthy controls, but lower reproducibility for T_r . The ICC provides an appropriate statistical method for measuring consistency and conformity of repeated measurements, but to date few studies have used this method to assess the reproducibility of aortic wave reflections. Bland-Altman analysis has been frequently reported in previous studies of aortic wave reflection indices (595, 675, 682, 685, 703-711). The between-observer SD's for AIx and AIx@HR75 were comparable to that reported in prior studies (675, 703, 704, 709-712), as were the SD's for AP (703, 712) and T_r (704, 711). Bland-Altman analysis however is more suitable for assessing systematic bias and random error in repeated measurements, than reproducibility.

Several previous trials have reported CVs for AIx@HR75 and AP (682, 685, 696); however, the CV is considered an inappropriate index of variability when values of the measured parameter range close to zero as for AIx and AP uncorrected and heart rate

corrected, particularly in healthy controls (675, 682). In such cases, high CV values that have been observed may not necessarily indicate poor reproducibility. Furthermore, the use of the Pearson correlation coefficient (r) may not be appropriate in reproducibility studies because it measures association and not concordance, and thus a high correlation between repeated measurements does not always reflect good reproducibility (713). On the basis of the limitations in these statistical approaches, the CV and r were not calculated.

ICC values showed similar reproducibility of aortic wave reflection parameters between controls and subjects with the metabolic syndrome. The high ICC values (≥ 0.90) observed for within-observer repeated measurements of AIx in controls and metabolic subjects concur with a prior study in an undefined patient group (709). ICC values for week-to-week assessments of AIx and AIx@HR75 were high (≥ 0.86 and ≥ 0.88 , respectively), similar to values obtained in other reproducibility studies where measurements were taken at hourly or weekly intervals (682, 685, 696). Week-to-week measurements of T_r showed higher ICC values than reported by Papaioannou et al. (2009) (685), but substantially lower values than reported in two earlier studies in healthy controls and patients with chronic kidney disease (682, 711). ICC values for between-observer measurements of AIx and AIx@HR75 were 1.00, indicating excellent reproducibility, consistent with two previous trials (709, 710). AP, AP@HR75 and T_r showed excellent between-observer reproducibility (ICC=1.00). Overall, the total ICC values, which incorporated all sources of within observer variation, showed excellent reproducibility for AP and AIx, both uncorrected and corrected, but lower reproducibility for T_r .

Few trials have established the within-observer reproducibility, both repeats and week-to-week, and between-observer reproducibility of aortic wave reflection parameters in any one investigation (703, 704, 711). On this basis, the variance components for measurements of aortic wave reflection were reported from all four sources. The within-subject variation for AIx, AIx@HR75, AP, and AP@HR75 was low, with the majority of the variability existing between-subjects, indicating good reproducibility of measurements. In contrast, T_r showed higher within-subject variability, resulting from higher variation between repeats and week-to-week measurements. However, between-observer variation in measurements was minimal for all aortic wave reflection parameters, reflecting the excellent reproducibility of this technique between operators.

RCVs were also established for wave reflection indices. From a clinical perspective, the RCV should be taken into account, when evaluating the effects of interventions on aortic

wave reflection indices in individuals to ensure responses are real and not due to biological variation. By performing several studies before and after treatment, the likelihood of showing a real effect is increased. The magnitude of clinically important changes in AIx is 10% (714), which is larger than the absolute RCV, even when measured once on two occasions, thus individuals could be confident of a significant change in AIx in an individual, highlighting the potential clinical utility of this technique.

Sample size requirements were established for future cross and parallel design trials by integrating all sources of within- (repeats, weeks, and operator) and between-subject variation obtained from repeated measurements of wave reflection indices. The sample size estimates also reflect the number of recordings taken per study visit, with an increase in the recordings, reducing the sample size requirement.

2.5.7.4.2 Endothelial function assessment

The present study demonstrated good short term reproducibility of endothelium-dependent and independent vasodilation by pulse wave analysis combined with pharmacological challenge for AIx@HR75 in healthy controls and metabolic subjects. GTN-mediated changes in AIx were also highly reproducible, but salbutamol-mediated changes in AIx showed lower reproducibility in subjects with and without the metabolic syndrome. AIx has previously been demonstrated to be influenced by heart rate, explaining the lower reproducibility of AIx uncorrected for heart rate observed (688).

The good reproducibility concurs with two previous studies (33, 695). Wilkinson *et al.* (2002) (33) assessed the reproducibility of changes in AIx after GTN and salbutamol challenge in healthy controls using Bland-Altman analysis. Hayward *et al.* (2002) (695) reported good reproducibility of GTN and salbutamol-mediated changes in the peripheral AIx using Bland-Altman analysis and correlation coefficients. The findings contrast to a recent study by Paul *et al.* (2009) (696) where ICC values for GTN- (0.58 and 0.17, respectively) and salbutamol-mediated (0.18 and 0.04) changes in AIx@HR75 showed poor reproducibility in controls and patients with heart failure (CHF). However, endothelial function assessments were repeated at hourly intervals, and the authors suggest the poor reproducibility may be due to diurnal variability - as has been observed with other established techniques - or alternatively due to a carry-over effect from the first study assessment (696).

To date, no studies have reported the reproducibility of T_r changes following GTN and salbutamol administration. Both GTN and salbutamol-induced changes in T_r revealed poor reproducibility. The ICC value for baseline T_r measurements was high for metabolic subjects, but low for the control group. A recent study reported similarly low ICC values for baseline T_r (685). Taken together, the current study demonstrates that the optimal parameter for estimating endothelial function from PWA is $AIx@HR75$.

RCVs were established for GTN and salbutamol-mediated responses in $AIx@HR75$, to allow assessment of the effect of interventions in individual patients. Performing several studies before and after an intervention will maximise the chances of showing a real benefit that is not due to spontaneous variation. Sample size tables were constructed from the variance estimates of GTN and salbutamol-mediated responses in $AIx@HR75$ to guide future clinical trials.

2.5.8 Advantages and clinical utility of applanation tonometry and pulse wave analysis

Applanation tonometry and pulse wave analysis of the radial artery provides a reproducible, non-invasive tool for the assessment of central blood pressures and wave reflection indices. The radial artery is easily accessible; the examination can be performed within a short-time frame and is well tolerated. Furthermore, operators require minimal training in the use of this technique. This methodology has the advantage of providing additional data regarding the status of the cardiovascular system, over and above that obtained from peripheral blood pressures. There is increasing evidence to support the clinical utility of applanation tonometry and pulse wave analysis for the estimation of central blood pressures and wave reflection indices. Physiologically, central blood pressures are more relevant to ventricular-vascular coupling compared to peripheral blood pressures (673). There is cumulating evidence to demonstrate the independent and often additional pathophysiological relevance of central blood pressure compared to peripheral blood pressure (673, 714-718). Furthermore, a differential impact of blood pressure-lowering drugs on central aortic pressure and clinical outcomes has been reported (718-721). This highlights the potential value of the pulse wave analysis methodology for elucidating beneficial mechanisms when clinical trials with similar blood pressure control have discordant outcomes depending on the drug assignment. There is also a growing evidence base to support the prognostic implications of

increased central arterial wave reflections (714, 722, 723). A number of studies have shown that central arterial wave reflections have the capacity to independently predict the risk of cardiovascular events and mortality in various populations (691, 722-724), including a recent meta-analysis (714).

A number of issues will need to be addressed prior to the implementation of the pulse wave analysis technology into clinical practice. Further studies would be required to establish whether central blood pressures provide incremental value over and above peripheral blood pressures, and to provide additional evidence to support the prognostic value of aortic wave reflection indices. Investigations must also determine the prospective clinical benefit that may be gained by the modification of central blood pressure and aortic wave reflection indices. In addition, normal values or treatment targets for central blood pressure and aortic wave reflection indices must to be ascertained. Recent data from the Anglo-Cardiff Collaborative trial (725), and the European Project on Genes in Hypertension, in addition to ongoing studies from the European Network for Non-invasive Determination of Large Arteries should contribute to a greater understanding of the pathophysiology of central blood pressures and aortic wave reflections (726). Although specific guidelines do not currently exist for pulse wave analysis measurements, several recommendations have been proposed (700). Despite this, strict guidelines need to be established to ensure the standardisation of the pulse wave analysis protocols across international research centres before this technique could be employed in the clinical setting. With further evidence from therapeutic and outcome trials, and following the establishment of normal values, it is likely that pulse wave analysis may become increasingly used by physicians in conjunction with peripheral blood pressure recordings to manage and monitor therapeutic responses in the clinical environment.

2.5.9 Limitations of applanation tonometry and pulse wave analysis

There are several limitations regarding the measurement of central pulse pressures and aortic wave reflection parameters using applanation tonometry and pulse wave analysis. Firstly, the currently available non-invasive techniques only provide an estimation of central blood pressure and aortic wave reflection indices and not a direct measurement. Although the validity of the radial to aortic transfer function is well established for the measurement of central blood pressure (679, 727), the validity of this approach has been questioned for

measurement of aortic wave reflection indices, such as the AIx (728, 729). Secondly, since brachial artery pressure is measured as a surrogate of radial artery pressure for calibration of central blood pressure, this can introduce errors in central blood pressure measurements (730), whereas indices such as the AIx is a relative measurement and thus independent of calibration errors (731, 732). A further issue is that pulse wave analysis cannot be reliably used on patients with arrhythmia or severe ventricular dysfunction (733). Furthermore, specific central pressure and aortic wave reflection treatment targets for radial artery applanation tonometry have yet to be well defined (734). While the pulse wave analysis technique has demonstrated good reproducibility for measurement of central pulse waveform and aortic wave reflection parameters (675, 682, 703-706, 735), the variability of the non-invasive assessment of endothelial dependent and independent vasodilation using pulse wave analysis combined with pharmacological challenge has not always been consistently reported (33, 695, 696). The reproducibility of this technique would need to be improved before being incorporated as a research tool to assess endothelial function in clinical studies. Furthermore, endpoint studies would be required to establish the prognostic value of this endothelial function technique for assessing cardiovascular risk to improve its potential clinical utility.

3 Effect of Coenzyme Q₁₀ Supplementation on Endothelial Function in Simvastatin-Treated Patients with the Metabolic Syndrome

3.1 Background

The metabolic syndrome, characterised by the presence of visceral obesity, dyslipidaemia, glucose dysregulation, and hypertension (736), is associated with an increased risk of cardiovascular morbidity and mortality (737-741). Cardiovascular disease is preceded by endothelial dysfunction, a pivotal yet potentially reversible step that has been shown to predict cardiovascular events (742, 743), and has been implicated in the pathogenesis of the metabolic syndrome (744-750) and its components (22-24, 751-753). Increased oxidative stress in patients with the metabolic syndrome (754), has been proposed as a mechanism leading to endothelial dysfunction through uncoupling of mitochondrial oxidative phosphorylation and endothelial nitric oxide synthase (eNOS) activity, resulting in reduced NO bioavailability (12).

Statins are widely used in the management of atherogenic dyslipidaemia in patients with the metabolic syndrome and significantly reduce cardiovascular events (63, 77, 755-757). However, residual cardiovascular risk still persists (540-542). Statins inhibit conversion of HMG-CoA to mevalonate, but also decrease production of other intermediates in the cholesterol biosynthetic pathway. One such intermediate is coenzyme Q₁₀ (CoQ₁₀), an endogenous lipophilic antioxidant and a key cofactor in the mitochondrial electron transport chain (Chapter 1, section 1.3.3.3.1). Plasma CoQ₁₀ concentrations are reduced in patients receiving statin treatment (186-203), with the magnitude of CoQ₁₀ depletion suggesting a dose-dependent effect (188, 196). In a previous study, the statin-induced reduction in plasma CoQ₁₀ levels was greatest in those patients with the most pronounced improvement in endothelial function after atorvastatin therapy (758). This has led to speculation that statin-induced reductions of CoQ₁₀ levels may be limiting the maximum favourable effect of statins on peripheral endothelial function (758).

CoQ₁₀ supplementation may improve endothelial function through reductions in oxidative stress (12, 363, 532). Studies have reported enhanced endothelium-dependent vasodilation with CoQ₁₀ in patients with high cardiovascular risk, including those with chronic heart failure (522), ischaemic heart disease (368, 370), and type 2 diabetes mellitus (369, 371). To date, two studies have investigated the effect of CoQ₁₀ supplementation on endothelial function in statin-treated patients, with contrasting findings. Keuttner *et al.* (2005) (759) found no further improvement in flow-mediated dilation (FMD) of the brachial artery in males with manifest endothelial dysfunction receiving treatment with combined CoQ₁₀ and cerivastatin compared with statin or CoQ₁₀ monotherapy. In contrast, Hamilton *et al.* (2010) (371) demonstrated an improvement in FMD following CoQ₁₀ supplementation in statin-treated type 2 diabetic patients with endothelial dysfunction despite LDL-cholesterol levels <2.0 mmol/L. However, these patients were not on standardised statin therapy. Further studies are required to confirm whether adjunctive CoQ₁₀ therapy can ameliorate endothelial dysfunction in statin-treated patients.

3.1.1 Study hypotheses

The hypothesis for this study was that CoQ₁₀ supplementation will improve endothelial dysfunction in statin-treated patients with the metabolic syndrome. A further hypothesis was that plasma CoQ₁₀ levels are a surrogate marker for the extent of improvement in endothelial function. Accordingly, the effect of CoQ₁₀ treatment on endothelial function in males with the metabolic syndrome was studied in a randomised, double-blind, placebo-controlled 12-week cross-over trial that assessed FMD of the brachial artery, arterial stiffness, aortic wave reflections, blood pressure, plasma concentrations of CoQ₁₀, lipid profiles, glycaemic indices, inflammation, and albumin excretion.

3.2 Methods

3.2.1 Subjects

Thirty males, aged 35–65 years, with the metabolic syndrome as defined by International Diabetes Federation (IDF) guidelines were recruited. All subjects had a waist circumference ≥ 94 cm, triglycerides ≥ 1.7 mmol/L, and a systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg or treatment for hypertension. Participants were

stabilised on all therapies for at least one month prior to screening. Patients were statin naïve or treated with low dose statins at study entry. Subjects were excluded from the study if they had a history of coronary angioplasty, coronary stent placement, coronary bypass surgery, myocardial infarction, or a history of cerebrovascular accident within the 6 months prior to screening, history of unstable angina pectoris, symptomatic chronic heart failure requiring treatment, atrial fibrillation, aortic valve stenosis, peripheral vascular disease, type 1 diabetes mellitus or type 2 diabetes mellitus requiring oral anti-diabetic agents or insulin. Further exclusions were pre-treatment LDL-cholesterol levels <1.5 mmol/L or >6.5 mmol/L, pre-treatment triglyceride levels >5 mmol/L, uncontrolled hypertension (blood pressure $>160/100$ mmHg), significantly deranged liver function tests (alanine aminotransferase ≥ 3 x upper level of normal), significant renal impairment (plasma creatinine >150 μ mmol/L), body mass index >40 kg/m², other significant co-morbidities, current smokers, intolerance to statin therapy, treatment with ≥ 40 mg simvastatin daily or equivalent, treatment with other lipid modifying agents, or antioxidant vitamin supplementation, including CoQ₁₀.

3.2.2 Study design

This was a randomised, double-blind, placebo-controlled 12-week cross-over study of CoQ₁₀ supplementation in statin-treated males with the metabolic syndrome (Figure 3.1). Patients attended a screening visit to assess eligibility. After a two week run-in period of simvastatin therapy (40mg/day), eligible patients were randomised to treatment with either CoQ₁₀ (ChewQ®, Tishcon Corp, USA), 200 mg twice daily or placebo for 12 weeks, followed by a four-week washout period, and then received the alternative treatment for a further 12 weeks. Compliance with treatment was assessed at the end of each intervention through tablet count. A tablet compliance of $\geq 80\%$ was considered acceptable. Assessments were performed at both pre and post treatment periods. Patients underwent testing at the same time of the morning after a 12-hr overnight fast, in a quiet, air-conditioned room with a stable temperature of 22⁰C for the following: pulse wave analysis, brachial ultrasound, blood and urine sample collection and haemodynamic and anthropometric measurements. Subjects refrained from taking medications and undertaking strenuous exercise on the morning of the visits. Vascular function measurements were undertaken by a dedicated investigator. Patients were asked to maintain their usual diets and physical activity and not to alter their medication or undertake any significant lifestyle changes during the interventional period. Any adverse events were documented. The study protocol was approved by the Upper South B Regional Ethics Committee (NZ), and written informed consent was obtained from all participants.

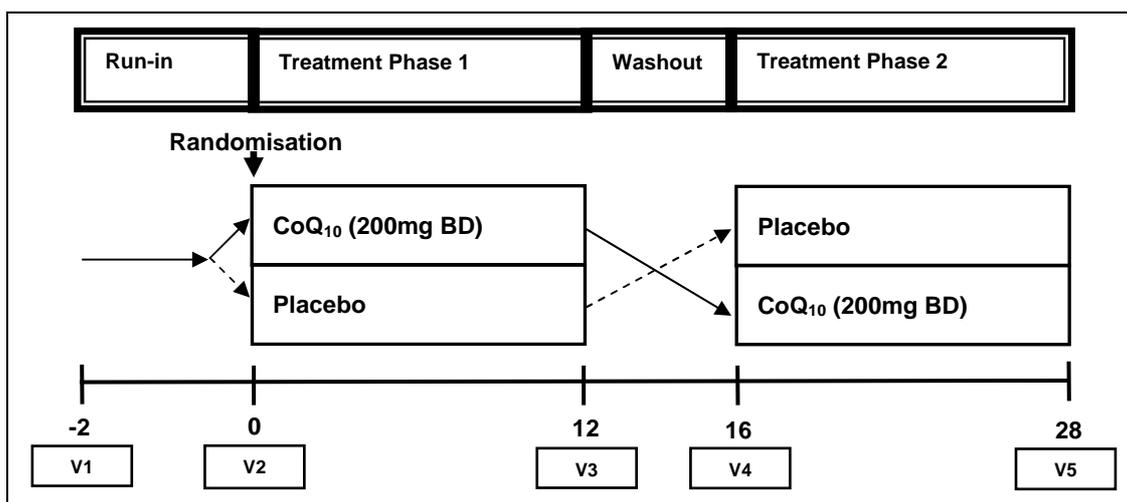


Figure 3.1 Summary outline of the 12-week randomised, double-blind, placebo-controlled, cross-over study of CoQ₁₀ supplementation in statin-treated males with the metabolic syndrome

3.2.3 Outcome measures

The primary outcome was 12-week changes in absolute FMD (%) of the brachial artery. Secondary outcomes were 12-week changes in absolute peak diameter change post-ischæmia, time to peak FMD, baseline and peak velocities and shear rates, mean EID, absolute peak diameter change post-GTN, peripheral and central haemodynamics, anthropometrics, and biochemical parameters, including plasma CoQ₁₀ concentrations.

3.2.4 Brachial artery ultrasound

3.2.4.1 Protocol

Conduit artery function was assessed by brachial ultrasound according to the protocol described in detail in Chapter 2, section 2.2.3. Briefly, the participant rested in a recumbent position for at least 20 minutes prior to commencement of vascular measurements. Endothelium-dependent vasodilation was assessed using the standard FMD protocol. Endothelium-independent vasodilation (EID) was assessed using a sublingual glyceryl trinitrate challenge.

To assess FMD, the left arm was placed in a specially designed cradle at approximately 80 degrees from the torso. A pneumatic cuff (Hokanson, Inc., Seattle, Washington) was

placed around the forearm, distal to the insonated artery. The ultrasound transducer was carefully positioned and fixed on the brachial artery using a specialised stereotactic clamp. After the 20 minute rest period, a baseline scan was recorded for one minute to assess resting brachial artery diameter and blood velocity. The occluding cuff was then rapidly (1-2 seconds) inflated to a pressure of approximately 50 mmHg above the systolic blood pressure for five minutes. Diameter and blood velocity recordings resumed 30 seconds before cuff deflation and continued for 2.5 minutes following cuff release.

To assess EID, 10 minutes rest followed the completion of the FMD test to allow arterial diameter to return to baseline. A second baseline recording was made for one minute. Maximum EID was then assessed after administration of 400 µg of sublingual glyceryl trinitrate (one spray). Diameter and blood velocity recordings recommenced two minutes after GTN administration and were continued for at least four minutes.

Systolic and diastolic blood pressures were measured in duplicate on the non-imaged arm using a validated oscillometric sphygmomanometer (BpTRU BPM-200, BpTRU Medical Devices, Coquitlam, BC Canada) following recordings obtained at baseline, post-ischæmia, second baseline, and post-GTN. Recordings were by the same investigator, and the data acquisition and frame analyses were completed prior to the unblinding of the study medication allocation.

3.2.4.2 Ultrasound diameter measurements

High resolution, B-mode ultrasound measurements were made using a duplex Acuson Antares™ Ultrasound System (Siemens Medical Solutions, USA), equipped with a 4-9 MHz linear array transducer (VFX 9-4). The brachial artery was imaged in the longitudinal plane 3-7 cm proximal to the antecubital fossa. A probe holding stereotactic clamp enabled precise positioning and ensured that pressure on the artery was minimised. Care was taken to ensure that the vessel clearly extended across the entire [un-zoomed] imaging plane to minimise the likelihood of skewing the vessel walls. Magnification and focal zone settings were adjusted to optimise imaging of the proximal and distal vessel walls. Ultrasound global (acoustic output, gain, dynamic range, gamma, and rejection) and probe-dependent (zoom factor, edge enhancement, frame averaging, and target frame rate) settings were standardised.

3.2.4.3 Diameter analysis

Ultrasound images were captured using a Toshiba Laptop equipped with a video capture device (XH3371, Dick Smith Electronics, New Zealand). Video files were collected at 25 frames per second and converted to Joint Photographic Experts Group (JPEG) images and subsequently used to make 25 diameter measurements per second. JPEG images provide comparable accuracy for ultrasound image measurements compared to the Digital Image and Communications in Medicine (DICOM) standard (582). Images were measured offline using semi-automated edge-detection software custom written to interface with the LabVIEW data acquisition platform (version 8.1, National Instruments, Austin, Texas) (583, 584). Custom written Excel Visual Basic code was used to fit peaks and troughs to diameter waveforms in order to calculate systolic, diastolic and mean diameters. Mean diameters were used for analysis. A recent study demonstrated that calculating FMD based on mean diameters, produces comparable results to calculations based on end-diastolic diameters (585). FMD and EID were expressed as the percentage increase in vessel diameter from the preceding baseline $[(\text{peak diameter post-ischaemia or GTN administration} - \text{baseline diameter}) / \text{baseline diameter}] \times 100$. The time to peak diameter (seconds) was calculated from the point of cuff deflation to the maximum diameter post-ischaemia. In our vascular laboratory, between-week coefficients of variation are 4.7 % for resting diameter measurements and 4.5 % and 4.1 % for peak diameter measurements post-ischaemia and -GTN, respectively.

3.2.4.4 Blood velocities

Continuous Doppler velocity measurements were also made using the Acuson Antares™ Ultrasound System. Sonication angle was kept constant at 45-60°. Pulse repetition frequency was adjusted between stages of testing to prevent aliasing. Blood flow velocities were calculated using the vascular package supplied with the Acuson Antares™ ultrasound machine and manually recorded offline from the JPEG images. Obvious outliers were removed and missing values were replaced using linear interpolation. In our vascular laboratory, between-week coefficients of variation for resting velocity measurements and peak velocity measurements are 27.6 % and 13.8 %, respectively.

3.2.4.5 Shear rate

The 25 diameter measurements/second were aggregated to 1 per second and synchronized with blood velocities. Shear rates were calculated as:

Shear rate (s^{-1}) = (8 * mean blood velocity) / diameter

Time averaged maximum velocities were used to calculate the shear rate. Time averaged maximum velocity is the average of the highest velocities throughout the cardiac cycle. Time averaged blood are more reliable when compared to time averaged mean blood velocities (586).

Shear rates were calculated at: resting baseline, peak post-ischaemia, second resting baseline and maximum GTN.

3.2.4.6 Arterial stiffness

The brachial artery β stiffness index, distensibility coefficient (DC) and compliance coefficient (CC) were determined from resting artery diameters and brachial blood pressures. The β arterial stiffness index was calculated as:

$$\beta \text{ stiffness index} = \ln (P_s/P_d) / (\Delta D/D_d)$$

where: P_s is the brachial systolic blood pressure in mmHg, P_d is the brachial diastolic blood pressure in mmHg, ΔD is the change in diameter during the cardiac cycle (or distension) in mm, and D_d the end-diastolic arterial diameter in mm (760).

The distensibility coefficient (DC) reflects the arterial elastic properties. The DC was calculated as:

$$DC (10^{-3}k/Pa) = (\Delta A/A) / \Delta P = (2\Delta D * D_d + \Delta D^2) / (\Delta P * D_d^2)$$

where: ΔD is the change in diameter during the cardiac cycle (or distension) in mm, and D_d the end-diastolic arterial diameter in mm, and ΔP the brachial pulse pressure (systolic minus diastolic blood pressure) in kPa (761).

The compliance coefficient (CC) reflects the local arterial compliance. The CC was calculated as:

$$CC (mm^2/kPa) = (\Delta A/A) / \Delta P = \pi(2\Delta D * D_d + \Delta D^2) / 4\Delta P$$

where: ΔD is the change in diameter during the cardiac cycle (or distension) in mm, and D_d the end-diastolic arterial diameter in mm, and ΔP the brachial pulse pressure (systolic minus diastolic blood pressure) in kPa (761).

3.2.5 Pulse wave analysis

Aortic wave reflections were assessed by pulse wave analysis using applanation tonometry of the radial artery with the SphygmoCor version 7.1 software (AtCor Medical, Sydney, Australia) according to Wilkinson *et al.* (675) (Chapter 2, section 2.5.5.1). After five minutes lying in the recumbent position, systolic and diastolic blood pressure was measured in duplicate in the dominant arm using a validated oscillometric sphygmomanometer (BpTRU BPM-200, BpTRU Medical Devices, Coquitlam, BC Canada). Briefly, a high-fidelity micromanometer (SPC-301; Millar Instruments, TX, USA) was used to obtain recordings of peripheral pressure waveforms by applying gentle pressure over the non-dominant radial artery. Data was collected directly into a microcomputer and recordings were assessed visually to ensure that the best possible recording was obtained and with minimisation of movement related artefacts. After 20 sequential waveforms were acquired, an averaged peripheral waveform was generated. A corresponding central waveform was then derived from a validated transfer function, and from this the augmentation index (AIx), ascending aortic pressure, and heart rate, were determined using the integral software. The AIx was defined as the difference between the first and second systolic peaks of the central arterial waveform (augmentation pressure (AP)), expressed as a percentage of the pulse pressure (PP), ($AIx = AP / PP \times 100\%$). Since the AIx is influenced by heart rate, an index normalised for a heart rate at 75 bpm ($AIx@HR75$) was used in accordance with Wilkinson *et al.* (2000) (688). Blood pressure and pulse wave analysis measurements were repeated after a two minute interval. All assessments were made in duplicate and mean values were calculated for analysis. In our vascular laboratory, between-week intra-class correlation coefficients for pulse wave analysis parameters are 0.92 for $AIx@HR75$, 0.91 for AIx, 0.90 for AP and $AP@HR75$, and 0.89 for T_r .

3.2.6 CoQ₁₀ formulation

Both Q-Gel® and placebo were supplied by Tischon Corp, USA. ChewQ® contained 100mg of CoQ₁₀, in a base (xylitol, orange flavour, silicon dioxide and magnesium stearate). This CoQ₁₀ preparation was used because it has similar absorption to Q-Gel®, which has been

shown to have significantly better bioavailability compared to other CoQ₁₀ formulations (416). Molyneux *et al.* (2007) have previously shown achievement of optimal plasma levels at 200 mg (408). Twice daily dosing with 200mg was selected in the hope of achieving higher plasma CoQ₁₀ levels. During the treatment phases, patients allocated to CoQ₁₀ took four CoQ₁₀ tablets per day (2 Bid), and those allocated to placebo took four placebo tablets daily (2 Bid). Randomisation into 1 of 2 sequences (active placebo or placebo active) was performed in permuted blocks of six from a computer generated randomisation list. The study treatments were dispensed by an independent hospital pharmacist in identical numbered bottles with the lowest available number allocated to each sequential participant. Participants and investigators administering the treatment and assessing outcomes were blinded to treatment assignment and to plasma CoQ₁₀ levels.

3.2.7 Biochemical parameters

All biochemical analyses were performed by Canterbury Health Laboratories, an ISO15189 accredited (human) pathology laboratory. Total plasma CoQ₁₀ was measured by reverse phase high performance liquid chromatography with electrochemical detection using a method described by Molyneux *et al.* (2007) (408). Plasma total cholesterol, triglycerides, and HDL-cholesterol were determined by an enzymatic colorimetric method (Architect c8000 analyser, Abbott Laboratories, Abbott Park, Illinois, U.S.A.). LDL-cholesterol was calculated from the Friedewald equation. High sensitivity C-reactive protein (hs-CRP) levels were determined by rate nephelometry. Safety markers, including electrolytes, plasma glucose, renal and liver function (Architect c8000 analyser), HbA1c (Biorad Variant HPLC), and a full blood count (Coulter Electronics, Luton, UK) were measured.

3.2.8 Statistical analyses

The sample size was based on published estimated treatment effects from a study examining the effect of CoQ₁₀ on endothelial function of the conduit arteries in patients with type 2 diabetes (369). A total of 30 patients in a cross-over design was calculated to provide sufficient power (90%) to detect a statistically significant (two-tailed $\alpha=0.05$) difference in the change from baseline between placebo and CoQ₁₀ of 1.8% for FMD with a standard deviation of 2%, allowing for 10% attrition rate.

All statistical analyses were performed using SPSS Base version 17.0 (SPSS, Inc., Chicago, Illinois). Comparison of changes from baseline for the primary and secondary variables, between placebo and treatment cross-over phases was tested using analysis of variance (ANOVA) with repeated measures. For *post-hoc* subgroup analyses, the influence of potential mitigating factors including the sequence of treatment, presence of cardiovascular disease, treatment with ACE inhibition, aspirin, beta blockade, CoQ₁₀ levels achieved, changes in CoQ₁₀ levels on treatment, and compliance to CoQ₁₀ treatment on changes in the primary outcome were analysed using these variables as between subject factors in ANOVA with repeated measures. If significant effects were found with the analyses (i.e. an interaction between subgroup and the effect of CoQ₁₀), subgroups could be further analysed using Fisher's protected LSD tests. Differences in changes are shown so that the positive value for any of the indices indicate an advantage to CoQ₁₀ in terms of brachial artery function, and haemodynamic, anthropometric and biochemical parameters. Correlations were performed using Pearson's correlation coefficient. Data are expressed as mean (SEM) and categorical data are presented as percentages. Statistical significance was inferred when $P < 0.05$.

3.3 Results

3.3.1 Baseline characteristics

Thirty eight patients were screened; of whom seven were not eligible to participate in the trial and one refused to participate. One participant was withdrawn from the study due to personal reasons, and thus a total of 29 patients completed the trial and were included in the analysis (Figure 3.2). Baseline characteristics are shown in Table 3.1. Overall compliance rates based on tablet counts were $96.4 \pm 1.0\%$ for CoQ₁₀ therapy and $95.6 \pm 1.2\%$ for placebo ($P=0.56$). Compliance to statin therapy was $96.3 \pm 2.1\%$ during the run-in phase, $95.3 \pm 1.4\%$ during the washout phase, $96.2 \pm 1.2\%$ during CoQ₁₀ supplementation, and $92.3 \pm 2.4\%$ during placebo administration ($P=0.32$). CoQ₁₀ treatment was well tolerated and not associated with any significant adverse effects. The sequence of treatment had no effect on the differences in the changes in FMD (i.e. there was no significant difference when comparing the results of the participants who were treated with the CoQ₁₀ first versus those who were first treated with the placebo (data not shown)).

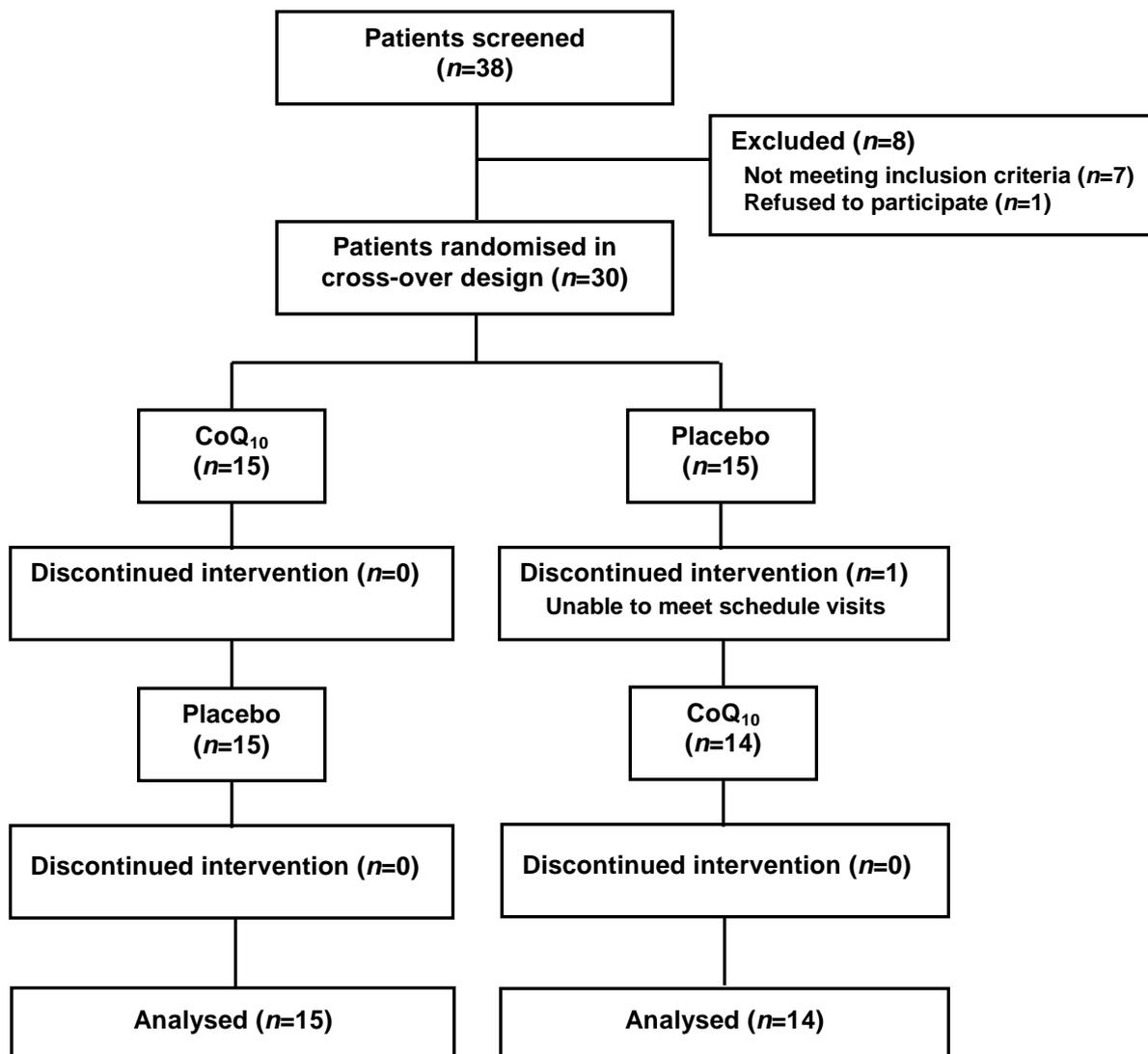


Figure 3.2 The flow of participants through the study

Table 3.1 Baseline characteristics of participants at screening

Parameter	(n=29)
Demographics and clinical characteristics	
Age, y	56 ± 8
SBP, mmHg	134 ± 11
DBP, mmHg	84 ± 8
Heart rate, bpm	68 ± 10
Weight, kg	94.1 ± 12.6
Body mass index, kg/m ²	30.0 ± 3.3
Waist circumference, cm	103.0 ± 6.6
Waist/hip ratio	0.96 ± 0.04
Body fat, %	30.5 ± 5.2
Total cholesterol, mmol/L	5.0 ± 1.3
Triglycerides, mmol/L	1.9 ± 0.6
LDL-cholesterol, mmol/L	3.0 ± 1.1
HDL-cholesterol, mmol/L	1.11 ± 0.20
Total cholesterol/HDL-cholesterol	4.6 ± 1.2
Plasma glucose, mmol/L	5.5 ± 0.6
HbA1c, %	5.7 ± 0.3
Insulin, pmol/L	88 ± 52
Creatinine, µmol/L	99 ± 17
Ex-smokers, n (%)	15 (51.7)
Alcohol intake, units per week	6 ± 7
Medical history, n (%)	
Treated hypertension	21 (72.4)
Coronary artery disease	9 (31.0)
Cerebrovascular disease	1 (3.4)
Type 2 diabetes (diet-controlled)	2 (6.9)
Drug therapy, n (%)	
Statin therapy	23 (79.3)
ACE inhibitor	14 (48.3)
Beta blocker	12 (41.4)
Diuretic	6 (20.7)
Calcium channel blocker	7 (24.1)
Angiotension II receptor blocker	1 (3.4)
Alpha blocker	2 (6.9)
Aspirin	16 (55.2)

Values are mean ± SD or number (percentage).

3.3.2 Plasma CoQ₁₀ levels

Plasma CoQ₁₀ levels pre and post 12 weeks of CoQ₁₀ and placebo treatment are shown in Figure 3.3. Plasma CoQ₁₀ concentrations increased 3.8 fold after 12 weeks on CoQ₁₀ therapy compared with placebo administration ($P<0.001$).

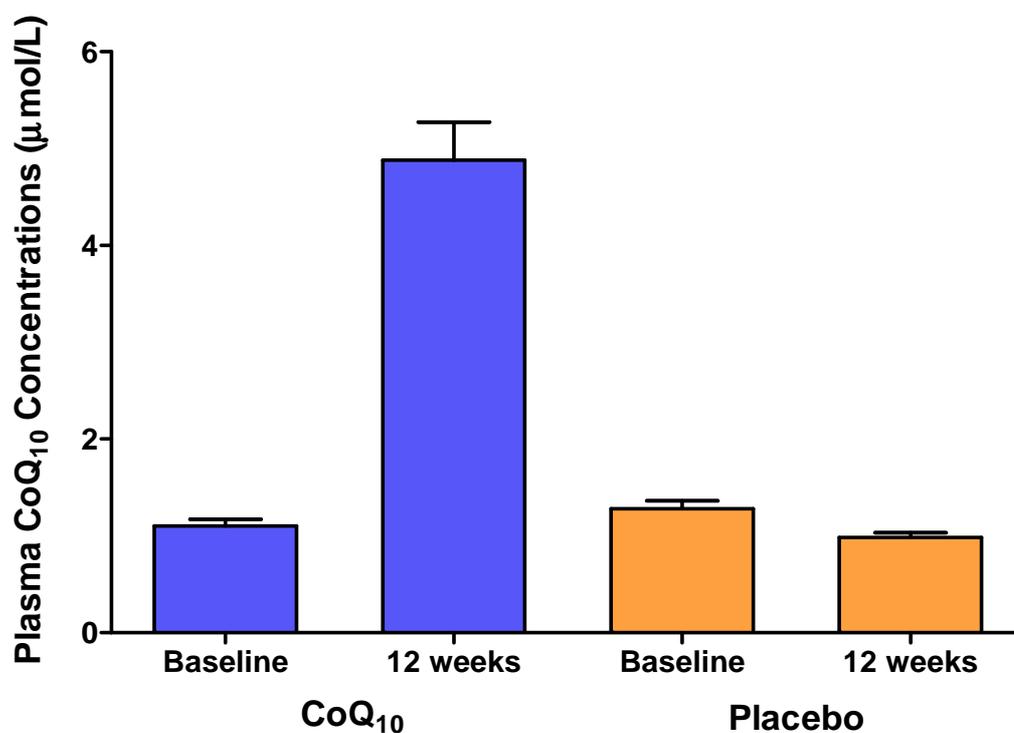


Figure 3.3 Mean plasma CoQ₁₀ concentrations at baseline and after 12 weeks of CoQ₁₀ and placebo treatment. $P<0.001$ for comparison of changes with CoQ₁₀ versus placebo

3.3.3 Endothelial function assessment by brachial ultrasound

Conduit vessel function pre and post placebo and CoQ₁₀ administration is presented in Table 3.2. Baseline brachial artery diameters were similar at all assessments and unchanged with CoQ₁₀ or placebo ($P>0.05$). CoQ₁₀ supplementation significantly increased brachial artery FMD compared with placebo administration (difference in changes 2.7%, 95% CI 1.2, 4.1, $P=0.001$) (Figure 3.4), but did not alter endothelium-independent vasodilation (EID) (-0.1%, -1.7, 1.5, $P=0.39$) (Figure 3.5). There was no effect of CoQ administration on time to peak brachial artery FMD compared to placebo ($P=0.64$). Baseline blood velocity, and baseline and peak shear rates were similar at all visits and were unchanged with CoQ₁₀ or placebo administration ($P>0.05$).

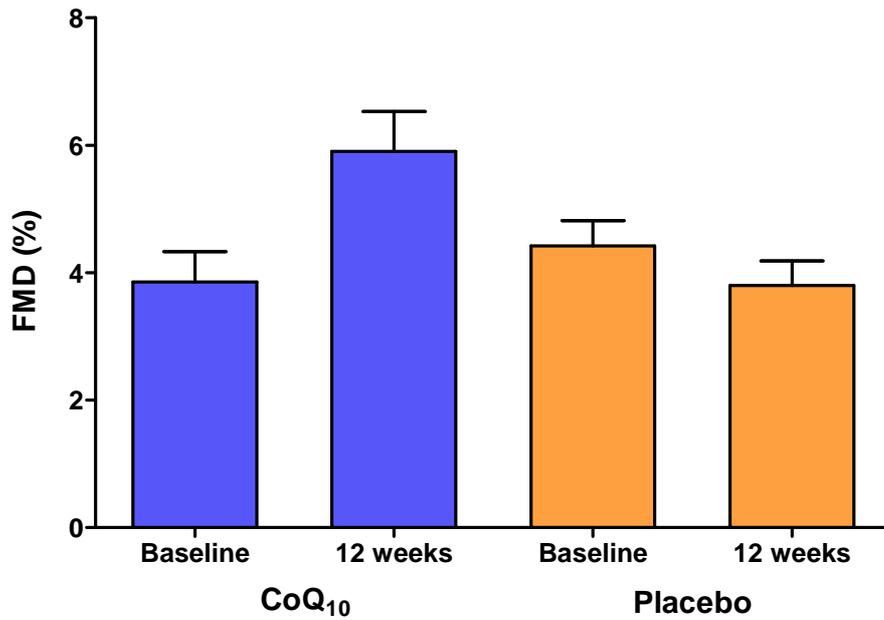


Figure 3.4 FMD of the brachial artery before and following 12 weeks of CoQ₁₀ and placebo administration. *P*=0.001 for comparison of changes with CoQ₁₀ versus placebo

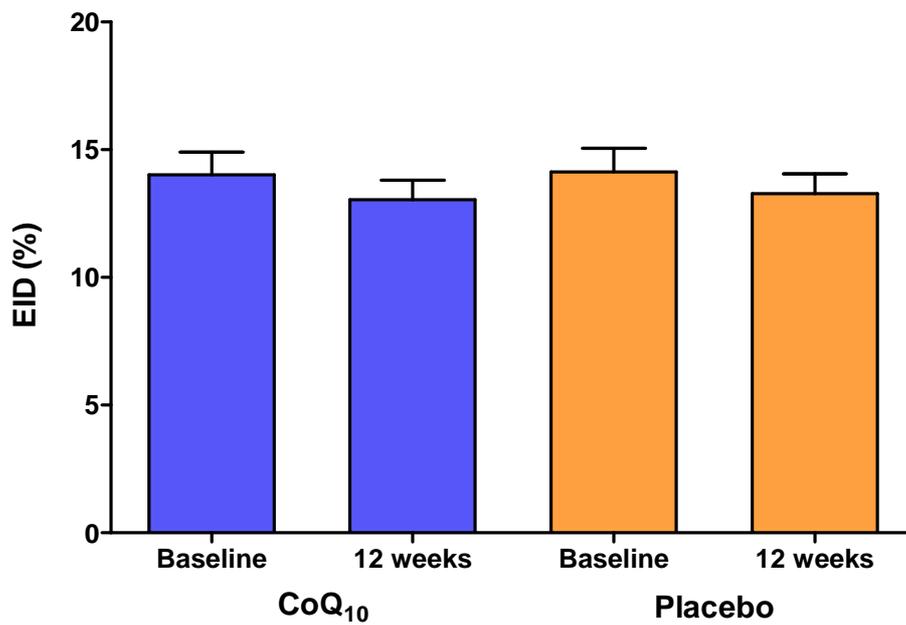


Figure 3.5 EID of the brachial artery before and following 12 weeks of CoQ₁₀ and placebo administration. *P*=0.39 for comparison of changes with CoQ₁₀ versus placebo

3.3.4 Haemodynamic and anthropometric parameters

CoQ₁₀ supplementation for 12 weeks did not have significant effects on peripheral or central blood pressures, heart rate, aortic wave reflection indices, the brachial artery stiffness index, distensibility coefficient or compliance coefficient compared to placebo administration ($P>0.05$, Table 3.3). Additionally, there was no significant differences in weight, BMI, body fat, waist and hip circumference or waist hip ratio changes between the CoQ₁₀ and placebo phases ($P>0.05$, Table 3.4).

3.3.5 Biochemical parameters

Biochemical parameters pre and post placebo and CoQ₁₀ administration are presented in Table 3.5. In addition to CoQ₁₀ concentrations, the CoQ₁₀ to total cholesterol and CoQ₁₀ to LDL-cholesterol ratios were significantly increased following 12 weeks of CoQ₁₀ therapy versus placebo administration ($P<0.001$). CoQ₁₀ supplementation was not associated with significant effects on plasma lipids, glycaemic control, hs-CRP levels or urinary microalbumin concentrations compared to placebo ($P>0.05$ Table 3.5).

3.3.6 *Post-hoc* subgroup analyses

Post-hoc subgroup analyses were performed to examine the treatment effects of CoQ₁₀ on the primary outcome variable in different patient subgroups. The therapeutic effect of CoQ₁₀ on brachial artery FMD was observed in patients with higher compliance to CoQ₁₀ therapy ($>$ median cutpoint) ($P=0.001$, comparing treatments within these patients), but not in those with lower compliance to CoQ₁₀ therapy ($<$ median cutpoint) ($P=0.35$). There were no differences in the CoQ₁₀ treatment effect in the following subgroups: patients with or without a history of cardiovascular disease, treatment with or without beta blockers or aspirin, but there was a trend for a more pronounced effect of CoQ₁₀ in patients who were treated with ACE inhibitors compared to those who were not ($P=0.06$). Despite the significant effect of CoQ₁₀ compliance on FMD response, no differences were observed according to CoQ₁₀ concentrations achieved on therapy or absolute changes in CoQ₁₀ levels on therapy defined by median cut points and achieved CoQ₁₀ concentrations of >2.9 $\mu\text{mol/L}$ ($P>0.05$).

Table 3.2 Effect of CoQ₁₀ and placebo supplementation on brachial artery function

	CoQ ₁₀		Placebo		Advantage to CoQ ₁₀ †	P-value‡
	Baseline	12-weeks	Baseline	12-weeks		
Resting characteristics						
Diameter, mm	4.6 ± 0.1	4.6 ± 0.1	4.6 ± 0.1	4.6 ± 0.1	0.0 (-0.2, 0.2)	0.70
Blood velocity, cm/sec	27.7 ± 1.7	31.2 ± 2.5	25.8 ± 1.2	30.1 ± 1.6	-0.8 (-6.8, 5.3)	0.80
Shear rate, s ⁻¹	496.8 ± 31.3	568.9 ± 56.2	466.5 ± 58.1	533.8 ± 38.5	4.9 (-112.9, 122.6)	0.93
Flow-mediated dilation						
FMD, %	3.9 ± 0.5	5.9 ± 0.6	4.4 ± 0.4	3.8 ± 0.4	2.7 (1.2, 4.1)	0.001
Absolute FMD, mm	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 (0.1, 0.2)	0.001
Peak diameter, mm	4.7 ± 0.1	4.9 ± 0.1	4.7 ± 0.1	4.8 ± 0.1	0.1 (-0.1, 0.3)	0.47
Time to peak FMD, s	45.7 ± 2.1	49.9 ± 3.9	48.1 ± 3.6	49.4 ± 2.5	-2.9 (-15.3, 9.6)	0.64
Peak shear rate, s ⁻¹	1555.8 ± 80.5	1466.8 ± 91.7	1668.6 ± 130.9	1648.2 ± 110.6	-68.6 (-426.3, 289.2)	0.69
EID-mediated dilation						
EID, %	14.0 ± 0.9	13.0 ± 0.8	14.2 ± 0.9	13.3 ± 0.8	-0.1 (-1.7, 1.5)	0.87
Absolute EID, mm	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	-0.0 (-0.1, 0.1)	0.91
Maximum diameter, mm	5.2 ± 0.1	5.3 ± 0.1	5.2 ± 0.1	5.3 ± 0.1	0.0 (-0.2, 0.3)	0.74

Values are mean ± SEM. †Difference between mean change after placebo and CoQ₁₀ (95% CI).

‡ANOVA with repeated measures for comparison of CoQ₁₀ and placebo changes. FMD, flow-mediated dilation; EID, endothelium independent vasodilation.

Table 3.3 Effect of CoQ₁₀ and placebo supplementation on haemodynamic parameters

	CoQ ₁₀		Placebo		Advantage to CoQ ₁₀ †	P-value‡
	Baseline	12-weeks	Baseline	12-weeks		
Brachial SBP, mmHg	134.5 ± 2.1	133.9 ± 2.4	135.6 ± 2.5	135.9 ± 2.3	1.0 (-4.8, 6.9)	0.72
Brachial DBP, mmHg	86.3 ± 1.1	85.1 ± 1.4	86.4 ± 1.3	86.0 ± 1.1	0.7 (-2.9, 4.3)	0.70
MAP, mmHg	104.2 ± 1.4	103.4 ± 1.7	104.6 ± 1.5	104.5 ± 1.3	0.6 (-4.1, 5.3)	0.79
Central SBP, mmHg	126.6 ± 2.0	125.9 ± 2.2	127.1 ± 2.3	127.8 ± 2.0	1.3 (-5.0, 7.5)	0.68
Central DBP, mmHg	87.1 ± 1.1	85.9 ± 1.4	87.2 ± 1.3	86.8 ± 1.1	0.7 (-2.8, 4.2)	0.68
Central PP, mmHg	39.6 ± 1.3	40.0 ± 1.4	39.8 ± 1.8	41.0 ± 1.8	0.7 (-2.8, 4.2)	0.69
Heart rate, bpm	63.3 ± 1.8	62.4 ± 1.9	64.8 ± 1.8	62.7 ± 1.7	-1.2 (-4.4, 2.1)	0.46
AIx@HR75, %	22.8 ± 0.9	22.9 ± 1.0	23.0 ± 0.9	22.8 ± 0.9	-0.3 (-2.4, 1.9)	0.81
AP@HR75, mmHg	8.4 ± 0.5	8.5 ± 0.5	8.6 ± 0.5	8.7 ± 0.5	-0.1 (-1.4, 1.3)	0.94
T _r , ms	147.2 ± 2.2	146.6 ± 2.2	145.1 ± 1.8	146.5 ± 2.1	1.7 (-2.1, 5.6)	0.27
Arterial stiffness index, β	35.7 ± 4.8	34.0 ± 2.7	31.7 ± 4.1	35.0 ± 4.0	5.0 (-4.8, 14.8)	0.31
DC, 10 ⁻³ /kPa	2.4 ± 0.5	5.0 ± 0.5	3.5 ± 1.0	5.3 ± 0.5	0.8 (-2.0, 3.6)	0.55
CC, mm ² /kPa	0.04 ± 0.01	0.08 ± 0.01	0.06 ± 0.02	0.09 ± 0.01	0.02 (-0.03, 0.06)	0.52

Values are mean ± SEM.

†Difference between mean change after placebo and CoQ₁₀ (95% CI).

‡ANOVA with repeated measures for comparison of CoQ₁₀ and placebo changes.

SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PP, pulse pressure; AIx, augmentation index; AI@HR75, augmentation index corrected for heart rate; AP, augmentation pressure; AP@HR75, augmentation pressure corrected for heart rate; T_r, time to reflection; DC, distensibility coefficient; CC, compliance coefficient.

Table 3.4 Effect of CoQ₁₀ and placebo supplementation on anthropometrics

	CoQ ₁₀		Placebo		Advantage to	
	Baseline	12-weeks	Baseline	12-weeks	CoQ ₁₀ [†]	<i>P</i> -value [‡]
Weight, kg	94.4 ± 2.5	94.5 ± 2.6	94.3 ± 2.4	94.3 ± 2.5	-0.2 (-1.3, 1.0)	0.79
Body mass index, kg/m ²	30.2 ± 0.7	30.2 ± 0.7	30.2 ± 0.7	30.1 ± 0.6	-0.1 (-0.5, 0.3)	0.66
Body fat, %	29.6 ± 1.4	30.0 ± 1.6	29.9 ± 1.5	29.7 ± 1.4	-0.6 (-1.7, 0.6)	0.33
Waist circumference, cm	102.5 ± 1.2	103.4 ± 1.3	102.9 ± 1.3	102.3 ± 1.0	-1.4 (-3.7, 0.82)	0.20
Hip circumference, cm	107.4 ± 1.3	107.7 ± 1.3	107.7 ± 1.3	107.3 ± 1.2	-0.7 (-1.9, 0.4)	0.22
Waist/hip ratio	0.96 ± 0.01	0.96 ± 0.01	0.96 ± 0.01	0.96 ± 0.01	-0.01 (-0.02, 0.01)	0.37

Values are mean ± SEM.

[†]Difference between mean change after placebo and CoQ₁₀ (95% CI).

[‡]ANOVA with repeated measures for comparison of CoQ₁₀ and placebo changes.

Table 3.5 Effect of CoQ₁₀ and placebo on biochemical parameters

	CoQ ₁₀		Placebo		Advantage to	
	Baseline	12-weeks	Baseline	12-weeks	CoQ ₁₀ [†]	<i>P</i> -value [‡]
CoQ ₁₀ μmol/L	1.1 ± 0.1	4.9 ± 0.4	1.3 ± 0.1	1.0 ± 0.1	4.1 (3.3, 4.8)	<0.001
CoQ ₁₀ / TC, μmol/mmol	0.25 ± 0.01	1.14 ± 0.08	0.29 ± 0.02	0.23 ± 0.01	0.96 (0.80, 1.11)	<0.001
CoQ ₁₀ / LDL-C, μmol/mmol	0.46 ± 0.02	2.08 ± 0.15	0.53 ± 0.03	0.42 ± 0.02	1.73 (1.44, 2.02)	<0.001
Total cholesterol, mmol/L	4.4 ± 0.1	4.3 ± 0.1	4.5 ± 0.2	4.3 ± 0.1	-0.1 (-0.4, 0.19)	0.51
Triglycerides, mmol/L	1.8 ± 0.1	1.8 ± 0.1	2.0 ± 0.1	1.8 ± 0.1	-0.2 (-0.5, 0.1)	0.14
LDL-cholesterol, mmol/L	2.5 ± 0.1	2.4 ± 0.1	2.5 ± 0.2	2.4 ± 0.1	0.0 (-0.2, 0.2)	1.00
HDL-cholesterol, mmol/L	1.08 ± 0.03	1.06 ± 0.04	1.07 ± 0.04	1.03 ± 0.03	0.02 (-0.04, 0.07)	0.55
TC/HDL-cholesterol	4.1 ± 0.1	4.1 ± 0.2	4.3 ± 0.2	4.3 ± 0.2	-0.1 (-0.3, 0.2)	0.69
Plasma glucose, mmol/L	5.5 ± 0.1	5.6 ± 0.1	5.4 ± 0.1	5.5 ± 0.1	0.1 (-0.1, 0.3)	0.53
Plasma HbA1c, %	5.7 ± 0.1	5.8 ± 0.1	5.7 ± 0.1	5.8 ± 0.1	-0.1 (-0.2, 0.1)	0.38
High-sensitivity CRP, mmol/L	1.75 ± 0.32	1.23 ± 0.19	1.76 ± 0.31	1.63 ± 0.35	0.39 (-0.41, 1.18)	0.32
Urinary ACR g/mol	6.4 ± 5.0	6.2 ± 5.0	7.9 ± 6.4	5.7 ± 4.5	-1.9 (-6.3, 1.9)	0.28

Values are mean ± SEM.

[†]Difference between mean change after placebo and CoQ₁₀ (95% CI).

[‡]ANOVA with repeated measures for comparison of between group changes.

ACR, albumin/creatinine ratio.

3.3.7 Correlation analysis

The increase in plasma CoQ₁₀ concentrations was not associated with the improvement in brachial artery FMD following 12 weeks of CoQ₁₀ treatment ($r=-0.15$, $P=0.46$). Furthermore, pre-treatment FMD values and achieved plasma CoQ₁₀ concentrations did not correlate with FMD responses following CoQ₁₀ supplementation ($P>0.05$).

3.4 Discussion

3.4.1 Effect of CoQ₁₀ on endothelial function

The present study demonstrated that 12 weeks of CoQ₁₀ supplementation at a dose of 400mg/day significantly improved FMD of the brachial artery in statin-treated patients with the metabolic syndrome. CoQ₁₀ supplementation resulted in a four-fold increase in plasma CoQ₁₀ concentrations from baseline during the treatment phase, which is comparable to other studies (368-371, 522). The study findings indicate a potential role for CoQ₁₀ in modifying endothelial dysfunction in patients on statin treatment. These findings are in contrast to a previous study by Keuttner *et al.* (2005) (759) where investigators observed no further improvement in endothelium-dependent vasodilation in 25 dyslipidaemic males with manifest endothelial dysfunction following combined treatment with CoQ₁₀ and cerivastatin, over and above that observed with statin or CoQ₁₀ alone. The lack of any additional benefit of combined CoQ₁₀ and statin treatment may reflect the relatively short treatment period (6 weeks), or low dose of CoQ₁₀ administered (150mg/day). Cerivastatin is no longer available due to the increased risk of mortality attributed to rhabdomyolysis (762). The present study corroborates the findings of a recent double-blind, placebo-controlled cross-over trial by Hamilton *et al.* (2008), who showed that 12 weeks of CoQ₁₀ supplementation increased FMD by 1% in 23 statin-treated type 2 diabetic patients (371). These patients presented with endothelial dysfunction, despite satisfactory control of blood pressure, glycaemia and lipids (LDL-cholesterol<2.5mmol/L) (371). Notably, these patients were not standardised for statin type or dose, whereas patients in the present study were standardised on 40mg/day of simvastatin therapy.

CoQ₁₀ has also been shown to improve conduit artery function in controlled intervention studies of patients with type 2 diabetes mellitus and coronary artery disease who

were not receiving concurrent statin therapy (368, 369), and in patients with chronic heart failure (CHF) where statin treatment was not a specific inclusion criterion (370, 522). In a double-blind, randomised placebo-controlled trial, Watts *et al.* (2002) (369) demonstrated an absolute increase in FMD of 1.6% following 12 weeks of oral CoQ₁₀ therapy (200mg/day) compared to placebo in 40 dyslipidaemic patients with type 2 diabetes mellitus. Belardinelli *et al.* (2006) (522) studied 23 patients with CHF in a double-blind, placebo-controlled cross-over study. Patients were randomised to treatment with CoQ₁₀ (100mg tid), CoQ₁₀ plus exercise training (ET), placebo, or placebo plus ET. Oral CoQ₁₀ supplementation resulted in a significant improvement in FMD after four weeks, and the combination of CoQ₁₀ and exercise training resulted in a more pronounced improvement in endothelial function (522). Tiano *et al.* (2007) (368) reported a positive effect of CoQ₁₀ therapy (300mg/day) on endothelium-dependent vasorelaxation of the brachial artery compared to placebo in 38 patients with coronary artery disease after four weeks of supplementation. Dai *et al.* (2011) (370) undertook a randomised controlled trial in 28 patients with ischaemic left ventricular systolic function and observed a significant improvement in the ischaemic vasodilator function after eight weeks of CoQ₁₀ therapy (300mg/day). Furthermore, a recent meta-analysis, comprising five randomised, double-blind, placebo-controlled trials (361, 368-371) in 194 patients with and without established cardiovascular disease, reported an absolute increase in FMD of 1.7% on CoQ₁₀ supplementation (763). In this meta-analysis, CoQ₁₀ did not improve GTN-mediated dilation of the brachial artery, suggesting no effect on endothelium-independent vasodilation (763).

Conversely, Raitakari *et al.* (2000) (361) studied 12 healthy hypercholesterolaemic subjects with endothelial dysfunction who received CoQ₁₀ (150mg/day) or placebo for four weeks in a double-blind cross-over study and did not find any significant effect of CoQ₁₀ on FMD. The reason for the inconsistency in this study with others is unknown, although it has been proposed that the mechanisms by which CoQ₁₀ may influence endothelial function could differ between patient populations (12). Two further randomised controlled trials in type 2 diabetic patients failed to show any improvement in microcirculatory function with CoQ₁₀ monotherapy, indicating that the effect of CoQ₁₀ may be specific to the vascular bed (487, 488). Playford *et al.* (2003) (487) did however; observe a significant increase in endothelium-dependent microcirculatory function in dyslipidaemic type 2 diabetic patients, with combined CoQ₁₀ and fenofibrate therapy, suggesting that CoQ₁₀ may have the potential to augment the

benefits of peroxisome proliferator-activated receptor (PPAR) alpha agonists on vascular function.

3.4.2 Effect of CoQ₁₀ on haemodynamic parameters

A number of clinical studies have described the potential of CoQ₁₀ to lower clinic blood pressure in hypertensive patients (480, 484, 489-498) and in patients with type 2 diabetes mellitus (485), as detailed in Chapter 4. In the present study, CoQ₁₀ supplementation was not associated with significant decreases in brachial or aortic blood pressures. This corroborates with the study described in Chapter 4, where CoQ₁₀ supplementation did not result in improved clinic blood pressures after 12 weeks of supplementation in patients with the metabolic syndrome and modest blood pressure elevations (559). A more obvious treatment effect might be expected in subjects with higher baseline blood pressure levels. These findings suggest that the ameliorating effects of CoQ₁₀ on endothelial function are mediated via alternative pathways to blood pressure reduction in this patient population. Furthermore, CoQ₁₀ supplementation did not significantly alter brachial artery stiffness or aortic wave reflection indices. Even though functional improvements were observed in the vascular endothelium, it is possible that the treatment period was not of sufficient duration for these observations to translate into reduced arterial stiffness or aortic wave reflections, both important prognostic indicators of cardiovascular risk (714, 722, 723). Consistent with the present findings, a recent study in obese subjects found no evidence for an effect of CoQ₁₀ therapy on arterial stiffness, as assessed by brachial-ankle pulse wave velocity (764).

3.4.3 Effect of CoQ₁₀ on biochemical parameters

The precise mechanisms for the vasoprotective effects of CoQ₁₀ are unknown. It has been proposed that CoQ₁₀ may exert beneficial effects on the endothelium through its bioenergetic properties as an obligatory cofactor in oxidative phosphorylation or through its antioxidant properties (363, 379). Tiano *et al.* (2007) (368) demonstrated that CoQ₁₀ supplementation resulted in significant improvements in peak oxygen consumption, oxygen pulse and endothelial function of the brachial artery in patients with coronary artery disease, providing indirect evidence that CoQ₁₀ improves mitochondrial bioenergetics. Furthermore, Dai *et al.* (2011) (370) showed that CoQ₁₀ therapy led to a significant reduction in the plasma lactate/pyruvate ratio, an indirect indicator of mitochondrial dysfunction, which correlated with the improvement in FMD. This finding suggests that CoQ₁₀ can improve endothelial

function in patients with ischaemic left ventricular systolic dysfunction via correction of mitochondrial function.

In addition to its role in the mitochondrial electron transport chain, CoQ₁₀ is also a potent antioxidant (443, 765), and may improve endothelial function by reducing oxidative stress through recoupling of eNOS and mitochondrial oxidative phosphorylation (12). *In vitro* and animal studies have provided some mechanistic insights into the potential role of CoQ₁₀ on ameliorating vascular function via its antioxidant properties. For example, in a rat model of the metabolic syndrome, CoQ₁₀ supplementation improved endothelial function in the mesenteric arteries, and attenuated the increase in oxidative and nitrosative stress markers in a dose dependent manner (364). CoQ₁₀ has also been shown to prevent oxidative stress in cultured endothelial cells under high glucose concentrations (365). However, there are conflicting data on the effects of CoQ₁₀ therapy on oxidative stress from clinical studies. Tiano *et al.* (2007) (368) showed an improvement in the activity of endothelium-bound extracellular superoxide dismutase (ecSOD) in subjects with coronary artery disease. Furthermore, the increase in ecSOD activity was significantly correlated with the improvement in FMD, suggesting that the endothelium-enhancing effects of CoQ₁₀ are related to improvements in local vascular oxidative stress (368). Conversely, other studies have failed to show any significant changes in markers of oxidative stress, including F2-isoprostane (369-371) and 24-hr urinary 20-hydroxyeicosatetraenoic acid (HETE) levels (370, 371), and serum SOD (370).

It is possible that CoQ₁₀ may have indirect effects on NO regulation by reducing lipid profiles, glycaemic indices or inflammation. CoQ₁₀ supplementation did not alter lipid profiles in the current patient population, consistent with previous studies that showed improvement in endothelial function was independent of lipid-lowering (369-371, 522). On the other hand, Raitakari *et al.* (2000) (361) reported a decrease in *ex-vivo* LDL oxidisability but no significant improvement in arterial endothelial function in patients with moderate hypercholesterolemia. CoQ₁₀ therapy was not associated with changes in plasma glucose or HbA1c levels in the present study. A small number of clinical trials have shown that CoQ₁₀ can improve plasma glucose and HbA1c, (484, 485, 487) and lower plasma insulin (484), whereas other studies have reported no effects of CoQ₁₀ on glycaemic indices (369-371). In the present study, CoQ₁₀ did not alter hs-CRP concentrations. Similarly, Dai *et al.* (2011) (370) did not find an effect of CoQ₁₀ supplementation on hs-CRP levels, despite improvements in endothelium-dependent vasodilation. Whether CoQ₁₀ may improve

endothelial function by modulating other mediators of endothelial dysfunction, such as endothelin-1 (372), and the eNOS inhibitor, asymmetric dimethylarginine (373) should be investigated. In the present study, urinary albumin/creatinine ratio, a marker of generalised endothelial dysfunction (766), was not significantly altered with CoQ₁₀ supplementation. These findings indicate that the benefits of CoQ₁₀ on endothelial function in statin-treated patients with the metabolic syndrome are independent of changes in lipids, inflammation, glycaemic indices and the urinary albumin/creatinine ratio. Further investigations are required into the mechanisms of CoQ₁₀-induced improvements in vascular function, particularly markers of oxidative stress, antioxidant activity and vasoactive mediators.

3.4.4 Subgroup analyses

Post-hoc analyses of subgroups in the study showed no significant differences in the FMD responses to 12 weeks of CoQ₁₀ therapy, except in patients with a higher compliance to CoQ₁₀ above the median cutpoint, although there was no association with increased CoQ₁₀ levels and FMD improvement. There was a trend for a more pronounced improvement in FMD in those patients who were not treated with ACE inhibitors, which may represent a Type 1 error. This observation is however, consistent with a meta-analysis of the effects of CoQ₁₀ on systolic function in patients with CHF, where studies that included patients taking ACE inhibitors showed no increase in ejection fraction, whereas studies with patients not taking ACE inhibitors found a 6.7% increase in ejection fraction (508). One suggested mechanism for the observed antihypertensive effect of CoQ₁₀ is reduction of total peripheral resistance (496), which in turn reduces left ventricular load and vascular tone. Sander *et al.* (2006) (508) postulated that CoQ₁₀ may also reduce afterload, and thus taking CoQ₁₀ in addition to ACE inhibitors may not produce an additional benefit on ejection fraction. ACE inhibition also improves endothelial function (767), which could explain the more profound beneficial effects on endothelial function in patients who are not taking ACE inhibitors. Further investigations are required to establish whether patients who are intolerant to ACE inhibitors may derive more cardiovascular benefit from CoQ₁₀ therapy.

Earlier trials in patients with CHF and ischaemic heart disease have reported significant subgroup effects with CoQ₁₀ supplementation (368, 370, 522). Belardinelli *et al.* (2006) (522) reported a six-fold greater improvement in endothelium-dependent relaxation after CoQ₁₀ therapy in CHF patients with plasma CoQ₁₀ levels above 2.4 µg/mL in comparison to those with plasma CoQ₁₀ levels less than 2.4 µg/mL. This finding indicates that CoQ₁₀ has more

benefit in patients who achieve postulated therapeutic concentrations of CoQ₁₀ with supplementation. Tiano *et al.* (2007) (368) showed that the improvement in brachial artery FMD was more pronounced in the subgroup of patients with low ecSOD levels, suggesting that CoQ₁₀ is more effective in patients with decreased levels of antioxidant defences, and presumably increased oxidative stress. Dai *et al.* (2011) (370) demonstrated that beneficial effects of CoQ₁₀ on endothelial function were more pronounced in patients with severe mitochondrial dysfunction, as evidenced by a higher lactate/pyruvate at baseline, and those with a history of prior myocardial infarction.

3.4.5 Relationship between changes in CoQ₁₀ and endothelial function

No significant associations were observed between the improvement in FMD and absolute or percentage increases in plasma CoQ₁₀ concentrations or achieved CoQ₁₀ levels following CoQ₁₀ supplementation. This contrasts with several previous therapeutic trials in CHF and ischaemic heart disease, where elevations in CoQ₁₀ levels were associated with enhancement of arterial function (368, 370). Notably, baseline CoQ₁₀ levels and achieved increases in CoQ₁₀ concentrations with therapy in the present study were comparable to these trials (368, 370). Tiano *et al.* (2007) (368) found a strong correlation between the percentage increase in plasma levels of CoQ₁₀ and the increase in brachial artery FMD following 300mg CoQ₁₀ daily for four weeks in patients with ischemic heart disease ($r=0.713$, $P<0.05$). Improvement in FMD was correlated with the increase in CoQ₁₀ levels in patients with CHF after CoQ₁₀ treatment or combined CoQ₁₀ and exercise training ($r=0.61$, $P<0.01$) (522). In a recent study, the absolute increase in CoQ₁₀ was significantly correlated with improvement in FMD in patients with left ventricular systolic dysfunction (370). These findings suggest, at least in patients with ischaemic heart disease and heart failure that there is a possible dose-dependent relationship (370).

3.4.6 Study limitations

In the current study only males were examined in order to exclude any effects of endogenous hormones, and therefore studies assessing vasodilatory responses to CoQ₁₀ therapy should also to be extended to include females. Improvement in FMD in patients with the metabolic syndrome may be mediated by reductions in local vascular oxidative stress (368, 532). However the present investigation into potential mechanisms by which CoQ₁₀

may contribute to improve arterial function was limited to glycaemic indices, and markers of inflammation and endothelial dysfunction. Further measurements of oxidative stress and/or antioxidant markers are warranted. Plasma CoQ₁₀ levels also do not necessarily reflect tissue CoQ₁₀ concentrations (393). There was limited statistical power for the *post-hoc* subgroup analyses, and therefore one can not exclude the possibility of significant differences in the response to CoQ₁₀ treatment within the subgroups examined.

3.4.7 Significance

This study has demonstrated that endothelium-dependent vasodilation of the brachial artery is significantly improved with short-term CoQ₁₀ supplementation compared to placebo in patients with the metabolic syndrome on concurrent simvastatin (40 mg/day) treatment. Despite treatment with simvastatin and concomitant medications for hypertension and vascular disease, the patients presented with endothelial dysfunction, indicating a residual risk of cardiovascular disease in this population. This study has expanded on the findings of Hamilton *et al.* (2008) (371) who demonstrated that conduit vessel function is improved in statin-treated patients with type 2 diabetes mellitus with manifest endothelial dysfunction and well controlled blood pressure, lipids and glycaemic indices, following CoQ₁₀ therapy. These studies support the hypothesis generated from a previous study (758), that statin-induced reductions in plasma CoQ₁₀ levels may be limiting the maximum beneficial effects of statins on the vasculature, and that adjunctive CoQ₁₀ supplementation can further augment endothelial function. Impaired FMD is a significant predictor of adverse cardiovascular events (38). The absolute increase in FMD of 2.7% observed with CoQ₁₀ administration compared to placebo is of clinical significance, since an absolute improvement in FMD, even as small as 1%, may translate into a 10-25% reduction in residual cardiovascular risk for these patients (38, 768, 769). Several interventions that improve FMD have also demonstrated improvement in cardiovascular outcomes (768, 769). Large cardiovascular outcome trials are, however, required to confirm the clinical relevance of improved endothelial function with CoQ₁₀ therapy in statin-treated patients. Furthermore, although CoQ₁₀ improved FMD in patients with the metabolic syndrome, conduit artery function was not completely restored. Investigations into the synergistic effects of CoQ₁₀ with other therapies that could further improve vascular function are warranted.

3.4.8 Conclusions

This study demonstrated that 12 weeks of supplementation with CoQ₁₀ significantly improves brachial artery endothelial function in simvastatin-treated males with the metabolic syndrome. A four-fold increase in plasma CoQ₁₀ concentrations was achieved on supplementation. CoQ₁₀ therapy was well tolerated and was not associated with any clinically relevant adverse effects. These findings support a potential role for the amelioration of endothelial function in statin-treated patients and further investigations are warranted to confirm the significance of these findings in large cardiovascular end point trials.

4 Effect of Coenzyme Q₁₀ Supplementation on 24-hr Ambulatory Blood Pressure in Hypertensive Patients with the Metabolic Syndrome

4.1 Background

Hypertension represents an increasing global burden of disease (770, 771), and is a significant modifiable risk factor for both adverse cardiovascular and renal outcomes (772, 773). Worldwide, hypertension is the leading cause of mortality and the third highest cause of morbidity (774). Lawes *et al.* (2008) (770) reported a total of 7.6 million premature deaths and 92 million disability adjusted-life years worldwide in 2001, attributable to high blood pressure. Suboptimal blood pressure control is estimated to be responsible for 54% of cerebrovascular disease and for 47% of ischemic heart disease (770). Furthermore, the relationship between blood pressure and cardiovascular disease is continuous and independent of other risk factors (775). In 2000, 26.4% of the world's adult population had hypertension. Within New Zealand, hypertension affects 1:3 to 1:5 adults, depending on ethnicity (776). Globally, the number of adults with hypertension in 2025 is predicted to increase by approximately 60% to a total of 1.56 billion (724), thus posing an important public health challenge.

The importance of lowering elevated blood pressure has been clearly demonstrated in numerous intervention trials and meta-analyses (777, 778). Major guidelines (779, 780) recommend the initiation of antihypertensive drugs in all patients with a clinic systolic blood pressure of 140 mmHg or greater, and/or a diastolic blood pressure of 90 mmHg or greater. There is now consensus that a more aggressive clinic blood pressure target of <130/80 mmHg should be pursued in patients with diabetes or a history cardiovascular or renal disease (779, 780). This more stringent blood pressure target is supported by evidence from the Hypertension Optimal Treatment (HOT) study (781), where intense blood pressure lowering was shown to confer a greater degree of cardiovascular protection in patients with type 2 diabetes mellitus. The Blood Pressure Lowering Treatment Trialists' (BPLTT) collaboration meta-analyses, which incorporated five trials on 22,000 patients (782, 783), also highlighted

significant benefits from more intensive blood pressure reductions in relation to stroke and major cardiovascular events. However, despite these targets, blood pressure control remains elusive for at least 50% of the hypertensive population (784, 785). Control of blood pressure in hypertensive patients often requires multiple drug therapy, but poor adherence is frequently due, in part, to undesirable side effects, such as renal or cardiac dysfunction, cough or depression (786). Accordingly, adjunctive therapy with agents such as coenzyme Q₁₀ (CoQ₁₀), with few side effects, have become increasingly popular.

CoQ₁₀ is an antioxidant and integral component of the mitochondrial electron transport chain (335) (detailed in Chapter 1, section 1.3.3.3.1). There is evidence that plasma CoQ₁₀ concentrations are reduced in patients with essential hypertension (480). Furthermore, increased oxidative stress has been observed in hypertensive states (787). Oxidative stress within the vascular wall, results in an increased production of superoxide that rapidly reacts with endothelial nitric oxide (NO) to form peroxynitrite, and thereby reduces NO bioavailability (363, 379, 425). This reduction impairs the ability of the endothelium to induce NO-mediated relaxation of vascular smooth muscle, resulting in vasoconstriction and increased blood pressure (363, 379, 425). It has been proposed that CoQ₁₀ supplementation has an antihypertensive action resulting from vasodilation via a direct effect on the endothelium and underlying vascular smooth muscle (363, 379, 425).

A number of clinical studies have described the potential of CoQ₁₀ to lower blood pressure in hypertensive patients (480, 484, 489-498). Rosenfeldt *et al.* (2007) (425) conducted a meta-analysis, comprising three randomised trials (484, 489, 490), one randomised cross-over study (491), and eight open label studies (480, 492-498) in 362 hypertensive patients, most of whom had essential hypertension or isolated systolic hypertension. They reported that CoQ₁₀ therapy had the potential to reduce blood pressure by up to 17/10 mmHg (425). Amongst treated patients, mean blood pressure was significantly decreased by 11/8 mmHg in the cross-over study (n=18), and by 17/8 mmHg in the randomised studies (n=120) (425) (Table 4.1). The meta-analysis was however, limited by the inclusion of studies which were open-labelled and not placebo-controlled. Furthermore, there were considerable differences in patient populations with respect to age, underlying disease and co-morbidities, CoQ₁₀ dose and duration, and the use of concomitant therapy between the trials. Finally, the meta-analysis did not make use of individual patient data from the component studies, which would have provided a more robust assessment of any effect of CoQ₁₀ on arterial pressure.

More recently, a Cochrane review addressed the evidence for CoQ₁₀ efficacy in the treatment of essential hypertension (499). This systematic review consisted of three randomised double-blind placebo-controlled studies with a total of 96 participants (499), including the cross-over study (491) and two of the randomised controlled studies (484, 489) in the Rosenfeldt *et al.* (2007) meta-analysis (425). Whilst this review found that CoQ₁₀ lowered blood pressure by 11/7 mmHg after 4 to 12 weeks of therapy in comparison to placebo, the authors cautioned that “Due to possible unreliability of some of the included studies, it is uncertain whether or not CoQ₁₀ decreases blood pressure in the long-term management of primary hypertension” (499). The reviewers concluded that larger properly conducted randomised controlled trials are warranted (499). Pertinent populations for such trials include patients with diabetes or the metabolic syndrome, since these disease states are associated with elevated oxidative stress (363).

4.1.1 Study hypothesis

The hypothesis for this study was that adjunctive CoQ₁₀ supplementation will lower 24-hr ambulatory blood pressure in inadequately treated hypertensive patients with the metabolic syndrome. Accordingly, the effect of CoQ₁₀ treatment on blood pressure in subjects with the metabolic syndrome and inadequate blood pressure control despite standard antihypertensive therapies was studied in a randomised, double-blind, placebo-controlled 28-week cross-over trial that assessed 24-hr ambulatory blood pressure parameters and clinic blood pressure. Biochemical and haematological indices were also included as safety markers.

Table 4.1 Randomised controlled trials of CoQ₁₀ in hypertension

Randomised controlled trials (n), period	CoQ ₁₀ /Placebo	SBP (mmHg)			DBP (mmHg)		
		Baseline	Final	ΔSBP	Baseline	Final	ΔDBP
Yamagami, 1986 (489) (n=20), 12 weeks	Treatment	167 (161.9 – 172.1)	148 (139.4 – 156.6)	-19	97 (93.5 – 100.5)	91 (83.8 – 98.3)	-6
	Placebo	168 (158.6 – 177.4)	164 (152.8 – 175.2)	-4	96 (92.7 – 99.4)	93 (83.8 – 102.2)	-3
Singh, 1999 (484) (n=59), 8 weeks	Treatment	168 (164.6 – 171.4)	152 (149.1 – 154.9)	-16	106 (104.3 – 107.7)	97 (95.5 – 98.5)	-9
	Placebo	166 (162.9 – 169.1)	164 (161.0 – 167.0)	-2	105 (103.3 – 106.8)	103 (101.3 – 104.7)	-2
Burke, 2001 (490) (n=41), 12 weeks	Treatment	165.1 (155.5 – 174.7)	147.3 (132.4 – 162.2)	-17.8	80.9 (77.8 – 84.1)	78.3 (72.6 – 84.0)	-2.6
	Placebo	164.4 (154.2 – 174.6)	162.7 (151.3 – 174.1)	-1.7	82.0 (77.7 – 86.3)	81.5 (74.5 – 87.6)	-0.5
Digiesi, 1990 (491) (n=18), 10 weeks†	Treatment	167 ± 2.6	156 ± 2.3	-11	103 ± 1.2	95 ± 1.0	-8
	Placebo		166 ± 2.4	-1		103 ± 1.0	0

†Cross-over study with two weeks washout between treatments. SBP, systolic blood pressure; DBP, diastolic blood pressure.

Adapted from Rosenfeldt *et al.* (2007) (425).

4.2 Methods

4.2.1 Subjects

Thirty-one patients, aged 25 – 75 years, with hypertension defined as an average clinic systolic blood pressure of ≥ 140 systolic mmHg or ≥ 130 mmHg for patients with type 2 diabetes mellitus, and receiving conventional antihypertensive medication which had been unchanged for at least one month before enrolment. All patients had the metabolic syndrome as defined by the International Diabetes Federation 2005 guidelines (590); a waist circumference ≥ 94 cm (males) or ≥ 80 cm (females), and at least one of the following in addition to treated hypertension: triglycerides ≥ 1.7 mmol/L or specific therapy for elevated triglycerides, HDL < 1.0 mmol/L (males) < 1.3 mmol/L (females) or specific therapy for a low HDL, or fasting plasma glucose > 5.6 mmol/L. Patients were predominantly Caucasian (90%) with 10% of Māori descent. Subjects were excluded from the study if they had uncontrolled hypertension (clinic blood pressure $> 160/100$ mmHg), a history of coronary angioplasty, coronary stent placement, coronary bypass surgery, or myocardial infarction within the 6 months prior to screening, or a history of cerebrovascular accident within the 12 months prior to screening. Further exclusions were unstable angina pectoris, symptomatic chronic heart failure requiring treatment, atrial fibrillation, type 1 diabetes mellitus, type 2 diabetes mellitus requiring insulin, HbA1c $> 9\%$, significantly deranged liver function tests (alanine aminotransferase ≥ 3 times the upper level of normal), significant renal impairment (plasma creatinine > 150 μ mmol/L), autonomic neuropathy, body mass index > 40 kg/m² or an upper arm circumference > 42 cm, other significant co-morbidities, current smoking, warfarin treatment, or antioxidant vitamin supplementation, including CoQ₁₀. Subjects were withdrawn if they were initiated on lipid lowering therapy during the study or had any alterations to their background antihypertensive medications.

4.2.2 Study design

This was a randomised, double-blind, placebo-controlled 12-week cross-over study with CoQ₁₀ supplementation in inadequately treated hypertensive patients with the metabolic syndrome (Figure 4.1). Sixty patients attended a screening visit to assess eligibility. After a two-week screening period, eligible patients were randomised to treatment with either CoQ₁₀ (Q-Gel®, Tishcon Corp, USA), 100 mg twice daily or matching placebo for 12 weeks, followed by a four week washout period, and then received the alternative treatment for a

further 12 weeks. Twenty-four hour ambulatory blood pressure and clinic blood pressure were taken at baseline and at the end of both treatment periods. Patients attended a follow-up visit halfway through each intervention phase. At the end of each 24-hr monitoring period and at the follow-up visits, fasting blood samples and urine samples were collected. Any adverse events were documented. Patients maintained their standard antihypertensive therapies and any lipid lowering treatment unchanged for the duration of the study period. Patients were asked to maintain their usual diets and physical activity and not to alter their lifestyle during the interventional period. The study protocol was approved by the Upper South B Regional Ethics Committee (NZ), and written informed consent was obtained from all participants.

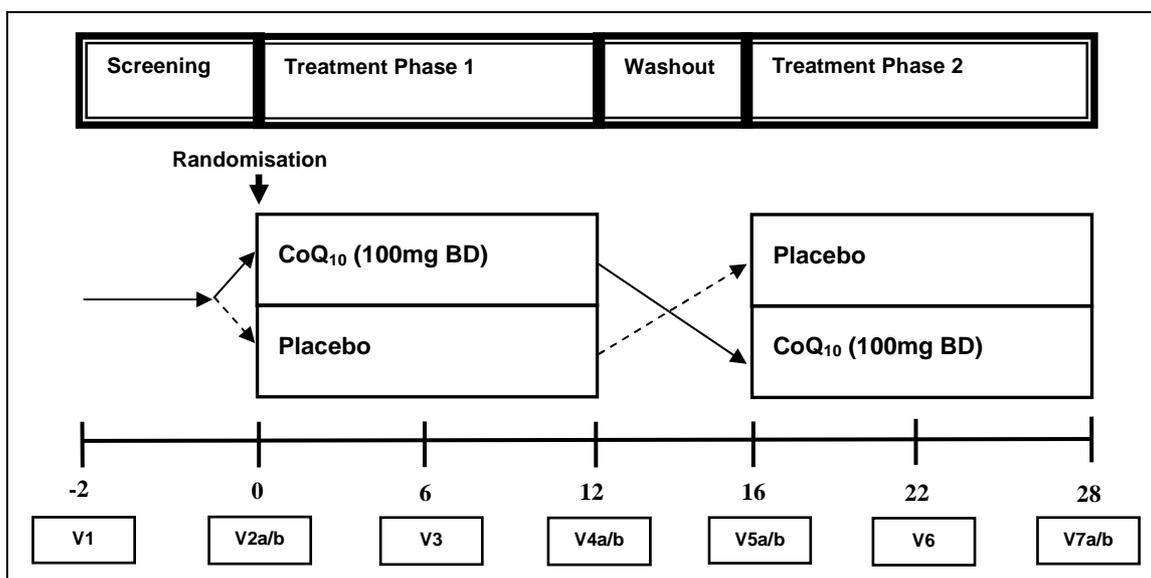


Figure 4.1 Summary outline of the 12-week randomised, double-blind, placebo-controlled, cross-over study of CoQ₁₀ supplementation in subjects with inadequately treated hypertension

4.2.3 Outcome measures

The primary outcomes were 12-week changes in mean 24-hr ambulatory systolic and diastolic blood pressure (Table 4.2). Secondary outcomes were 12-week changes in 24-hr mean arterial pressure, pulse pressure and heart rate, and the changes in mean daytime and night-time blood pressure and heart rate, minimum and maximum blood pressure and heart rate levels, morning surge and nocturnal fall in systolic and diastolic blood pressure, 24-hr and daytime and night-time blood pressure loads, blood pressure and heart rate variability, clinic blood pressure and heart rate, and plasma CoQ₁₀ levels (Table 4.2). Safety data,

including electrolytes, renal and liver function and a full blood count were assessed at the end of each treatment period and at each follow up visit and adverse events were documented.

4.2.4 Clinic blood pressure measurement

Clinic blood pressure was recorded using standard sphygmomanometry between 0700 and 1100 hours according to current guideline recommendations (788, 789). Blood pressure was assessed after five minutes of rest in the sitting position using appropriately sized cuffs. Three blood pressure measurements were obtained at two minute intervals and the mean of these recordings were calculated as the final clinic blood pressure values. Serial blood pressure measurements were performed by the same trained operator using the same calibrated sphygmomanometer throughout the study.

4.2.5 Ambulatory blood pressure monitoring

All subjects underwent 24-hour ambulatory blood pressure monitoring with the validated TM-2430 device, A&D Co. Ltd, Japan (accuracy ± 3 mmHg for blood pressure, $\pm 5\%$ for pulse) and suitably sized cuffs. Patients were asked to refrain from drinking alcoholic or caffeinated beverages within eight hours prior to the visit and to withhold short acting nitrates within four hours of the visit. The monitoring started between 0700 and 1100 hours after office blood pressure measurements had been recorded, with ambulatory blood pressure readings obtained at 20-minute intervals over the 24-hour period. Participants were instructed to engage in their usual physical activity levels, but to avoid strenuous exercise during the monitoring period and to record bed and rise times. Datasets with $<80\%$ valid readings were excluded from analysis. Data were analysed using Doctory ProTM ambulatory blood pressure monitor data analysis software (TM-2430-13, A&D Co. Ltd, Japan) (Figure 4.2). Daytime and night-time were defined by three established methods: 1) narrow fixed time intervals described in the European Society of Hypertension Guidelines 2005 (ESH, 2005) (788) (daytime 0900 – 2059 hrs, night-time 0100 – 0559 hrs), 2) the participant's reported retiring to bed and rising times, and 3) arbitrarily (daytime 0600 – 2059 hrs, night-time 2100 – 0559 hrs). Thresholds for normal ambulatory blood pressure levels were defined according to the European Society of Cardiology Task Force Guidelines for the Management of Arterial Hypertension (ESH/ESC 2007) (779), the ESH Guidelines, 2005 (ESH 2005) (788), the American Heart Association Recommendations for Blood Pressure Measurement, 2005 (AHA

2005) (789), and the International Database on Ambulatory Blood Pressure Monitoring in Relation to Cardiovascular Outcomes (IDACO 2007) study data (790).

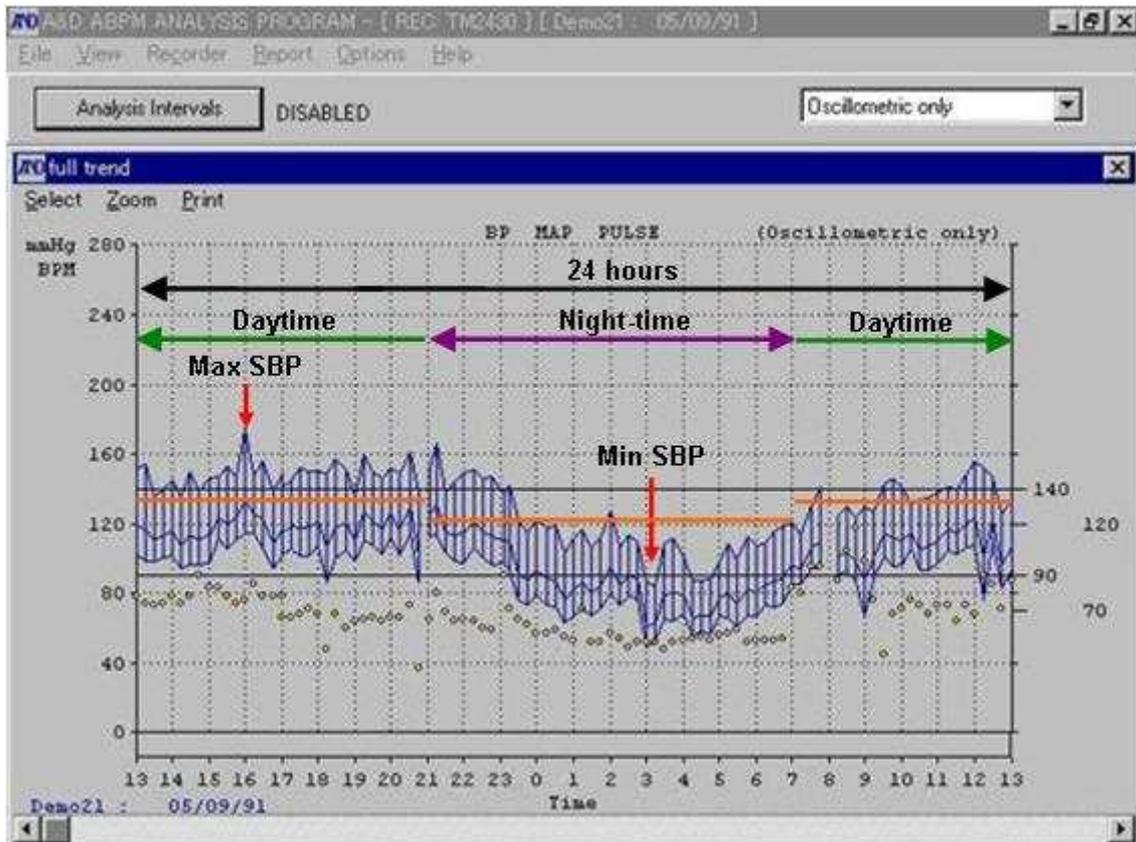


Figure 4.2 Example of a 24-hr ambulatory blood pressure and heart rate output using Doctor Pro™ ABPM data analysis software (TM-2430-13). Systolic and diastolic blood pressures are indicated by the top and bottom blue lines, respectively, and heart rate is indicated by circles. This individual's recordings show the normal diurnal rhythm in blood pressure, with a nocturnal fall and morning surge. As indicated, mean 24-hr and day-time and night-time parameters can be assessed, in addition to minimum and maximum levels, and blood pressure loads, which are expressed as the percentage of recordings above a defined threshold

Table 4.2 Twenty-four hour blood pressure parameters assessed before and after CoQ₁₀ and placebo treatment phases

Parameter	Description of 24-hr blood pressure parameters
Mean	Mean 24-hr SBP, DBP, PP, MAP, HR Mean daytime SBP, DBP, PP, MAP, HR†‡§ and mean night-time SBP, DBP, PP, MAP, HR†‡§ Mean night-time/day-time ratio SBP, DBP, PP, MAP, HR†‡§
Min/Max	Min for SBP, DBP, PP, MAP, HR†‡§, Max for SBP, DBP, PP, MAP, HR†‡§ and Min/Max ratio for SBP, DBP, PP, MAP, HR†‡§
Morning Surge	Sleep trough morning surge in SBP, DBP (Mean BP first two hrs after arising – mean of lowest night-time BP ± 2 readings) Pre-awakening morning surge in SBP, DBP (Mean BP first two hrs after arising – mean BP two hrs prior to arising)
Nocturnal Fall	Nocturnal fall in SBP, DBP (Mean daytime BP– mean night-time BP)†‡§ % Nocturnal fall in SBP, DBP ((Nocturnal fall/mean daytime BP)*100)†‡§
Load#	% SBP readings >135mmHg, ≥125mmHg for 24hrs and % DBP readings >85mmHg, ≥80mmHg for 24hrs % SBP readings >140mmHg, ≥130mmHg during daytime†‡§ and % DBP readings >90mmHg, ≥85mmHg during daytime†‡§ % SBP readings >125mmHg, ≥120mmHg during night-time†‡§ and % DBP readings >75mmHg, ≥70mmHg during night-time†‡§
Variability	SD for mean 24hr SBP, DBP, HR; mean daytime SBP, DBP, HR and mean night-time for SBP, DBP, HR†‡§ Average real variability, ARV ₂₄ for mean of SBP, DBP, HR
	$ARV = \frac{1}{N-1} \sum_{k=1}^{N-1} BP_{k+1} - BP_k $ where: <i>N</i> is the number of BP readings, <i>k</i> ranges from 1 to <i>N</i> -1.

†Defined by the participant’s recorded retiring to bed and rising times. ‡Defined arbitrarily as 0600-2059 hrs for daytime and 2100-0559 hrs for night-time. §Defined by the narrow fixed time interval method described in the ESH 2005 guidelines as 0600-2059 hrs for daytime and 0100-0559 hrs for night-time. #Thresholds for normal ambulatory blood pressure levels defined by the ESH/ESC 2007, ESH 2005, AHA 2005 and IDACO 2007 guidelines. SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; HR; heart rate; SD, standard deviation; ARV₂₄, average real variability in 24-hr ABP recordings (weighted for the time interval between consecutive recordings).

4.2.6 CoQ₁₀ formulation

Both Q-Gel® and placebo were supplied by Tischon Corp, USA, and were identical in appearance. Q-Gel® contained 100mg of CoQ₁₀, vitamin E 150 IU, annatto seed extract, Biosolv® base (lecithin, polysorbate, sorbitin monoleate, and medium chain triglycerides). Q-Gel® was selected as it has been shown to have superior bioavailability in comparison to other CoQ₁₀ formulations (416), and twice daily dosing with 100mg was selected, since Molyneux *et al.* (2007) have previously shown achievement of optimal plasma levels at 200 mg (408). Randomisation into 1 of 2 sequences (active placebo or placebo active) was performed in permuted blocks of six from a computer generated randomisation list. Compliance with treatment was assessed at the end of each intervention through capsule count. The study treatments were dispensed by an independent hospital pharmacist in identical numbered bottles with the lowest available number allocated to each sequential participant. Participants and investigators administering the treatment and assessing outcomes were blinded to treatment assignment and to plasma CoQ₁₀ levels.

4.2.7 Biochemical parameters

All biochemical analyses were performed by Canterbury Health Laboratories, an ISO15189 accredited (human) pathology laboratory. Total plasma CoQ₁₀ was measured by reverse phase high performance liquid chromatography with electrochemical detection using a method described by Molyneux *et al.* (2007) (408). Plasma total cholesterol, triglycerides, and HDL cholesterol were determined by an enzymatic colorimetric method (Architect c8000 analyser, Abbott Laboratories, Abbott Park, Illinois, U.S.A.). LDL-cholesterol was calculated from the Friedewald equation. High sensitivity C-reactive protein levels (hs-CRP) were determined by rate nephelometry. Safety markers, including electrolytes, plasma glucose, renal and liver function (Architect c8000 analyser), HbA1c (Biorad Variant HPLC), and a full blood count (Coulter Electronics, Luton, UK) were measured. Urine concentrations of creatinine and sodium were also determined (Architect c8000 analyser).

4.2.8 Statistical analyses

The sample size was based on published estimated treatment effects included in the meta-analyses examining the blood pressure lowering efficacy of CoQ₁₀ in patients with hypertension (425, 499). A total of 30 patients in a cross-over design was calculated to provide sufficient power (>80%) to detect a statistically significant (two-tailed $\alpha=0.05$)

difference in the change from baseline between placebo and CoQ₁₀ of 8 mmHg for systolic blood pressure and 4 mmHg for diastolic blood pressure, assuming SDs of 13 mmHg and 7 mmHg, respectively,. This allowed for a 10% attrition rate.

All statistical analyses were performed using SPSS Base version 17.0 (SPSS, Inc., Chicago, Illinois). Comparison of changes from baseline for each primary and secondary variable, between placebo and treatment cross-over phases was tested using analysis of variance (ANOVA) with repeated measures. For *post-hoc* subgroup analyses, the influence of potential mitigating factors including presence of type 2 diabetes or cardiovascular disease, baseline ambulatory systolic and diastolic blood pressure levels, dipper status, treatment with statins, metformin, and ACE inhibition, beta blockade, and CoQ₁₀ levels achieved on changes in primary and secondary outcomes were analysed using these variables as between subject factors in ANOVA with repeated measures. If significant effects were found with the analyses (i.e. an interaction between subgroup and the effect of CoQ₁₀), subgroups could be further analysed using Fisher's protected LSD tests. Differences in changes are shown so that the positive value for any of the indices indicate an advantage to CoQ₁₀ in terms of the blood pressure and heart rate parameters. The treatment effect size was calculated as the absolute value of the difference between the changes in variables following CoQ₁₀ and placebo treatment divided by the standard deviation of the change. Variables are summarised as mean \pm SEM and categorical data are presented as percentages. Statistical significance was inferred when $P < 0.05$.

4.3 Results

4.3.1 Baseline characteristics

Sixty patients were screened, of which 29 were not eligible to participate in the trial. One participant was withdrawn from the study as a result of an alteration to their antihypertensive therapy, and thus a total of 30 patients completed the trial and were included in the analysis (Figure 4.3). Baseline characteristics are shown in Table 4.3. Seventy three percent of participants were taking two or more antihypertensive therapies. Overall compliance rates based on tablet counts were 96% during CoQ₁₀ therapy and 95% during placebo administration.

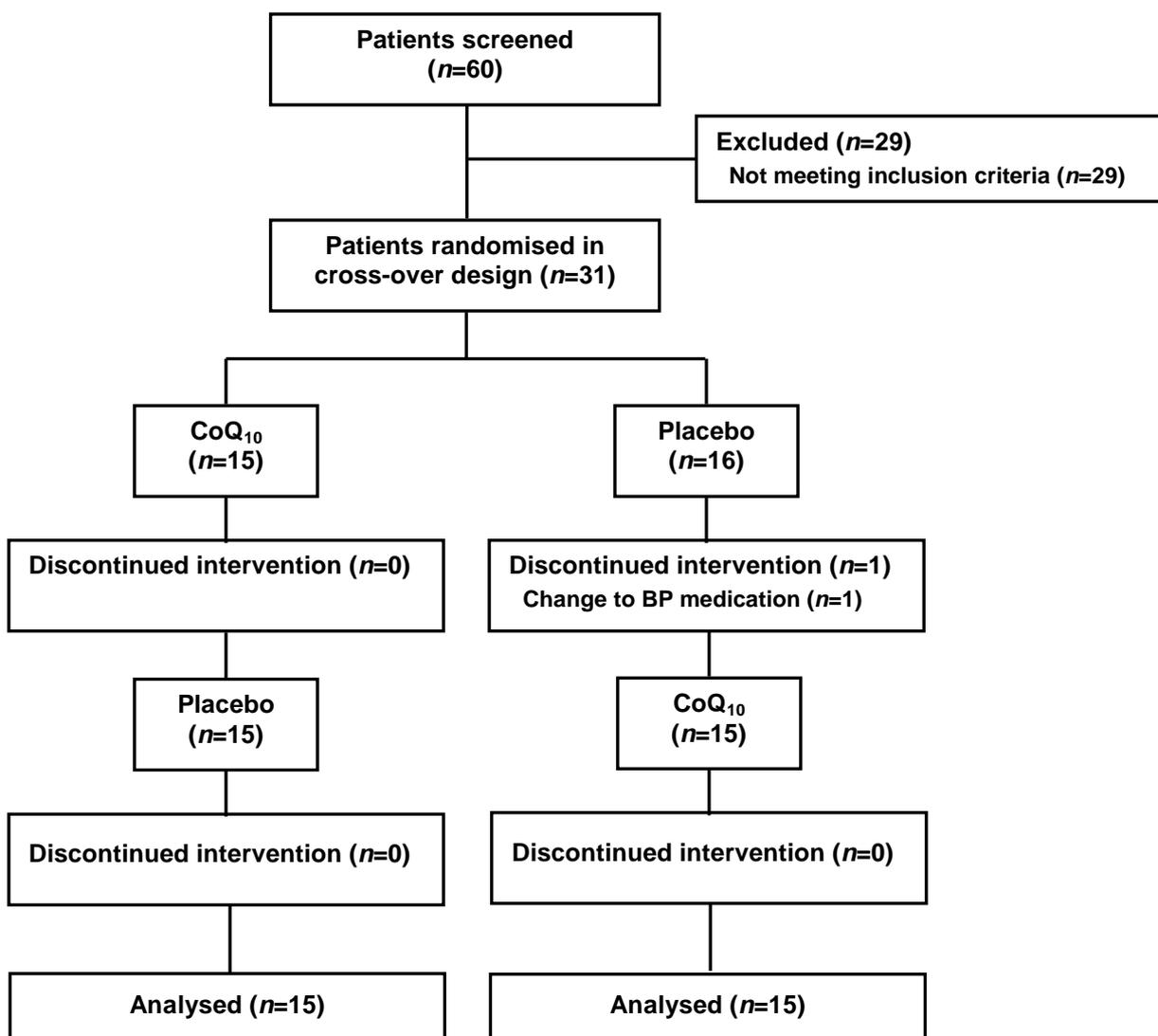


Figure 4.3 The flow of participants through the study

Table 4.3 Baseline characteristics of participants

Parameter	(n=30)
Demographics and clinical characteristics	
Age, y	64 ± 1
Female, n (%)	15 (50.0)
Clinic SBP, mmHg	147.8 ± 2.1
Clinic DBP, mmHg	77.4 ± 2.2
Heart rate, bpm	71.3 ± 2.8
Waist circumference, cm	110.2 ± 2.1
Weight, kg	94.1 ± 2.9
Body mass index, kg/m ²	32.1 ± 0.9
Cardiovascular disease, n (%)	9 (30.0)
Type 2 diabetes, n (%)	16 (53.3)
Total cholesterol, mmol/L	5.0 ± 0.2
Triglycerides, mmol/L	2.1 ± 0.3
HDL-cholesterol, mmol/L	1.18 ± 0.06
Plasma glucose, mmol/L	6.1 ± 0.2
Plasma creatinine, μmol/L	87 ± 3
Antihypertensive therapy, n (%)	
ACE inhibitor	21 (70.0)
Beta blocker	16 (53.3)
Diuretic	13 (43.3)
Calcium channel blocker	11 (36.7)
Angiotension II receptor blocker	5 (16.7)
Alpha blocker	4 (13.3)
No of antihypertensives, n (%)	
1	8 (26.7)
2	10 (33.3)
3	7 (23.3)
4	4 (13.3)
5	1 (3.3)
Concomitant therapy, n (%)	
Aspirin	14 (46.7)
Metformin	13 (43.3)
Sulfonylurea	7 (23.3)
Statins	16 (53.3)
Fibrate	2 (6.7)

Values are mean ± SEM or number (percentage).

4.3.2 Plasma CoQ₁₀ levels

Plasma CoQ₁₀ levels pre and post six weeks and 12 weeks of CoQ₁₀ and placebo treatment are shown in Figure 4.4. Baseline plasma CoQ₁₀ levels were similar prior to the placebo and CoQ₁₀ treatment phases ($P=0.18$). CoQ₁₀ levels were significantly increased from baseline after six and 12 weeks of CoQ₁₀ supplementation ($P<0.0001$), although the increase from six to 12 weeks was non-significant ($P=0.29$). No changes were observed following placebo treatment ($P=0.57$). Plasma CoQ₁₀ concentrations increased 3.7 fold after 12 weeks on CoQ₁₀ therapy compared with placebo administration ($P<0.0001$).

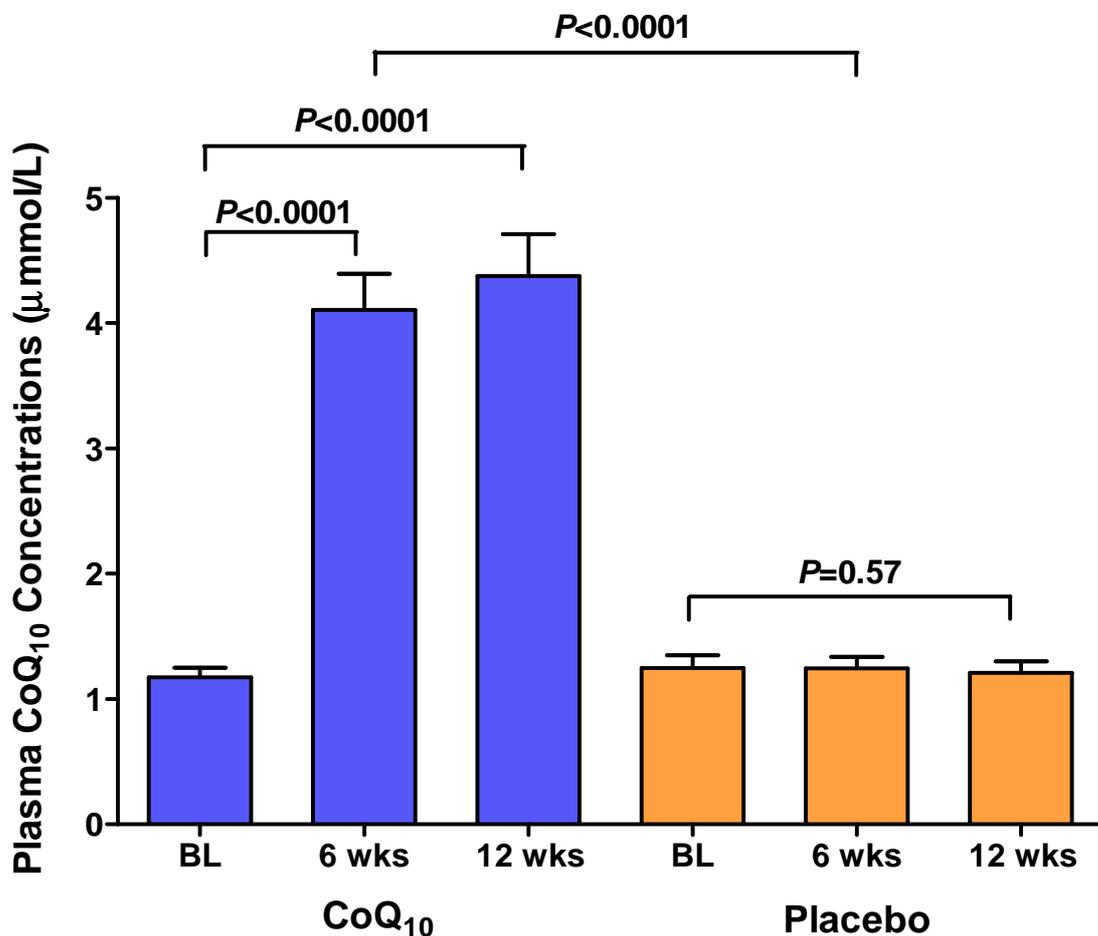


Figure 4.4 Mean plasma CoQ₁₀ concentrations at baseline and after six and 12 weeks of CoQ₁₀ and placebo treatment

4.3.3 Mean 24-hr ambulatory blood pressure and heart rate

There were no significant reductions in mean 24-hr systolic or diastolic blood pressure following treatment with CoQ₁₀ compared with placebo (Table 4.4). Furthermore, no significant decreases were observed in 24-hr pulse pressure, mean arterial pressure or heart rate during CoQ₁₀ therapy compared with placebo administration.

4.3.4 Clinic blood pressure and heart rate

No changes were observed in mean clinic systolic or diastolic blood pressure with 12 weeks of CoQ₁₀ therapy in comparison with placebo administration, however subjects manifested a significant increase in clinic heart rate during the placebo phase compared with the CoQ₁₀ phase ($P=0.04$) (Table 4.4).

4.3.5 Mean daytime and night-time blood pressure and heart rate

Mean daytime and night-time blood pressures and heart rate following CoQ₁₀ and placebo treatment, with day and night-time defined by the ESH 2005 guidelines, are summarised in Table 4.5. No significant effects of CoQ₁₀ were observed on mean daytime or night-time blood pressures in comparison with placebo treatment, although there was a small increase in daytime diastolic blood pressure during placebo administration compared with CoQ₁₀ ($P=0.14$). Daytime and night-time heart rates were not altered with CoQ₁₀ therapy, whereas night-time heart rate increased significantly on placebo treatment compared with CoQ₁₀ treatment ($P=0.006$). Furthermore, there were no changes in the mean daytime/night-time blood pressure ratios or night-time/daytime blood pressure ratios with CoQ₁₀ versus placebo therapy (data not shown). Minimum and maximum daytime and night-time blood pressure readings were not significantly different following CoQ₁₀ and placebo supplementation (data not shown). Minimum and maximum daytime heart rate levels were also unchanged, but there was an increase in minimum night-time heart rate during the placebo phase (2.7 bpm) compared with the CoQ₁₀ phase (-0.4), ($P<0.05$). These effects of CoQ₁₀ treatment on daytime and night-time blood pressure and heart rate were very similar to those classified by the arbitrary and participant definitions of daytime and night-time (Appendices 3 and 4).

4.3.6 Ambulatory blood pressure load

There were no significant differences in 24-hr systolic or diastolic blood pressure loads with CoQ₁₀ compared with placebo administration (Table 4.6). Similarly, there was no effect of CoQ₁₀ therapy on daytime systolic blood pressure loads; however a significant reduction was observed in daytime diastolic blood pressure loads, with thresholds set at >90 mmHg ($P=0.007$) and ≥ 85 mmHg ($P=0.03$) during CoQ₁₀ treatment compared with placebo (Figure 4.5). Night-time blood pressure loads were similar during the two treatment phases. The same pattern was observed according to arbitrary and participant defined daytime and night-time for blood pressure loads (Appendices 5 and 6).

4.3.7 Morning surge in ambulatory blood pressure

The effects of CoQ₁₀ supplementation on morning surge in blood pressure are presented in Table 4.7. There were no differences in pre-awakening morning surge and sleep through morning surge in systolic or diastolic blood pressure between CoQ₁₀ and placebo phases.

4.3.8 Nocturnal effect on blood pressure

The nocturnal fall in systolic and diastolic blood pressure with CoQ₁₀ supplementation and placebo is shown in Table 4.7. No effects of CoQ₁₀ on nocturnal fall or the percentage nocturnal fall in systolic and diastolic blood pressure were observed in comparison with placebo treatment. Similarly, there were no effects of CoQ₁₀ versus placebo on nocturnal fall in blood pressure for the arbitrary and participant definitions of daytime and night-time (Appendices 7 and 8).

Table 4.4 Effect of CoQ₁₀ and placebo on 24-hr ambulatory and clinic blood pressure and heart rate

	CoQ ₁₀		Placebo		Advantage to CoQ ₁₀ †	P-value‡
	Baseline	12-weeks	Baseline	12-weeks		
24-hr						
SBP, mmHg	144.3 ± 2.7	143.2 ± 2.5	143.9 ± 2.7	143.6 ± 2.4	0.9 (-2.4, 4.1)	0.60
DBP, mmHg	79.0 ± 1.7	78.7 ± 1.7	78.1 ± 1.7	79.2 ± 1.7	1.3 (-0.3, 2.9)	0.12
PP, mmHg	65.3 ± 1.8	64.5 ± 1.7	65.7 ± 1.9	64.5 ± 1.7	-0.4 (-3.0, 2.1)	0.73
MAP, mmHg	100.8 ± 1.9	100.2 ± 1.8	100.1 ± 1.9	100.6 ± 1.8	1.2 (-0.8, 3.1)	0.25
HR, bpm	69.5 ± 1.8	69.2 ± 1.6	69.2 ± 1.9	70.9 ± 2.0	2.1 (-0.5, 4.6)	0.10
Clinic						
SBP, mmHg	142.8 ± 2.0	141.8 ± 3.1	140.1 ± 2.1	142.2 ± 2.5	3.0 (-2.7, 8.7)	0.30
DBP, mmHg	74.2 ± 2.1	73.3 ± 2.3	72.2 ± 2.0	73.4 ± 1.9	2.0 (-1.1, 5.1)	0.21
HR, bpm	72.0 ± 2.8	72.6 ± 1.8	70.1 ± 2.5	75.7 ± 2.4*	4.8 (0.2, 9.4)	0.04

Values are mean ± SEM.

† Difference between mean change after placebo and CoQ₁₀ (95% CI).

‡ ANOVA with repeated measures for comparison of between group changes.

*P<0.05 using paired t-test for within-group changes from baseline.

SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; HR, heart rate.

Table 4.5 Effect of CoQ₁₀ and placebo on daytime and night-time ambulatory blood pressure and heart rate

	CoQ ₁₀		Placebo		Advantage to CoQ ₁₀ †	P-value‡
	Baseline	12-weeks	Baseline	12-weeks		
Daytime§						
SBP, mmHg	148.6 ± 2.8	148.4 ± 2.5	148.6 ± 2.8	150.0 ± 2.3	1.6 (-3.1, 6.3)	0.50
DBP, mmHg	82.6 ± 1.7	82.6 ± 1.8	81.1 ± 1.7	83.3 ± 1.7	2.2 (-0.7, 5.1)	0.14
PP, mmHg	66.1 ± 2.1	65.9 ± 1.8	67.5 ± 2.0	66.6 ± 1.7	-0.6 (-4.3, 3.1)	0.73
MAP, mmHg	104.6 ± 1.8	104.5 ± 1.9	103.6 ± 1.9	105.5 ± 1.7	2.0 (-1.2, 5.2)	0.21
HR, bpm	73.3 ± 2.0	73.0 ± 1.9	73.2 ± 2.3	74.7 ± 2.3	1.8 (-1.3, 4.8)	0.25
Night-time§						
SBP, mmHg	133.6 ± 3.6	132.1 ± 3.6	132.7 ± 3.4	130.7 ± 3.5	-0.5 (-5.2, 4.2)	0.84
DBP, mmHg	72.1 ± 2.2	71.6 ± 2.1	71.1 ± 2.2	70.4 ± 2.1	-0.2 (-3.7, 3.2)	0.90
PP, mmHg	61.5 ± 2.1	60.6 ± 2.1	61.6 ± 2.2	60.4 ± 2.2	-0.3 (-2.5, 2.0)	0.81
MAP, mmHg	92.6 ± 2.6	91.7 ± 2.5	91.6 ± 2.5	90.5 ± 2.5	-0.3 (-4.1, 3.5)	0.87
HR, bpm	62.5 ± 1.7	62.2 ± 1.8	60.8 ± 1.7	64.1 ± 2.1*	3.7 (1.2, 6.2)	0.006

Values are mean ± SEM.

†Difference between mean change after placebo and CoQ₁₀ (95% CI).

‡ANOVA with repeated measures for comparison of between group changes.

§Daytime and night-time defined by the narrow fixed time interval method described in the ESH 2005 guidelines.

*P<0.05 using paired t-test for within-group changes from baseline.

SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; HR, heart rate.

Table 4.6 Effect of CoQ₁₀ and placebo on ambulatory blood pressure load

	CoQ ₁₀		Placebo		Advantage to CoQ ₁₀ †	P-value‡
	Baseline	12-weeks	Baseline	12-weeks		
24hr BP loads						
% SBP >135 mmHg	65.8 ± 4.8	64.2 ± 4.3	64.5 ± 4.1	65.4 ± 4.1	2.5 (-4.3, 9.3)	0.46
% SBP ≥125 mmHg	81.0 ± 3.2	79.0 ± 3.4	79.2 ± 3.3	80.3 ± 3.2	3.1 (-2.1, 8.4)	0.23
% DBP >85 mmHg	35.4 ± 4.7	33.5 ± 4.6	32.8 ± 4.3	34.5 ± 4.5	3.7 (-1.9, 9.2)	0.19
% DBP ≥ 80 mmHg	47.7 ± 5.4	46.7 ± 4.8	45.5 ± 4.9	46.9 ± 4.8	2.4 (-2.9, 7.7)	0.37
Daytime BP loads§						
% SBP >140 mmHg	62.8 ± 5.3	65.2 ± 4.5	63.9 ± 4.6	68.1 ± 4.5	1.8 (-9.0, 12.6)	0.74
% SBP ≥130 mmHg	81.6 ± 3.5	82.5 ± 3.5	81.2 ± 3.1	85.0 ± 3.1	2.8 (-6.5, 12.1)	0.55
% DBP >90 mmHg	30.6 ± 4.8	26.3 ± 4.5	24.7 ± 4.3	30.2 ± 4.8	9.7 (2.8, 16.6)	0.007
% DBP ≥ 85 mmHg	42.3 ± 5.4	40.1 ± 5.1	38.3 ± 4.9	44.5 ± 5.2	8.3 (0.8, 15.8)	0.03
Night-time BP loads§						
% SBP >125 mmHg	63.8 ± 6.9	60.5 ± 5.9	61.2 ± 6.5	58.7 ± 6.5	0.8 (-10.8, 12.3)	0.90
% SBP ≥120 mmHg	69.8 ± 6.1	69.8 ± 5.9	70.0 ± 6.0	67.2 ± 6.1	-2.7 (-12.7, 7.2)	0.58
% DBP >75 mmHg	43.4 ± 7.4	40.8 ± 6.4	41.0 ± 6.6	35.6 ± 6.5	-2.9 (-14.6, 8.8)	0.62
% DBP ≥ 70 mmHg	55.1 ± 7.0	51.6 ± 6.7	54.3 ± 6.6	48.6 ± 7.0	-2.3 (-14.0, 9.5)	0.70

Values are mean ± SEM. †Difference between mean change after placebo and CoQ₁₀ (95% CI).

‡ANOVA with repeated measures for comparison of between group changes.

§Daytime and night-time defined by the narrow fixed time interval method described in the ESH 2005 guidelines.

SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; HR, heart rate.

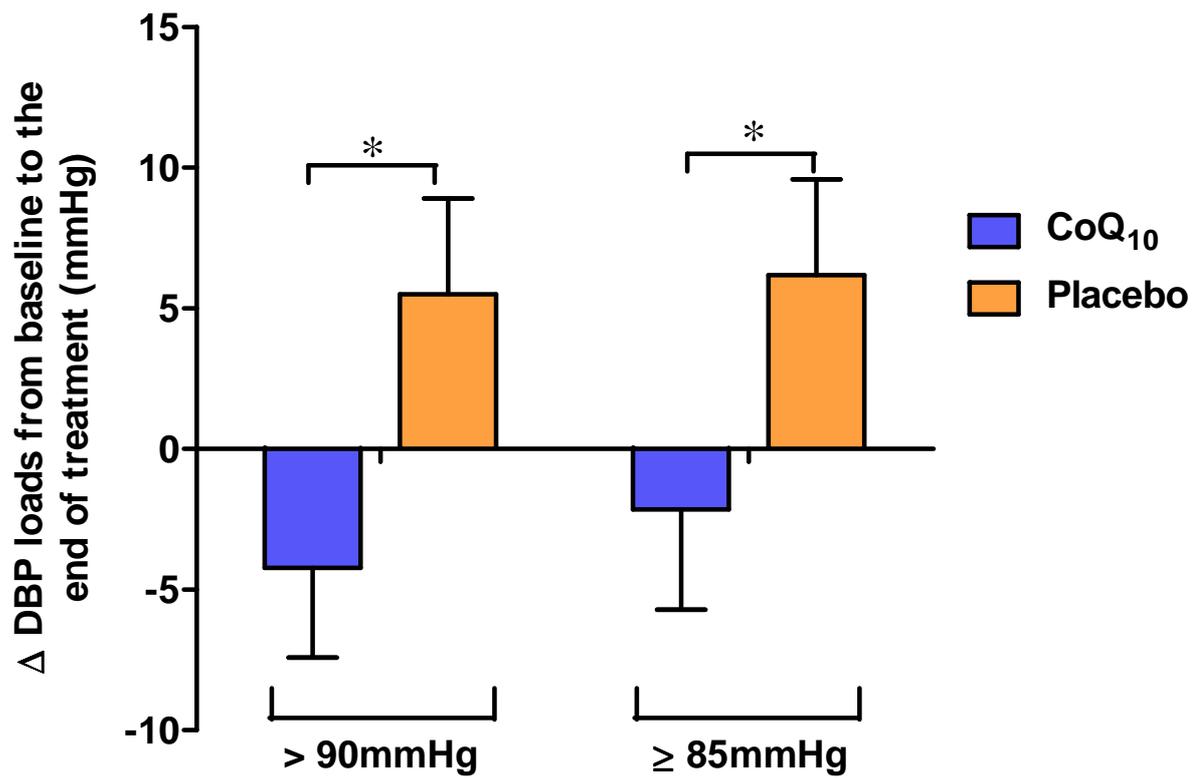


Figure 4.5 Changes in daytime diastolic blood pressure loads >90 mmHg and ≥85 mmHg from baseline following 12 weeks of treatment with CoQ₁₀ or placebo (**P*<0.05 for comparison of between group changes)

Table 4.7 Effect of CoQ₁₀ and placebo on morning surge and nocturnal fall in blood pressure

	CoQ ₁₀		Placebo		Advantage to CoQ ₁₀ [†]	P-value [‡]
	Baseline	12-weeks	Baseline	12-weeks		
Pre-awakening morning surge						
SBP, mmHg	14.3 ± 3.7	15.3 ± 2.8	16.9 ± 2.7	17.5 ± 3.0	-0.4 (-8.3, 7.5)	0.91
DBP, mmHg	10.8 ± 2.1	13.0 ± 1.8	12.1 ± 1.7	12.6 ± 1.9	-1.8 (-6.3, 2.8)	0.44
Sleep through morning surge						
SBP, mmHg	28.2 ± 3.4	26.0 ± 2.8	30.4 ± 2.6	31.2 ± 3.5	3.0 (-5.6, 11.5)	0.48
DBP, mmHg	18.0 ± 1.5	19.4 ± 1.6	19.2 ± 1.8	21.2 ± 1.9	0.6 (-5.0, 6.1)	0.84
Nocturnal fall [§]						
SBP, mmHg	15.1 ± 3.0	16.3 ± 3.1	15.9 ± 3.0	19.2 ± 3.1	2.1 (-4.9, 9.0)	0.55
DBP, mmHg	10.5 ± 1.5	11.0 ± 1.9	10.0 ± 1.6	13.0 ± 1.6	2.4 (-2.9, 7.8)	0.36
% Nocturnal fall [§]						
SBP, %	10.0 ± 2.0	10.9 ± 2.1	10.5 ± 1.9	12.8 ± 2.0	1.4 (-3.0, 5.7)	0.53
DBP, %	12.8 ± 1.8	13.0 ± 2.2	12.4 ± 2.0	15.6 ± 1.8	3.1 (-2.9, 9.0)	0.30

Values are mean ± SEM.

[†] Difference between mean change after placebo and CoQ₁₀ (95% CI).

[‡] ANOVA with repeated measures for comparison of between group changes.

[§] Daytime and night-time defined by the narrow fixed time interval method described in the ESH 2005 guidelines.

SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; HR, heart rate.

4.3.9 Ambulatory blood pressure and heart rate variability

There were no significant differences in the 24-hr average real variability (ARV₂₄) for systolic or diastolic blood pressure or heart rate by CoQ₁₀ versus placebo treatment (Table 4.8). The standard deviation of mean 24-hr blood pressure and heart rate were also unchanged by CoQ₁₀ versus placebo administration. Similarly, there was no effect of CoQ₁₀ therapy on the standard deviations of mean daytime or night-time blood pressures or heart rate compared with placebo administration. Blood pressure and heart rate variability for arbitrary and participant definitions of daytime and night-time also did not differ between the CoQ₁₀ and placebo phases (Appendices 9 and 10).

4.3.10 Subgroup analysis

In subgroup analyses, that included baseline systolic and diastolic blood pressure levels defined by the median cut points, presence of type 2 diabetes, presence of cardiovascular disease, concentration of CoQ₁₀ achieved on therapy (>2.5 µg/ml and >3.5 µg/ml), nocturnal fall in systolic blood pressure >10% and diastolic blood pressure >10%, or treatment with statins, metformin, ACE inhibitors or beta blockers, there was no evidence of any treatment effect of CoQ₁₀ on 24-hr ambulatory blood pressure parameters or heart rate compared with placebo. A small number of subgroup-variable combinations showed a significantly different response to CoQ₁₀ treatment but there were no consistent patterns, indicating that they were not anymore than spurious potential Type I errors (Appendix 11).

4.3.11 Tolerability and adverse events

CoQ₁₀ was well tolerated and there were no reported serious adverse events following either CoQ₁₀ or placebo treatment. There were no clinically or statistically significant changes in biochemistry and haematology safety parameters included assessment of electrolytes, liver and renal function, and complete blood count (data not shown).

Table 4.8 Effect of CoQ₁₀ and placebo on ambulatory blood pressure and heart rate variability

	CoQ ₁₀		Placebo		Advantage to CoQ ₁₀ [†]	P-value [‡]
	Baseline	12-weeks	Baseline	12-weeks		
24hr ARV ₂₄						
SBP	13.6 ± 0.5	13.9 ± 0.6	14.5 ± 0.5	13.9 ± 0.6	-0.9 (-2.6, 0.8)	0.28
DBP	10.2 ± 0.6	10.3 ± 0.7	10.7 ± 0.6	10.4 ± 0.7	-0.4 (-2.0, 1.2)	0.59
HR	7.1 ± 0.6	6.8 ± 0.6	6.5 ± 0.5	6.0 ± 0.4	-0.2 (-1.8, 1.4)	0.79
24hr SD						
SBP	18.7 ± 0.7	18.9 ± 0.9	19.6 ± 0.7	19.7 ± 0.8	-0.2 (-2.5, 2.1)	0.87
DBP	13.3 ± 0.6	14.1 ± 0.8	13.3 ± 0.6	14.3 ± 0.8	0.2 (-1.8, 2.2)	0.81
HR	11.7 ± 0.8	10.6 ± 0.7	11.0 ± 0.8	10.3 ± 0.8	0.3 (-1.7, 2.3)	0.75
Daytime SD [§]						
SBP	16.2 ± 0.6	16.6 ± 0.9	17.0 ± 0.7	17.0 ± 0.8	-0.5 (-3.2, 2.2)	0.72
DBP	13.3 ± 0.9	13.0 ± 0.9	12.5 ± 0.8	13.3 ± 0.9	1.0 (-1.8, 3.8)	0.47
HR	12.1 ± 1.0	9.9 ± 0.8	9.7 ± 0.8	9.7 ± 0.9	2.1 (-1.4, 5.6)	0.24
Night-time SD [§]						
SBP	13.0 ± 0.8	13.3 ± 1.1	14.3 ± 0.7	13.3 ± 0.8	-1.3 (-5.2, 2.7)	0.52
DBP	9.1 ± 0.7	10.2 ± 1.3	9.0 ± 0.6	8.0 ± 0.5	-2.1 (-5.5, 1.3)	0.22
HR	4.8 ± 0.4	4.9 ± 0.6	4.4 ± 0.4	4.6 ± 0.3	0.0 (-1.2, 1.2)	0.99

Values are mean ± SEM.

[†]Difference between mean change after placebo and CoQ₁₀ (95% CI). [‡]ANOVA with repeated measures for comparison of between group changes.

[§]Daytime and night-time defined by the narrow fixed time interval method described in the ESH 2005 guidelines. ARV₂₄, 24 hr average real variability; SD, standard deviation of the mean; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate.

4.4 Discussion

4.4.1 Effect of CoQ₁₀ on mean blood pressure

There is considerable enthusiasm for the use of CoQ₁₀ as an antihypertensive agent. The concept has attraction since patients with essential hypertension and/or diabetes mellitus reportedly have evidence of oxidative stress (787) and low circulating levels of CoQ₁₀ (480), a number of clinical studies have documented an antihypertensive action of CoQ₁₀ supplementation (480, 484-486, 489-498), and CoQ₁₀ has been demonstrated experimentally to induce vasodilation through effects on the endothelium and vascular smooth muscle (363, 379). Support for the concept comes from the meta-analysis of Rosenfeldt *et al.* (2007) (425), which concluded that until the results of further trials are available, “it would seem acceptable to add CoQ₁₀ to conventional antihypertensive therapy....’ and that it “...may have a particular therapeutic role in hypertensive patients with consistently increased levels of oxidative stress as in diabetes or renal failure”. As mentioned however, this meta-analysis is open to a number of questions and criticisms. Furthermore, a subsequent, more selective analysis by the Cochrane Hypertension Group concluded that it was uncertain whether CoQ₁₀ reduces blood pressure in the long-term management of primary hypertension (499). In view of this uncertainty, the present study was carried out to determine whether CoQ₁₀, when added to conventional antihypertensive therapy, reduces arterial pressure in patients with the metabolic syndrome and inadequately controlled hypertension.

Compared to placebo, 12 weeks of CoQ₁₀ supplementation was not associated with clinically significant reductions in clinic or 24-hr ambulatory blood pressure in patients with the metabolic syndrome and inadequately treated hypertension. Furthermore, there were no changes in mean daytime and night-time ambulatory blood pressures with CoQ₁₀ compared with placebo treatment. Although there were trends for a small reduction in 24-hr systolic and diastolic blood pressures and mean arterial pressure in favour of CoQ₁₀, these effects were not statistically significant.

These observations in hypertensive patients with the metabolic syndrome may not necessarily apply to other patient groups. For example some studies reporting an antihypertensive effect of CoQ₁₀ enrolled patients with considerably higher baseline levels of blood pressure (systolic blood pressure >160 mmHg) than in the present study (425, 499) (Figure 4.6). A more obvious treatment effect might be expected in subjects with higher

baseline blood pressure levels. Consistent with this, less pronounced blood pressure lowering effects of CoQ₁₀ have been reported in modestly hypertensive patients with type 2 diabetes (485, 486). Hodgson *et al.* (2002) (485) reported a blood pressure reduction of 6 mmHg systolic and 3 mmHg diastolic after 12 weeks of CoQ₁₀ supplementation in type 2 diabetic patients, where baseline levels were modestly elevated (485). Smaller treatment effects were also demonstrated in a more recent controlled trial of type 2 diabetic subjects with left ventricular diastolic dysfunction, in which CoQ₁₀ was shown to reduce 24-hr diastolic blood pressure by 2.2 mmHg (486). Again, these patients had modest blood pressure elevations (486). However a recent meta-analysis found no evidence that the efficacy of blood pressure lowering treatment depends substantively upon baseline blood pressure levels (791). It is possible also that there are significant interactions of CoQ₁₀ with other therapies. For example, Chew *et al.* (2008) demonstrated a more pronounced antihypertensive effect of CoQ₁₀ when administered in combination with fenofibrate to patients with type 2 diabetes mellitus (486). Only two of the patients in the present study were on fibrate therapy.

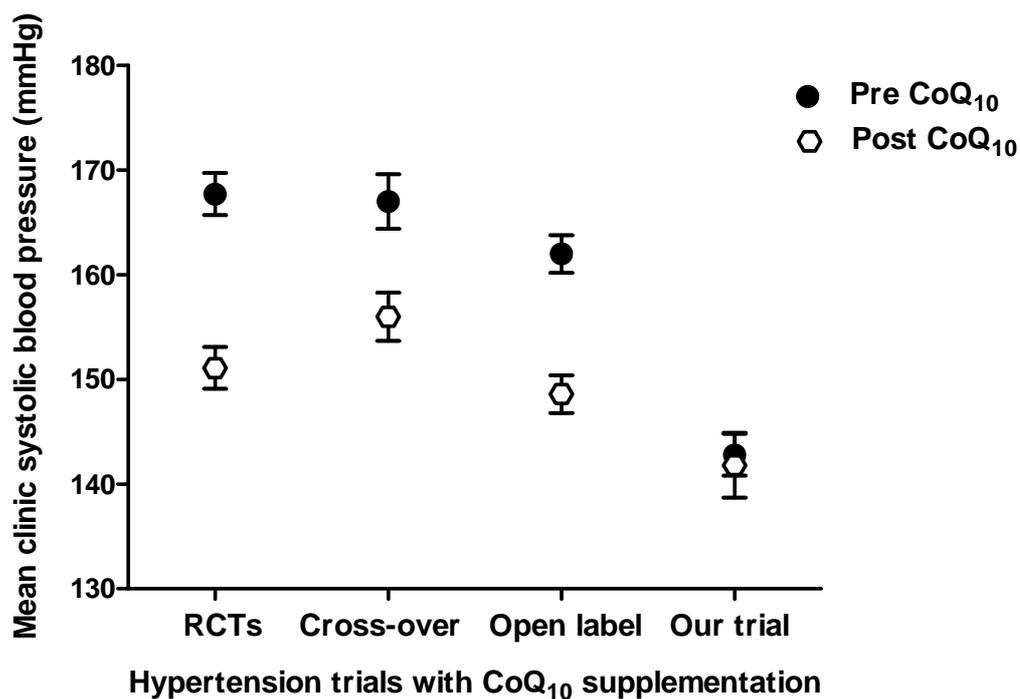


Figure 4.6 Mean \pm SEM pre and post clinic systolic blood pressure levels of hypertensive patients in the randomised controlled trials (n=120), cross-over study (n=18), and open label trials (n=214) from in the meta-analysis and the present study (n=30)

The present findings concur with one other double-blind, placebo-controlled intervention trial by Mori *et al.* (2009) (792) who found eight weeks of CoQ₁₀ administration had no effect on 24-hr ambulatory blood pressure in patients with chronic kidney disease. In that study, treated blood pressure levels were 125/73 mmHg prior to randomisation. As noted above, however, any antihypertensive action of CoQ₁₀ is likely to be less obvious the lower the baseline level of blood pressure. In this regard, it has been shown that CoQ₁₀ does not have vasodilatory effects in normotensive animals or humans (425).

4.4.2 Effect of CoQ₁₀ on blood pressure load

Blood pressure loads can be defined as the proportion of 24-hour, daytime or night-time blood pressure readings that are increased relative to pre-determined thresholds as described by White *et al.* (1989) (793). Since there is no standardised definition of what represents “normal” ambulatory blood pressure (794), thresholds defined from current guidelines were used to assess 24-hr, daytime and night-time blood pressure loads (779, 788-790). In hypertensive patients, blood pressure loads have been reported to be more closely related to cardiac function and left ventricular hypertrophy than mean blood pressure (793, 795-797). The key study by White *et al.* (1989) (793) established that there was a marked increase in the prevalence of left ventricular hypertrophy in untreated hypertensive patients, with systolic blood pressure loads greater than 50% and diastolic blood pressure loads exceeding 40%. Recently, Andrade *et al.* (2010) (798) demonstrated that a daytime systolic load of 24.5% or greater (>135mmHg) independently predicted cardiovascular events in elderly hypertensive patients. To my awareness this is the first study to examine the effect of CoQ₁₀ on blood pressure loads in treated hypertensive patients. Compared with placebo, CoQ₁₀ treatment had favourable effects on daytime diastolic blood pressure loads >90 mmHg and ≥85 mmHg, but no effect on 24-hour or night-time loads. The mechanisms underlying this effect of CoQ₁₀ may involve changes in cardiac and/or vascular (large or small vessel) function but this study was not designed to assess these possibilities. Whatever the underlying mechanisms, the current data raise the possibility that although CoQ₁₀ supplementation did not lead to reductions in absolute blood pressure levels, patients may nevertheless benefit through a decrease in daytime arterial load. It is possible, however, that these statistically significant and rather small effects on blood pressure load may reflect a type 1 error, given the large number of variables examined in this study.

4.4.3 Effect of CoQ₁₀ on morning surge in blood pressure

Blood pressure exhibits a well defined circadian rhythm, with a nocturnal decline during sleep, and a surge at the time of awakening (799-801). Accordingly, the incidence of cardiovascular events and sudden cardiac death displays similar diurnal variation with a morning peak (802, 803). Normal morning blood pressure surge is a physiological event, but an exaggerated morning surge in blood pressure increases cardiovascular risk (804). The association between the degree of morning blood pressure surge and cardiovascular risk is therefore not linear but has a threshold (804). To date, six prospective studies have demonstrated a significant association between exaggerated morning blood pressure surge and cardiovascular events (805-810). However, there is currently no consensus on the definition or threshold of pathological morning surge in blood pressure (811), although Li *et al.* (2010) (810) and Stolarz-Skrzypek *et al.* (2010) (812) indicate that a sleep-through or pre-awakening morning surge in systolic blood pressure <20 mmHg is not likely to be associated with an increased cardiovascular risk. Control of morning surge in blood pressure following bedtime administration of an alpha adrenergic blocker, angiotension II receptor blocker or therapy with a calcium channel blocker has been reported to reduce urinary albuminuria (811, 813). These results indicate that selective suppression of morning blood pressure surge may prevent target organ damage and subsequent cardiovascular events (811). However, there was no evidence of a hypotensive effect of CoQ₁₀ on pre-awakening or sleep-through morning surge in systolic or diastolic blood pressure in the present study population. This suggests that CoQ₁₀ does not selectively target the morning blood pressure surge, although it should be noted that the mean pre-awakening morning blood pressure surge at baseline was not likely to be pathological as the mean sleep-through morning blood pressure surge was not markedly elevated.

4.4.4 Effect of CoQ₁₀ on nocturnal fall in blood pressure

The normal circadian rhythm of blood pressure has a nocturnal decrease of 15% to 25% of daytime values, whereas 25% to 40% of hypertensive patients exhibit a non-dipping pattern. A blunted decrease in nocturnal blood pressure is associated with target organ damage (814), and is a strong independent risk factor for cardiovascular mortality (815, 816). Ohkubo *et al.* (2002) (817) reported a linear and inverse relationship between cardiovascular mortality and the nocturnal decrease in blood pressure which was independent of 24-hr blood pressure load and additional cardiovascular risk factors. The “non-dipper” pattern has been

arbitrarily defined as a blood pressure reduction of less than 10% during night-time, although current data point towards the importance of looking at the nocturnal dip as a continuous variable (794). In view of the pathological evidence for an attenuated nocturnal fall in blood pressure, it is important to note that 50% of the patients in this study were ‘non-dippers’ and this remained unchanged with CoQ₁₀ in comparison to placebo treatment, indicating no improvement in the non-dipper status of these patients.

4.4.5 Effect of CoQ₁₀ on blood pressure variability

It has been hypothesised that cardiovascular prognosis is not only related to absolute 24-hr blood pressure levels but also to blood pressure variability. In early trials, intra-arterial blood pressure monitoring of variability measured as a standard deviation (SD) of reading to reading blood pressures was shown to be associated with target organ damage in hypertensive patients (818, 819). A number of prospective trials have explored the prognostic significance of blood pressure variability but results have been inconsistent (820-826). In the majority of these trials, the investigators used the SD of the daytime, night-time, or 24-hr blood pressure as an index of variability; however the 24-hr SD in particular is limited by the diurnal blood pressure profile. To address this issue, Bilo *et al.* (2007) (827) proposed the SD over 24 hours weighted for the time interval between consecutive readings (SD₂₄), and the average of the daytime and night-time weighted for the duration of the daytime and night-time interval duration of day and night (SD_{dn}). Also, Mena *et al.* (2003) derived the average real variability (ARV₂₄), which is weighted for the time interval between consecutive readings (828). In a recent meta-analysis of over 8000 people from 11 trials, blood pressure variability assessed by SD_{dn} and ARV₂₄, was shown to be a significant and independent predictor of mortality and cardiovascular events, after adjustment for 24-hr blood pressure levels and other co-variables (829). Although, the proportion of the risk explained by the variability was low, the blood pressure variability did add to risk stratification (829). This prompted us to examine whether CoQ₁₀ may have an effect on blood pressure variability. No changes in SD or ARV₂₄ were observed with 12 weeks supplementation, suggesting that CoQ₁₀ does not influence blood pressure variability in this treated hypertensive population.

4.4.6 Effect of CoQ₁₀ on clinic blood pressure and heart rate

Heart rate may be an important therapeutic target, since it has been shown to independently predict cardiovascular risk (830). There was no change in clinic heart rate with

CoQ₁₀, but a significant increase with placebo after 12 weeks. There was also an increase in the adjusted night-time heart rate of 3.6 bpm with placebo compared with CoQ₁₀, which may represent a type 1 error. A similar observation was made in a controlled trial in type 2 diabetes patients with left ventricular diastolic dysfunction, where CoQ₁₀ administration had no effect on 24-hr heart rate (486). Conversely, in the double-blind, placebo controlled study of Singh *et al.* (1999) (484), CoQ₁₀ (120 mg daily) reduced heart rate in 58 patients with coronary artery disease who were receiving antihypertensive medication. After eight weeks of follow-up, a significant reduction in heart rate of 12 (95% CI 9, 15) bpm was observed in the CoQ₁₀ group compared with the control group. By contrast, in a controlled intervention trial of 74 patients with chronic kidney disease, eight weeks of CoQ₁₀ treatment (200 mg daily) was associated with a small but significant increase in 24-hr heart rate ($p < 0.03$) (792). Given the inconsistent effects of CoQ₁₀ observed in these studies, further trials are required to establish whether or not CoQ₁₀ supplementation leads to reductions in heart rate.

4.4.7 *Post-hoc* subgroup analyses

Post-hoc analyses of subgroups in the study showed no consistently significant differences in the response to adjunctive CoQ₁₀ therapy. Of the 3108 subgroup variable combinations examined, 86 (2.8%) showed a significantly different response to CoQ₁₀ treatment, probably reflecting a type I error rate.

4.4.8 Safety and tolerability of CoQ₁₀

CoQ₁₀ treatment was well tolerated and was not associated with any clinically relevant changes in safety parameters. These findings are consistent with a growing number of clinical studies that have reported good tolerability of CoQ₁₀, with relatively few adverse effects (409, 410, 424-431). Most clinical trials have not described significant adverse effects that necessitated stopping therapy, and to date, no serious adverse side effects have been reported (433). However, gastrointestinal effects such as abdominal discomfort, nausea, vomiting, diarrhoea, and anorexia, in addition to allergic rash and headache have been documented (433). It has been suggested that the effect of CoQ₁₀ on platelet function may increase the risk of bleeding in patients taking antiplatelet drugs such as aspirin or clopidogrel (437). Since CoQ₁₀ is structurally related to vitamin K, it has been postulated to have pro-coagulant effects (831), with reports suggesting that CoQ₁₀ may counteract the anticoagulant effects of warfarin, and increase the risk of inadequate anticoagulation in warfarin-treated patients

(438). Thus, on the basis of available evidence, patients on warfarin therapy were excluded from the present study. The long-term effects of CoQ₁₀ on tolerability are yet to be elucidated.

4.4.9 Study strengths

This study population was representative of patients who might be considered for treatment with adjunctive therapy, in addition to conventional antihypertensive therapies, since patients with the metabolic syndrome and/or diabetes mellitus reportedly have evidence of oxidative stress. The assessment of changes in large number of blood pressure variables over a 24-hour period, in addition to absolute levels allowed a more accurate assessment of the potential hypotensive effects of CoQ₁₀. A CoQ₁₀ formulation (Q-Gel®) was used which has been demonstrated to have superior bioavailability in comparison to other CoQ₁₀ supplement brands (416). In this study, twenty nine of the 30 patients attained CoQ₁₀ levels > 2.9 µmol/L (2.5 µg/mL) on therapy. These achieved levels have previously been associated with significant reductions in blood pressure (425). There was sufficient statistical power to detect the anticipated effects of CoQ₁₀ on 24-hr ambulatory systolic and diastolic blood pressure and mean arterial pressure which is shown in Table 4.9. The standard deviation of the mean changes in systolic and diastolic blood pressures following CoQ₁₀ versus placebo treatment was less than anticipated from previous studies. It is clear that any potential effect of CoQ₁₀ therapy in this study population is less than the 8 mmHg systolic and 4 mmHg diastolic reductions seen in earlier trials, as highlighted in Table 4.4. Based on the observed effect sizes, 1042 patients and 772 patients would be needed to show a significant difference in systolic and diastolic blood pressure, respectively (Table 4.9).

4.4.10 Study limitations

Although this study was sufficiently powered to detect an 8/4 mmHg change in blood pressure, one cannot rule out the possibility of smaller antihypertensive effects of CoQ₁₀ treatment in this patient group. Further trials would require larger numbers in order to confirm whether supplementation with CoQ₁₀ confers a less significant blood pressure lowering effect in similar populations. For example, on the basis of the observed effect sizes in the present study (0.10 and 0.30 for 24-hr systolic and diastolic blood pressure, respectively), a sample size of approximately 190 in a cross-over design would be required to detect a differential reduction from CoQ₁₀ of 2mmHg in systolic and diastolic blood pressure as statistically significant (Table 4.9).

Table 4.9 Effect size and power calculations for primary outcome variables in studies with a cross-over design

	Observed advantage to CoQ ₁₀ (mmHg)	SD	Effect size	Number of subjects required to detect various BP differences for $\beta=0.80$ at $\alpha=0.05$ (n)				
				Observed advantage	1 mmHg	2 mmHg	4 mmHg	8 mmHg
SBP	0.86	8.78	0.10	1042	771	193	48	12 [†]
DBP	1.30	4.38	0.30	114	192	48	12 [†]	3
MAP	1.15	5.33	0.22	215	284	71	18	4

[†]Blood pressure differences assumed in the original power calculation for the present study, although the observed SD was smaller than anticipated on the basis of previous trials.

SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure.

There is a strong likelihood of type 1 errors, given the multiple blood pressure parameters examined in this trial. There was limited statistical power for the *post-hoc* subgroup analyses, and therefore the possibility of significant effects of CoQ₁₀ treatment within the subgroups examined cannot be excluded. Although the current recommendation is to target systolic blood pressure to <130 mmHg in patients with type 2 diabetes mellitus (779, 780), recently published findings from the ACCORD (Action to Control Cardiovascular Risk in Diabetes) trial showed that there was no additional benefit of lowering systolic blood pressure to less than 120 mmHg versus less than 140 mmHg, and thus there is currently no evidence from randomised-controlled trials to provide support for this recommendation (832).

4.4.11 Significance

The study findings do not support a current role for adjunctive CoQ₁₀ treatment in patients with moderate hypertension on multiple antihypertensive agents. This study was powered to detect significant blood pressure lowering effects of 8/4 mmHg with CoQ₁₀ compared to placebo, with an estimated treatment effect similar to that seen in the Cochrane review (499) and equivalent to half of that observed in the Rosenfeldt meta-analysis (425). Less significant blood pressure reductions with CoQ₁₀ may be relevant at a population level. Since the possibility of a smaller treatment effect in the study patients cannot be excluded, further trials are warranted that would require larger numbers (shown in Table 4.9). Such trials could include patients with borderline hypertension, a direct comparison of CoQ₁₀ therapy with other antihypertensive agents, or combination treatment with other agents such as fenofibrate where interactive effects with CoQ₁₀ have been observed (486), and further exploration of the effects on diastolic blood pressure loads.

4.4.12 Conclusions

This adequately powered, randomised controlled study demonstrated that compared with placebo, CoQ₁₀ does not result in clinically significant reductions in systolic or diastolic 24-hr ambulatory blood pressure or heart rate in patients with the metabolic syndrome and inadequately treated hypertension, although there was a significant reduction in daytime diastolic blood pressure loads. CoQ₁₀ was well tolerated and was not associated with any clinically relevant changes in safety parameters. Whereas the possibility that CoQ₁₀ may have clinically useful antihypertensive effects in selected populations cannot be ruled out, the current data does not support a role in the routine management of patients with the metabolic

syndrome. However, one cannot exclude a small hypotensive effect, which may still have clinical benefits at the population level, in this group. The present study highlights the need for further randomised controlled trials to establish whether CoQ₁₀ has any role as an adjunct or alternative to conventional therapy in hypertensive patients.

5 Effect of Coenzyme Q₁₀ Supplementation on Simvastatin Tolerance

5.1 Background

Statins are well established as one of the most effective measures for lowering LDL-cholesterol levels and modifying the risk of cardiovascular disease (reviewed in Chapter 1, section 1.3.1.1). Statin therapy is generally fairly well tolerated, with a low incidence of serious adverse events. Progressively lower targets for cholesterol reduction (72, 116, 117), have necessitated higher statin doses, bringing an increased risk of side effects, particularly muscle related myopathies (119). Although serious rhabdomyolysis is very rare (125-127), prospective studies such as the Prediction of Muscular Risk in Observational conditions (PRIMO) survey have demonstrated that up to 10 – 15% of statin-treated patients develop myalgia (128-130), and thus there is often a requirement for a reduction in statin dose, or cessation of statin therapy. This potentially compromises cardiovascular risk management (120-123), thereby creating a need for approaches to prevent statin-induced myopathy.

The underlying pathophysiology of statin-induced myopathy is unknown, but one postulated mechanism is mitochondrial dysfunction secondary to depletion of CoQ₁₀ (119). Statins competitively inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting step in cholesterol biosynthesis (833), but this also limits synthesis of CoQ₁₀ (Chapter 1, section 1.3.3.3.6.5). Accordingly, there is substantial evidence that statin therapy reduces plasma or serum CoQ₁₀ concentrations by up to 54% (186-203). The magnitude of plasma CoQ₁₀ depletion is dose related (188, 196), and reversible on cessation of statin therapy (191). This, taken together with the essential role of CoQ₁₀ in mitochondrial function (335) (Chapter 1, section 1.3.3.3.1.1), and normal skeletal muscle function (500, 501), has prompted the hypothesis that CoQ₁₀ reductions may contribute to statin-related myopathy. Circulating concentrations of CoQ₁₀, however, do not necessarily reflect tissue CoQ₁₀ concentrations (393), and clinical studies evaluating the effect of statin treatment on skeletal muscle CoQ₁₀ levels are contradictory (192, 193, 475, 476). Impaired mitochondrial function has been demonstrated in some patients with statin-induced myopathy (151, 194, 204), although reports have been inconsistent (476).

Oral CoQ₁₀ supplementation has been shown to restore plasma CoQ₁₀ levels in patients on statin therapy without affecting the cholesterol lowering effect (190, 191, 477). Prior to initiation of the present study, no trials examining the effects of CoQ₁₀ on statin myalgia had been reported. Randomised placebo-controlled trials were therefore needed to evaluate whether there is a role for adjunctive CoQ₁₀ therapy in reducing or eliminating statin-induced myopathy.

5.1.1 Study hypothesis

The hypothesis for this study was that treatment with CoQ₁₀ in combination with upward dose titration of simvastatin would improve statin tolerability and reduce myalgia symptoms in patients with a history of statin-induced myalgia. Accordingly, the effects of CoQ₁₀ on statin tolerance in dyslipidaemic patients with prior statin-induced myalgia were studied in a double-blind, placebo-controlled trial that assessed the simvastatin dose tolerated and changes in myalgia scores. Presuming that any improvement in statin tolerability might be reflected by changes in CoQ₁₀, mitochondrial function or muscle damage, plasma levels of CoQ₁₀, the lactate/pyruvate ratio and creatine kinase were measured. Creatine kinase was also included as a safety marker and as a means of guiding statin dose.

5.2 Methods

5.2.1 Subjects

Forty-four patients, aged 18 – 75 years, with self reported myalgia that were unable to continue on an adequate dose of statin therapy were recruited from the Lipid Disorders and Cardiovascular Prevention Outpatient Clinic at Christchurch Hospital, Christchurch, New Zealand, through referrals from general practitioners and from advertisements in local newspapers. Subjects were excluded from the study if they had an acute myocardial infarction or cerebral vascular accident within three months, significantly deranged liver function tests (alanine aminotransferase or aspartate aminotransferase >3 times upper level of normal), significant renal impairment (calculated glomerular filtration rate <45 ml/min), decompensated heart failure, other significant co-morbidities, warfarin treatment, or antioxidant vitamin supplementation.

5.2.2 Study design

This was a randomised, double-blind, placebo-controlled 12-week parallel study with CoQ₁₀ supplementation in combination with upward dose titration of simvastatin as outlined in Figure 5.1. Primary outcomes were the number of patients who tolerated 40 mg/day of simvastatin at 12 weeks, the number of patients remaining on simvastatin therapy, and the change in myalgia score from baseline to the end of treatment. Patients attended a screening visit to assess eligibility. Prior to randomisation, patients underwent a two-week washout of CoQ₁₀ supplements and lipid modifying therapies, except for ezetimibe (n=4). Eligible patients were then stratified according to prior myalgia symptoms as either severe (n=9) or moderate (n=25). Severe myalgia was defined as the inability to tolerate a statin dose of 20 – 40 mg/day within one month of commencement, and moderate myalgia was defined as the development of myalgia at 20 mg/day or higher doses after longer than one month. Within each stratum, patients were randomised to treatment with CoQ₁₀ capsules (Q-Gel®, Tishcon Corp, USA), 200 mg/day or placebo for 12 weeks in combination with open label simvastatin treatment that was titrated up from a starting dose of 10 mg/day to 20 mg/day then to 40 mg/day simvastatin at four weekly intervals. Patients were seen at baseline and every four weeks during the study. For all visits, blood pressure and weight were recorded and fasting blood samples were collected for the measurement of plasma levels of CoQ₁₀, lipids, creatine kinase and the lactate/pyruvate ratio. Liver and renal function and haematological indices were assessed at baseline and at the final visit. Myalgia symptoms were recorded daily by patients by a visual analogue scale (detailed in section 5.2.3). Patients who experienced significant myalgia reduced their simvastatin dose or discontinued the study medication if unable to tolerate the lowest simvastatin dose (10 mg/day). Patients who exited the study earlier than 12 weeks attended a final visit within three days of cessation of treatment. The total standard 10 mg simvastatin doses tolerated was calculated and defined as the total number of equivalent 10 mg simvastatin doses taken by each participant during the 12 week study period. Compliance to treatment was assessed at each visit through capsule and tablet count. The study protocol was approved by the Upper South B Regional Ethics Committee (NZ), and written informed consent was obtained from all participants.

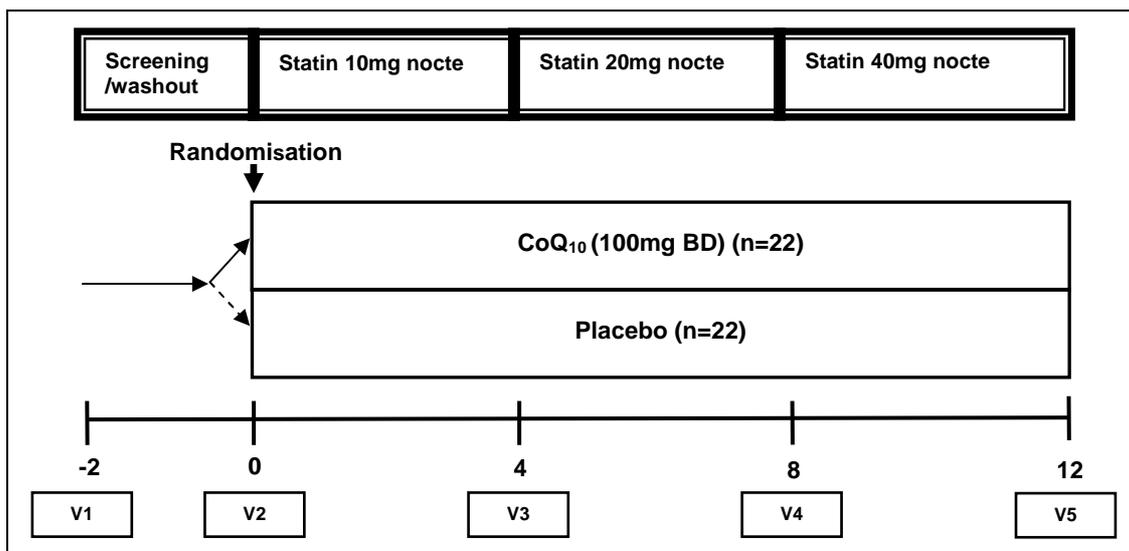


Figure 5.1 Summary outline of the 12-week randomised, double-blind, placebo-controlled study of CoQ₁₀ supplementation in combination with upward titration of simvastatin in patients with prior statin-induced myalgia

5.2.3 Myalgia scores

Myalgia was assessed using a 0 – 100 mm visual analogue scale adapted from Landstad *et al.* (2001) (834), with 0 mm indicating no pain and severe pain at 100 mm as shown in Figure 5.2. Participants were required to record the absence and presence and severity of myalgia symptoms on the visual analogue scale for each anatomical site affected by myalgia, at baseline and daily for the duration of the study. Visual analogue scales were analysed by one person to ensure consistency. The severity of myalgia for the total number of sites affected by myalgia per day (median myalgia score) and the severity of myalgia adjusted for the number of affected sites per day (adjusted median myalgia score) were calculated for all participants. Myalgia scores were presented as the change from baseline to the end of treatment.

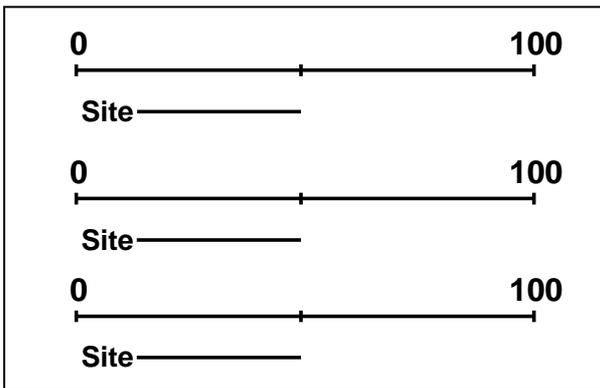


Figure 5.2 Example of the visual analogue scale for daily myalgia scoring of each anatomical site affected with 0 mm representing no muscle pain and 100 mm representing severe muscle pain

5.2.4 CoQ₁₀ formulation

Both Q-Gel® and placebo were supplied by Tischon Corp, USA, and were identical in appearance. Q-Gel® contained 100 mg of CoQ₁₀, vitamin E 150 IU, annato seed extract, Biosolv® base (lecithin, polysorbate, sorbitin monoleate, and medium chain triglycerides). This CoQ₁₀ preparation was used because it has been shown to have significantly better bioavailability in comparison to other CoQ₁₀ supplement brands when given to healthy male subjects (416). Randomisation was performed in permuted blocks of size 6 from a computer generated randomisation list. The study treatments were dispensed by an independent hospital pharmacist in identical numbered bottles with the lowest available number allocated to each sequential participant. Participants and investigators administering the treatment and assessing outcomes were blinded to treatment assignment and to plasma CoQ₁₀ levels.

5.2.5 Biochemical analysis

All biochemical analyses were performed by Canterbury Health Laboratories, an ISO15189 accredited (human) pathology laboratory. Total plasma CoQ₁₀ was measured by reverse phase high performance liquid chromatography with electrochemical detection using a method adapted from Molyneux *et al.* (2007) (408). Plasma total cholesterol, triglycerides, and HDL cholesterol were determined by an enzymatic colorimetric method (Aeroset analyser Model LN, Abbott Laboratories, Illinois IL, USA). LDL-cholesterol was calculated from the Friedewald equation. Whole blood lactate and pyruvate concentrations were determined by a spectrophotometric enzymatic method (835). Safety data, including electrolytes, renal and

liver function and creatine kinase (Aeroset analyser Model LN) and a full blood count (Coulter Electronics, Luton, UK) were measured at baseline and the end of treatment.

5.2.6 Statistical analyses

The sample size (n=44) was based on the following assumptions: 1) <10% of the placebo group would tolerate the highest simvastatin dose of 40 mg/day and concomitant CoQ₁₀ treatment would increase this to 50% or more; 2) 50% of the placebo group would tolerate at least 10 mg/day of simvastatin and >90% in the CoQ₁₀ supplemented group; 3) a difference of 9 mm in myalgia scores between treatment groups represented a clinically significant effect. On the basis of these anticipated treatment effects and allowing for a withdrawal rate of 10%, 22 people in each treatment group provided the study with 80% statistical power at two-tailed $\alpha=0.05$ for the primary outcome measures.

All statistical analyses were performed using SPSS version 17.0 (SPSS, Inc., Chicago, Illinois). Data are expressed as mean (SEM) or median (inter-quartile range). Categorical variables were compared using Chi-square or Fisher's exact test. Between-treatment group changes were compared using the Mann-Whitney U test, and within group changes were compared using the Wilcoxon-signed rank for continuous variables. Correlation analysis was performed using Spearman's correlation coefficient. Statistical significance was inferred when $P<0.05$.

5.3 Results

5.3.1 Baseline characteristics

Baseline characteristics of the patients are shown in Table 5.1. All 44 patients who were screened met the eligibility criteria and were stratified according to the severity of prior myalgia symptoms as mild (n=25) or severe (n=19). Patients were then randomised: 22 patients to CoQ₁₀ (mild myalgia group n=13, severe myalgia group n=9) and 22 patients to placebo (mild myalgia group n=12, severe myalgia group n=10) in combination with upward dose titration of simvastatin therapy for up to 12 weeks (Figure 5.3). One patient in the simvastatin alone group had a significant elevation in creatine kinase levels (894 U/L), despite an absence of muscle symptoms, and therefore the statin dose was reduced from 40 mg/day to

20 mg/day, with a resultant decrease in creatine kinase levels by 289 U/L after four weeks. There were no significant differences in baseline parameters between the two treatment groups except for the number of patients with myalgia symptoms, and therefore myalgia scores were expressed as the change from baseline to the end of the treatment.

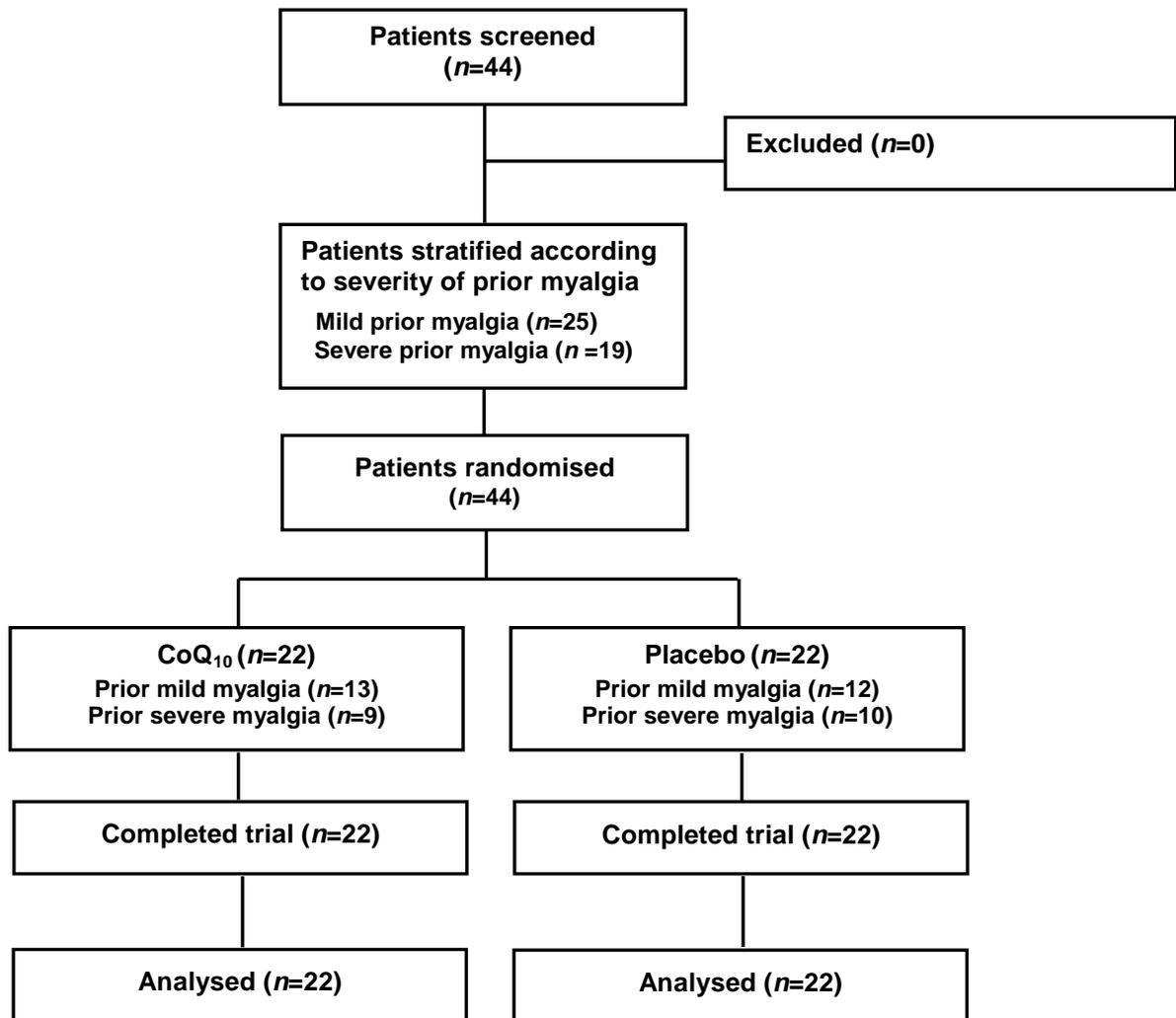


Figure 5.3 The flow of participants through the study

Table 5.1 Baseline characteristics of study participants

	CoQ ₁₀ + Simvastatin Therapy (n=22)	Simvastatin Therapy (n=22)	<i>P</i> -value‡
M/F, n	12/10	10/12	0.55
Age, yr	59 ± 2	59 ± 2	0.80
Weight, kg	81.7 ± 3.5	80.6 ± 3.5	0.83
Systolic blood pressure, mmHg	134 ± 4	138 ± 4	0.48
Diastolic blood pressure, mmHg	79 ± 2	83 ± 2	0.20
Heart rate, bpm	67 ± 3	68 ± 2	0.85
Current smoker, n (%)	1 (5%)	3 (14%)	0.61
Type 2 diabetes mellitus, n (%)	3 (14%)	2 (9%)	1.00
Glucose, mmol/L	5.6 ± 0.4	5.1 ± 0.6	0.29
Creatinine, mmol/L	0.08 ± 0.0	0.09 ± 0.0	0.28
Total cholesterol, mmol/L	6.9 ± 1.0	7.1 ± 1.4	0.56
LDL-cholesterol, mmol/L	4.5 ± 0.2	4.6 ± 0.2	0.65
Triglycerides, mmol/L	2.7 ± 0.6	2.2 ± 0.2	0.42
HDL-cholesterol, mmol/L	1.44 ± 0.09	1.50 ± 0.08	0.62
TC/HDL-cholesterol ratio	5.1 ± 0.3	4.9 ± 0.2	0.61
Plasma CoQ ₁₀ levels, μmol/L†	1.3 (1.0 – 1.4)	1.4 (1.1 – 1.8)	0.08
Creatine kinase, U/L	106 ± 15	130 ± 25	0.41
Blood lactate / pyruvate ratio	12.6 ± 1.2	13.1 ± 1.3	0.75
Statin prior to wash out, n (%)	13 (59%)	12 (55%)	1.00
Myalgia symptoms, n (%)	5 (23%)	0 (0%)	0.02

Values are mean ± SEM, †median (inter-quartile range) or number (percentage).

‡t-test for between group comparisons.

5.3.2 Simvastatin tolerability

The effect of concomitant CoQ₁₀ supplementation compared to placebo on the dose of simvastatin tolerated is summarised in Table 5.2. There were no significant differences between treatment regimes in the number of patients who tolerated 40 mg/day simvastatin ($P=0.34$), or in the number of patients remaining on any simvastatin dose ($P=0.47$). When stratified according to severity of prior statin-induced myalgia, the number of patients who tolerated 40 mg/day simvastatin and the number of patients remaining on simvastatin did not differ between treatments ($P\geq 0.32$ for both). Furthermore, there was no effect of concomitant CoQ₁₀ supplementation on the total number of days [median (inter-quartile range)] for which simvastatin was tolerated in comparison to statin alone [84 (44 – 86) vs 83 (69 – 85), $P=0.83$]. The total standard 10 mg simvastatin doses tolerated did not differ between the combined CoQ₁₀ and simvastatin and the simvastatin alone groups [195 (60 – 201) vs 189 (93 – 195) dose days, $P=0.29$]. The overall compliance to simvastatin administration was 98% in both treatment groups and compliance with CoQ₁₀ supplementation was 93%.

Table 5.2 Simvastatin dose tolerated at 12 weeks

Tolerated Dose (mg/day)	CoQ ₁₀ + Simvastatin Therapy (n=22)	Simvastatin Therapy (n=22)
40	16 (73%)	13 (59%)
20	0 (0%)	3 (14%)
10	0 (0%)	2 (9%)
0	6 (27%)	4 (18%)

Values are number (%).

$P=0.34$ for comparison of patients who tolerated 40 mg/day simvastatin.

$P=0.47$ for comparison of patients remaining on simvastatin (Chi-square).

5.3.3 Myalgia scores

The changes in myalgia scores from baseline to end of treatment are presented in Table 5.3. There was a small but significant increase in myalgia from baseline after both treatment regimes ($P < 0.001$). However, there was no effect of concomitant CoQ₁₀ supplementation during simvastatin therapy in comparison to statin alone on the changes in myalgia scores and the myalgia scores adjusted for the number of affected anatomical sites. Additionally, the increase in myalgia score and the adjusted myalgia score did not differ between the two treatment groups, within those patients who tolerated the top dose of 40 mg/day simvastatin ($P = 0.33$ for both).

Table 5.3 Changes in myalgia score from baseline to the end of the study

	CoQ ₁₀ + Simvastatin Therapy (n=22)	Simvastatin Therapy (n=21)	P-value†
Δ Myalgia score, mm	6.0 (2.1 – 8.8)*	2.3 (0 – 12.8)*	0.63
Δ Adjusted myalgia score, mm	4.2 (1.0 – 6.4)*	2.1 (0 – 11.4)*	0.73

Values are median (inter-quartile range).

†Mann Whitney U Test for comparison of between-group changes.

* $P = 0.001$ for within group changes from baseline (Wilcoxon-signed rank test).

Myalgia score from 0 (no pain) to 100 (severe pain).

5.3.4 Biochemical parameters

The changes in biochemical parameters from baseline to the end of treatment are summarised in Table 5.4. Plasma CoQ₁₀ levels were increased after combined CoQ₁₀ and simvastatin therapy (131% (24% – 181%); $P < 0.001$) and decreased with simvastatin alone (-34% (-46% – -24%); $P < 0.001$) as shown in Figure 5.4. Improvements in lipid profiles were similar between treatment regimes. There was a trend for an increase in plasma creatine kinase levels with combined therapy ($P = 0.14$) and a small but significant increase with simvastatin alone ($P < 0.05$), but the lactate/pyruvate ratio remained unchanged in both groups. Parameters of liver and renal function, electrolytes, and full blood count were not altered with either treatment regime (data not shown).

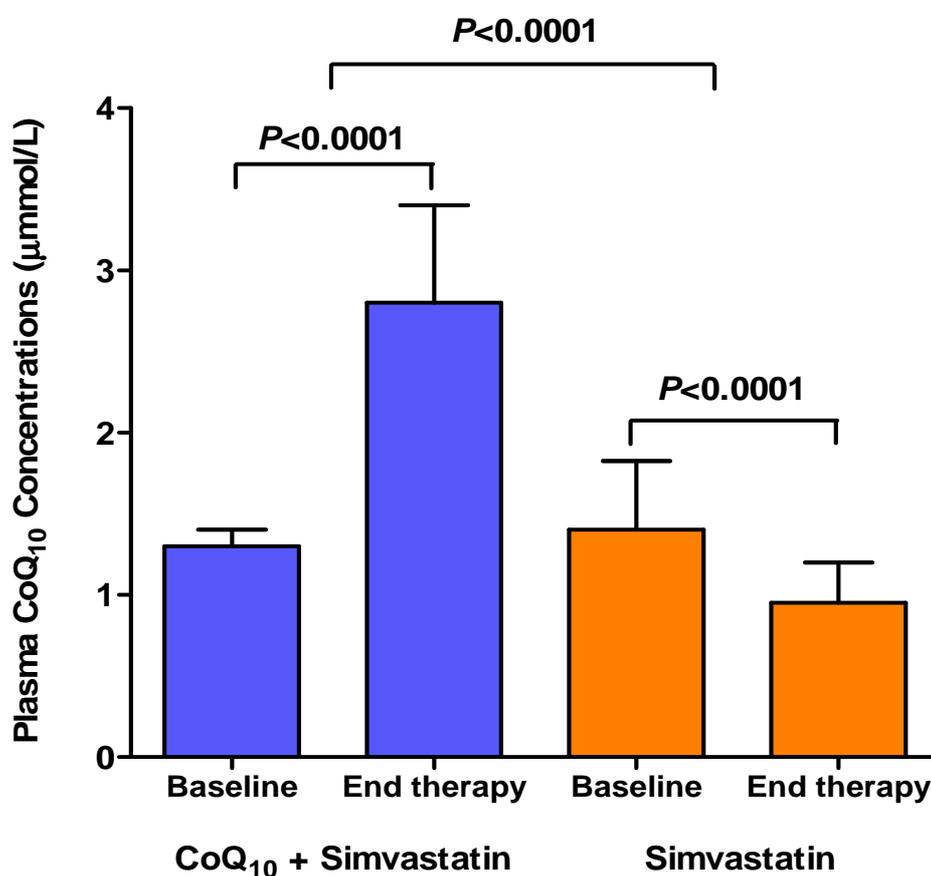


Figure 5.4 Median plasma CoQ₁₀ levels at baseline and following supplementation with placebo or with CoQ₁₀ in combination with the maximum tolerated dose of simvastatin

Table 5.4 Changes in biochemical parameters from baseline to the end of the study

	CoQ ₁₀ + Simvastatin Therapy (n=22)	Simvastatin Therapy (n=22)	P-value†
Changes in plasma CoQ ₁₀ levels			
Δ CoQ ₁₀ , μmol/L	1.7 (0.3 – 2.2)**	-0.5 (-0.6 – -0.3)**	<0.001
Δ CoQ ₁₀ / LDL-cholesterol ratio, μmol/mmol	0.65 (-0.26 – 0.92)**	-0.05 (-0.15 – 0.01)	0.01
Changes in plasma lipoprotein levels			
Δ Total cholesterol, mmol/L	-1.5 (-2.4 – -0.7)**	-1.6 (-2.4 – -0.9)**	0.57
Δ LDL-cholesterol, mmol/L	-1.7 (-2.4 – -1.0)**	-1.3 (-2.1 – -0.9)**	0.53
Δ Triglycerides, mmol/L	-0.4 (-0.6 – 0.3)	-0.3 (-0.6 – 0.03)*	0.90
Δ HDL-cholesterol, mmol/L	0.01 (-0.08 – 0.15)	-0.02 (-0.09 – 0.15)	0.65
Change in marker of mitochondrial function			
Δ Lactate / pyruvate ratio	-0.8 (-3.8 – 2.9)	-0.8 (-6.8 – 2.1)	0.50
Change in marker of muscle damage			
Δ Creatine kinase, U/L	17 (-15 – 46)	19 (-3 – 28)*	0.85

Values are median (inter-quartile range).

†Mann Whitney U Test for comparison of between-group changes.

* $P < 0.05$, ** $P < 0.001$, Wilcoxon-signed rank test for within-group changes from baseline.

5.3.5 Correlation analysis

At baseline, plasma CoQ₁₀ levels were positively correlated with total cholesterol ($r=0.575$, $P<0.001$), and LDL-cholesterol concentrations ($r=0.471$, $P=0.002$). There was a negative correlation between total cholesterol and creatine kinase concentrations at baseline ($r=-0.370$, $P<0.013$). Baseline CoQ₁₀ levels were not associated with the change in myalgia score ($r=0.055$, $P=0.82$), ($r=-0.119$, $P=0.60$), or change in myalgia score adjusted for the number of affected anatomical sites ($r=0.034$, $P=0.88$), ($r=0.67$, $P=0.77$) following either statin alone or concomitant CoQ₁₀ therapy, respectively. The increase in CoQ₁₀ levels with combined therapy was positively correlated with the total standard 10mg simvastatin doses tolerated ($r=0.491$, $P<0.024$), as shown in Figure 5.5, however this association was not observed in the statin alone group ($r=-0.215$, $P<0.338$). The total standard 10mg simvastatin doses tolerated with statin therapy alone was negatively correlated with the change in myalgia score from baseline ($r=-0.517$, $P=0.016$) (Figure 5.6) and change in adjusted myalgia score from baseline ($r=-0.518$, $P=0.016$), but these associations were eliminated following combined CoQ₁₀ and statin therapy ($r=-0.196$, $P=0.381$ for myalgia score and $r=0.002$, $P=0.994$ for adjusted myalgia score). No correlations were observed between creatine kinase at baseline or following either treatment regimen with the change in myalgia scores or total standard 10 mg simvastatin doses tolerated.

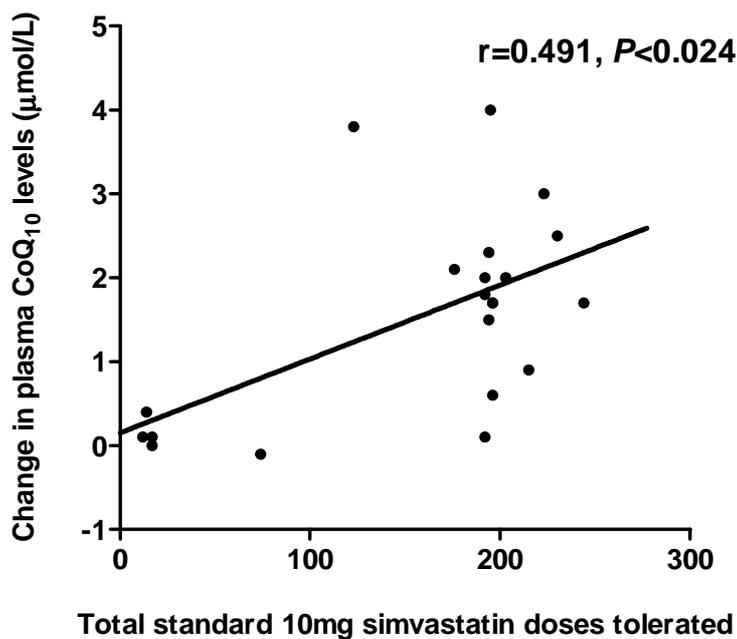


Figure 5.5 Association between the increase in plasma CoQ₁₀ concentrations following upward titration of simvastatin in combination with CoQ₁₀ and the total standard 10 mg simvastatin doses tolerated

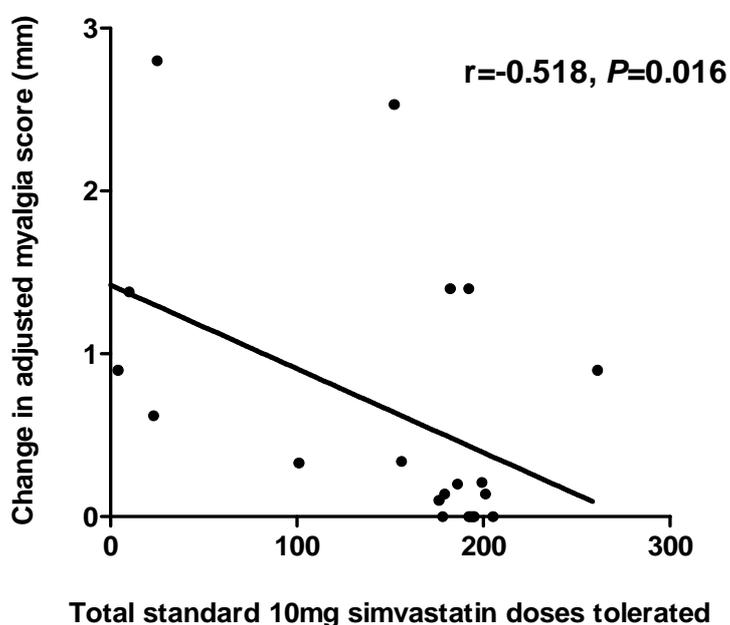


Figure 5.6 Association between the change in adjusted myalgia score from baseline following upward titration of simvastatin alone and the total standard 10 mg simvastatin doses tolerated

5.4 Discussion

5.4.1 Effect of CoQ₁₀ on simvastatin tolerability and myalgia symptoms

In this study, 12 weeks supplementation with CoQ₁₀ in combination with upward titration of simvastatin therapy from 10 mg/day to 40 mg/day was not associated with improved simvastatin tolerability and reduced muscle symptoms in patients with a history of statin-related myalgia in comparison to statin therapy alone. Although, the number of patients achieving the 40 mg/day simvastatin dose during the study was higher with combined CoQ₁₀ therapy, this was not significant. Interestingly, the number and dose of simvastatin tolerated by participants was increased in both treatment groups compared to baseline, suggesting a profound placebo effect of therapy. This was the first double-blind, placebo-controlled randomised study to evaluate the impact of CoQ₁₀ on simvastatin-induced myalgia. Although simvastatin therapy resulted in a significant increase in myalgia scores and adjusted myalgia scores over the treatment period, combined CoQ₁₀ treatment did not attenuate the increase in myalgia scores. The finding of a lack of effect of CoQ₁₀ on improving statin tolerance in this study in comparison to placebo contrasts with the first comparator trial by Caso *et al.* (2007) (502) who reported a decrease in pain severity by 40% ($P < 0.001$) and a decrease in pain interference with daily activities by 38% ($P < 0.02$) after the addition of CoQ₁₀ (100mg/day) to current statin treatment for 30 days in patients with myopathic symptoms. No changes in pain severity or decrease in pain interference with daily activities were observed in the comparator group treated with vitamin E (400 IU/day) (502). Their study, however, was not placebo-controlled and patients were not on a standardised dose or type of statin treatment, whereas in the present trial patients were standardised to simvastatin with the goal of achieving a daily dose of 40 mg, as used in the Heart Protection Study (68). Myalgia was evaluated using the Brief Pain Inventory questionnaire in the study by Caso *et al.* (2007) (502), which is a more detailed instrument for assessing myopathy than the visual analogue scale used in the present study. A further difference between study protocols was that patients were administered CoQ₁₀ supplements for four weeks duration to allow adequate absorption of CoQ₁₀ in the trial by Caso *et al.* (2007) (502), but in the present trial, patients were initiated on CoQ₁₀ at the same time as the statin but were able to discontinue statin treatment at any time if considerable myalgia was experienced. Therefore, it is feasible to hypothesise that the ability of CoQ₁₀ to provide protection from myalgia may have been limited by a delay in the

accumulation of CoQ₁₀ in those patients who withdrew early from treatment. Taken together, it could be speculated that these key differences in study design and the lack of a more comprehensive myalgia pain scoring scale in the current trial, may explain why CoQ₁₀ supplementation was associated with a reduction in statin-induced myalgia symptoms in the study by Caso *et al.* (2007) (502) but the findings were not replicated in the present study. To date, these are the only published two randomised controlled trials that have investigated the effects of CoQ₁₀ in patients with statin-related myalgia, albeit with contrasting findings. A randomised, double-blind, placebo-controlled clinical trial (ClinicalTrials.gov identification number NCT00716612) is currently recruiting 80 patients to address whether CoQ₁₀ supplementation can decrease the incidence of muscle pain in patients with previous statin-related myalgia (380).

5.4.2 Effect of CoQ₁₀ and simvastatin compared to simvastatin alone on CoQ₁₀ levels

Treatment with increasing doses of simvastatin monotherapy for up to 12 weeks resulted in a median reduction of 34% in plasma CoQ₁₀ levels, consistent to that seen in a large number of observational studies (186-188, 191-194, 197, 200-202), and randomised controlled trials (190, 195, 196, 198, 199, 380). Conversely, a small number of studies have not shown any changes in plasma CoQ₁₀ concentrations following statin treatment (50-52), in part explained by the small number of participants on low doses of statin used in these trials (836). It is possible the reduction in circulating CoQ₁₀ may reflect decreased LDL-cholesterol concentrations, although in the present study the CoQ₁₀ to LDL-cholesterol ratio was not significantly lowered. Several studies have, however, reported a lower CoQ₁₀ to LDL-cholesterol ratio after statin treatment (188, 194, 197), suggesting that CoQ₁₀ depletion may not only be due to a reduction in LDL carriers, although this has not been consistently shown in all trials (191-193). In the present study, patients in the combined treatment group were administered a CoQ₁₀ preparation, Q-Gel® that has been demonstrated to have significantly better bioavailability, in comparison to other CoQ₁₀ supplement brands when given to healthy male subjects (416), and which achieved clearly elevated levels in this trial, with a 131% median increase in plasma CoQ₁₀ levels. This concurs with previous trials reporting restoration of plasma CoQ₁₀ concentrations in statin treated patients on concomitant CoQ₁₀ therapy (190, 191, 203, 477).

While baseline plasma CoQ₁₀ levels did not predict changes in myalgia with either treatment regime in the present study, the increase in CoQ₁₀ levels in patients receiving combined treatment was positively correlated with the statin tolerability. This could imply that those patients who were better absorbers of CoQ₁₀ were able to tolerate statin therapy, or alternatively that those patients who withdrew early from treatment did not allow sufficient time for adequate accumulation of CoQ₁₀ in order to provide protection from the myopathic effects of statin therapy. Furthermore, the reduction in plasma CoQ₁₀ concentrations in those treated with simvastatin alone, was associated with an increase in muscle symptoms after therapy, suggesting that changes in plasma CoQ₁₀ following statin treatment may be predictive of simvastatin intolerance. Certainly, statin-induced CoQ₁₀ depletion has been shown to be dose-dependent (188, 196), and more aggressive lipid lowering with statins is associated with increased risk of myopathy (837, 838).

Nonetheless, circulating concentrations of CoQ₁₀ do not necessarily reflect tissue CoQ₁₀ concentrations, and both animal models and clinical studies evaluating the effect of statin treatment on skeletal muscle CoQ₁₀ levels are contradictory. In one animal study, simvastatin or pravastatin administered to rabbits reduced skeletal muscle CoQ₁₀ by up to 72%, along with pathological muscle changes (839), whereas, high-dose simvastatin therapy was not associated with decreases in muscle CoQ₁₀ concentrations, despite producing severe lesions in skeletal muscles of rabbits (840). An experimental study of rats administered varying doses of cerivastatin did not show any significant reductions in muscle CoQ₁₀ levels compared to untreated controls (841). In an early clinical trial, four weeks of simvastatin 20 mg/day in 20 hypercholesterolaemic patients resulted in a 47% increase in muscle CoQ₁₀ concentrations, despite a reduction in serum levels of CoQ₁₀ (193). Subsequently, six months of treatment with 20 mg/day of simvastatin in a study with 19 patients gave a similar result (192). A further trial comparing the effect of eight weeks of treatment with simvastatin 80 mg/day versus atorvastatin 40 mg/day on mean muscle CoQ₁₀ concentrations, reported a 34% reduction in those treated with simvastatin, but no change with atorvastatin (475), suggesting the effect of statin therapy on intramuscular CoQ₁₀ levels may be drug and dose dependent (842).

Few published studies have examined intramuscular CoQ₁₀ levels in patients with symptomatic myopathy; however, one trial measured CoQ₁₀ levels in muscle biopsies from patients with statin-related myopathy or high serum creatine kinase or both. No significant difference was seen in the mean intramuscular CoQ₁₀ concentration in patients with statin-

related myopathy compared to controls (476). Given the conflicting data, it is possible that the decrease in plasma CoQ₁₀ concentrations observed in the present study may not be reflected by a similar reduction in intracellular CoQ₁₀ or conversely that the increase whilst on CoQ₁₀ may not reflect an increase in intramuscular or mitochondrial levels.

5.4.3 Effect of CoQ₁₀ and simvastatin compared to simvastatin alone on mitochondrial function

There was no evidence of statin-induced mitochondrial dysfunction in the present study, since the lactate/pyruvate ratio, an indicator of anaerobic metabolism, were unaltered with both combined CoQ₁₀ and statin treatment and statin alone. In contrast, a previous study by De Pinieux *et al.* (1996) (194) reported significantly increased blood lactate/pyruvate ratios by 16% ($p < 0.05$) in statin-treated hypercholesterolaemic patients compared to untreated hypercholesterolaemic patients. Since the lactate/pyruvate ratio only provides an indirect measure of mitochondrial function, it may have been more informative to examine skeletal muscle biopsies for biochemical abnormalities in the patients of the present study, to assess the impact of statin-induced myalgia on mitochondrial function. In addition to histochemical studies of skeletal muscle biopsies, biochemical analyses of mitochondrial function should include an assessment of oxygen utilization by polarography, and measurement of enzymatic activity of individual complexes of the electron transport chain and ATPase by spectrophotometry.

There have been further trials providing support for mitochondrial dysfunction as a feature of statin-induced myopathy, but others resulted in contrasting findings. Gambelli *et al.* (2004) (843) demonstrated mitochondrial alterations in biopsies from nine patients on statin therapy with various myopathic syndromes. Furthermore, elevated phosphodiesterase was observed in the Phosphorus-31 magnetic resonance spectroscopy (³¹P-MRS) spectra of patients on statin therapy compared to controls, indicating altered mitochondrial function (844). However, in a further trial, patients with severe statin-induced myalgia and rhabdomyolysis did not show altered phosphocreatine recovery of ³¹P-MRS or mitochondrial defects on histology, suggesting normal mitochondrial function (845). Although ³¹P-MRS did show slowed pH recovery, the authors indicated this may be due to a defect in ion transport rather than an index of mitochondrial impairment (844).

Schick *et al.* (2007) (846) observed significantly decreased mitochondrial DNA levels in skeletal muscle biopsies from patients undergoing treatment with simvastatin 80 mg/day for eight weeks, but not in those treated with 40 mg/day atorvastatin, the implication being that simvastatin and atorvastatin may differ in their effects on muscle metabolism. Muscle CoQ₁₀ concentrations were also significantly reduced, but only in those patients on simvastatin treatment. Interestingly, there was a significant overall correlation between the changes in muscle CoQ₁₀ and the change in mitochondrial DNA/nuclear DNA ratios ($r=0.63$, $P>0.01$), and a stronger association within the simvastatin treated group ($r=0.76$, $P>0.002$). Notably, these patients had normal creatine kinase levels and no muscle symptomatology (846). Another trial reported increased respiratory exchange ratios in patients with statin-induced rhabdomyolysis or myositis compared to controls, supporting the possibility of a mitochondrial deficit in statin-related muscle effects (847). Additionally, a recent investigation found significant mitochondrial abnormalities or impaired fat metabolism in biopsies from 52% of patients with statin-induced myopathies, with the majority of individuals having elevations in creatine kinase ranging from 4 to >10 ULN (151). Muscle biopsies from four patients with statin-related myopathy but without creatine kinase elevations however, demonstrated findings consistent with mitochondrial dysfunction, including increased intramuscular lipid stores, ragged red fibres and cytochrome oxidase negative fibres (204). After discontinuation of statin therapy, three patients underwent repeat biopsies that showed resolution of pathologic abnormalities (204). These features are suggestive of a defect in the mitochondrial electron transport chain and have been reported previously in patients with primary CoQ₁₀ deficiency, where CoQ₁₀ supplementation resolved symptoms (447).

However, there is limited evidence linking mitochondrial dysfunction to reduced intramuscular CoQ₁₀ in patients with statin-induced myopathy. In one trial, muscle biopsies from 18 patients with statin-associated myopathy revealed only two patients with decreased intramuscular CoQ₁₀ levels and some morphologic evidence of mitochondrial dysfunction (476). By contrast, patients on 80 mg simvastatin for eight weeks showed reduced muscle CoQ₁₀, in addition to reduced respiratory enzyme and citrate synthase activity, and reduced mitochondrial volume (475).

A history of plasma creatine kinase elevation has been shown to be a strong independent predictor of muscle symptoms in patients on statin therapy (129). In addition, more intensive statin therapy has been associated with larger increases in creatine kinase

concentrations, along with a further increased risk of adverse muscle events (154). In the majority of patients enrolled in the present study, creatine kinase was within normal ranges at baseline. There was a non-clinically significant increase in creatine kinase levels after adjunctive CoQ₁₀ supplementation and a statistically significant increase with statin alone. However, no correlation could be detected between creatine kinase and myalgic symptoms or statin tolerance before or after the intervention period. These results confirmed previous observations that statin-myopathy can occur without a concomitant increase in plasma creatine kinase (204). Caso *et al.* (2007) (502) suggests this lack of association of muscle symptoms and creatine kinase indicates that plasma creatine kinase concentration is not a sensitive marker to detect or assess statin-induced myopathies. In a recent review of biochemical markers of muscle damage, it was recommended that assessment of more than one marker would provide a better estimation of muscle stress (848). Serum carbonic anhydrase III (CA-III) may be a useful diagnostic marker of muscle damage following statin therapy, since it is present in skeletal muscle but not myocardial tissue (849). Furthermore, CA-III is found in the circulation after muscular injury and has been shown to have greater sensitivity than creatine kinase (849).

5.4.4 Study limitations

The overall changes in myalgia scores were small with both combined CoQ₁₀ and simvastatin treatment or statin alone in the study, suggesting patients experienced rather mild myalgia symptoms. Although patients were representative of those commonly encountered in clinical practice, it is possible that they did not have sufficiently severe myalgia to observe a benefit from CoQ₁₀ supplementation. Certainly, the sample size of 44 participants was based on the assumption that there would be a difference of 9 mm in myalgia scores between groups after therapy (favouring combined therapy); however the difference in myalgia score changes favoured simvastatin alone. Additionally, it was assumed that greater than 10% of the placebo group would tolerate 40 mg/day of simvastatin compared to 50% or more in the combined therapy group, but this assumption did not hold for the group receiving statin alone, where 59% of patients tolerated the 40 mg/day simvastatin dose. The study population was also very heterogeneous in terms of statin type and dose tolerated and the severity of the muscle side effects experienced prior to study enrolment.

There was no evidence of statin-induced mitochondrial dysfunction, since lactate pyruvate ratios were unaltered. Plasma CoQ₁₀ levels were used as a surrogate measure for

skeletal muscle CoQ₁₀ levels, but the direct measurement of CoQ₁₀ from muscle biopsies would have been more informative, as plasma CoQ₁₀ levels do not necessarily reflect tissue CoQ₁₀ concentrations (393). Accumulation of CoQ₁₀ and early withdrawal of statin treatment may have limited this study, since patients were initiated on CoQ₁₀ at the same time as simvastatin therapy but were able to discontinue statin treatment at any time if considerable myalgia was experienced. Furthermore, myalgia was assessed using a visual analogue scale to score pain intensity, but a more comprehensive pain scale such as the validated Brief Pain Inventory questionnaire may have been more informative.

5.4.5 Significance

There is as yet no clear answer as to whether depletion of CoQ₁₀ in muscle mitochondria has an etiologic role in statin-induced myopathy and can be mitigated by CoQ₁₀ supplementation. Although one small trial has shown some benefit from oral CoQ₁₀ therapy in eliminating muscle symptoms (47), the present study does not support the use of CoQ₁₀ as an adjunctive therapy in all patients with statin-induced myopathy.

It is possible that statin-associated myopathy may be due to direct effects on the cell membrane or to the depletion of other metabolites in the mevalonate pathway resulting from statin HMG-CoA reductase inhibitory activity, and unrelated to CoQ₁₀ depletion. Statins have been shown to affect skeletal muscle membrane physiology impairing Na⁺/K⁺ ATPase, Na⁺/Ca²⁺, as well as Na⁺/Ca²⁺ ATPase pump activity, leading to an increased calcium influx, which may result in the development of myotoxicity (164). Another proposed mechanism is that impaired cholesterol synthesis leads to changes in cholesterol in myocyte membranes, inducing membrane instability (850, 851). However, this pathway is unlikely to be an important mechanism, since inherited disorders of the cholesterol biosynthesis pathway that reduce cholesterol concentrations are not associated with myopathy (165), and non-statin lipid lowering agents in animal models, induce myopathy through distinctly separate pathways (174). Furthermore, inhibition of squalene synthase, which catalyses the first step in cholesterol synthesis uncommitted to isoprenoid synthesis does not induce myotoxicity in primary cultures of rat myotubes (167) or in human skeletal myotubes (168).

More recently, it has been proposed that statins may induce myopathy by reducing the availability of the isoprenoid co-metabolites, farnesyl pyrophosphate (F-PP) and geranylgeranyl PP (GG-PP), leading to the depletion of the key isoprenoids, which control

myofiber apoptosis (170). A study in neonatal rat skeletal myotubes, found that administration of mevalonate, a precursor to F-PP and GG-PP, restored protein synthesis and reversed the myopathic changes induced by statins, supporting this theory. This was confirmed in a report, where inhibition of protein synthesis and loss of differentiated myotubes in neonatal rat skeletal muscle was reversed after farnesol and geranylgeraniol supplementation (167). Enhanced apoptosis of myofibres has been implicated in statin-associated myopathy as a result of reduced prenylation of small GTP binding proteins leading to elevated levels of cytosolic calcium and activation of mitochondrial-mediated apoptotic signalling (77). Indeed, this hypothesis is supported by studies of vascular smooth muscle cells demonstrating that statin-induced apoptosis is prevented by supplementation with isoprenoids, including F-PP and G-PP (78, 79). Other potential mechanisms for statin-related myopathy linked to decreased isoprenoid synthesis include reduction in prenylation-dependent processing of lamins (176-178, 180), and reduction in selenoproteins (84, 85), and glycoproteins (86). There is also evidence to suggest that deficiency of vitamin D, a metabolite of cholesterol synthesis, may contribute to statin-induced myopathy. A recent study by Ahmen *et al.* (2009) (169) reported a significant correlation between low serum 25 (OH) vitamin D (D2 1 D3) (32 ng/mL) and myalgia in statin treated patients. Furthermore, this statin-related myalgia was largely reversed in this patient cohort by vitamin D supplementation. This has led to speculation that vitamin D deficiency reversibly augments statin-induced myalgia (169).

5.4.6 Conclusions

Despite achieving significant increases in plasma CoQ₁₀ levels on supplementation, there was no improvement in statin tolerance and myalgia symptoms in patients with prior statin related muscle complaints, suggesting that CoQ₁₀ may not have an etiological role in statin-induced myalgia. Although the study findings do not provide support for the routine use of oral CoQ₁₀ supplementation to mitigate myalgia associated with statin therapy, CoQ₁₀ might still be beneficial in specific subpopulations, dependent on such factors as age, severity of myalgia, concomitant disease, or genetic susceptibility to statin intolerance. The present study highlights the need for further adequately powered randomised controlled trials to establish whether there is a role for CoQ₁₀ in reducing or eliminating statin myopathy and one such trial is currently underway, which should help to address this issue. Considerations for future trials should include patients with more severe and clearly defined myopathy, initiation of CoQ₁₀ prior to statin therapy, a more objective myopathic pain score and muscle biopsy studies.

6 Genetic Risk Factors Associated with Statin Myopathy

6.1 Background

The benefits of statins on cardiovascular risk reduction are well established (852). Despite their proven efficacy, statins have also gained attention as a result of adverse side effects, with myopathy, the most common cause of statin withdrawal (119). Not surprisingly, discontinuation of statin therapy has been shown to result in a significant increase in cardiovascular events (120-123). Recently a disproportionate increase in the incidence of rhabdomyolysis with high-dose simvastatin compared with superior LDL-cholesterol lowering doses of rosuvastatin and atorvastatin has been highlighted (155). Accordingly, the FDA have made recommendations that use of the 80mg dose of simvastatin be restricted to patients who have been taking it for 12 months or longer without signs or symptoms of muscle toxicity. Adverse musculoskeletal effects can result from individual risk factors, such as drug interactions, in particular with simvastatin, and family history of myopathy, the patient's age and gender, and underlying disease (133). There is also growing evidence that genetic variability is an important risk factor for statin-related myopathy (Table 6.1). Candidate genes include those with variants affecting coenzyme Q₁₀ (CoQ₁₀) synthesis (205), statin pharmacokinetics (143-149), and pharmacodynamics, (150), vascular function (206), pain perception (207), and inherited metabolic muscle diseases (151-153), including mitochondrial myopathies (853-855) and muscular dystrophies (856).

6.1.1 Metabolic muscle disease risk factors

There is evidence that statins may uncover myalgia in patients with previously asymptomatic inherited muscle myopathies (151-153, 857). For example, one study demonstrated that patients with statin-related muscle side effects who underwent mutation testing were four times more likely to have mutant alleles for metabolic muscle myopathies in comparison to asymptomatic patients on statin therapy (151).

6.1.1.1 Coenzyme Q₁₀ deficiency

Coenzyme Q₁₀ (CoQ₁₀) is produced via the mevalonate pathway and has a key role in electron transport during mitochondrial oxidative phosphorylation (335). Statins block the production of farnesyl pyrophosphate, an intermediate in CoQ₁₀ synthesis. Consequently statin therapy has been shown to lower plasma CoQ₁₀ levels by 16 to 75%, reflecting a reduction in CoQ₁₀ synthesis and/or lipoprotein carriers (186-203, 758, 858) (reviewed in Chapter 1 Section 1.3.3.3.6.5). Evidence that muscle CoQ₁₀ levels are reduced is less conclusive, although some studies have reported decreased muscle CoQ₁₀ in statin treated subjects (475). One postulated mechanism for statin-induced myopathy is mitochondrial dysfunction, secondary to CoQ₁₀ depletion (119), given the important role of CoQ₁₀ for normal skeletal muscle (500, 501). Mutations in the *COQ2* gene, which encodes para-hydroxybenzoate-polyprenyl transferase, the second enzyme in the biosynthetic pathway of CoQ₁₀ (859), have been associated with severe inherited myopathy (459, 460, 463). The *COQ2* gene is 24,986 base pairs in length, and consists of 7 exons, located on chromosome 4 (859) (Figure 6.1). Quinzii *et al.* (2006) (455, 460) were first to identify a homozygous missense mutation in *COQ2* (Y297C) in two siblings with infantile encephalopathy and steroid resistant nephrotic syndrome, causing primary CoQ₁₀ deficiency. Mollet *et al.* (2007) (459) reported a homozygous single-base frameshift deletion in the *COQ2* gene in an infant with early-onset nephrosis, who died at 12 days of multi-organ failure. Furthermore, Diomedi-Cassadei *et al.* (2007) (463) identified two infants with early-onset glomerular lesions that harboured mutations in the *COQ2* gene. The first patient presented with steroid-resistant nephrotic syndrome at 18 months and was found to be a compound heterozygote for mutations in the *COQ2* gene (463). The second patient, with an identified homozygous *COQ2* mutation (S146N), presented at five days with oliguria, rapidly developed end-stage renal failure and died at age of 6 months (463). Interestingly, both patients who were homozygous for the Y297C mutations (455, 460) and the compound heterozygote for R197H and N228S mutations (463) responded dramatically to CoQ₁₀ supplementation.

In view of the fact that *COQ2* mutations have been identified causing CoQ₁₀ deficiency, Oh and colleagues (205) rationalised that more subtle genetic variation in the *COQ2* gene may be associated with statin myopathy. They investigated two informative single nucleotide polymorphisms (SNPs), the synonymous polymorphism, rs6535454 (SNP-1), and the non-coding polymorphism, rs4693075 (SNP-2) of the *COQ2* gene in 133 patients with statin myopathy on monotherapy and 158 statin tolerant controls that remained asymptomatic for

more than one year (205). A significant association was found between statin intolerance and SNP1 and SNP2 genotypes and the 2-SNP haplotype (combinations of polymorphisms on a single chromosome) (205), indicating that polymorphisms of the *COQ2* gene may have a role in predicting increased risk to myopathic symptoms induced by statin therapy.

6.1.1.2 Myoadenylate deaminase deficiency

Myoadenylate deaminase deficiency is an autosomal recessive disorder of skeletal muscle, characterised by exercise intolerance and myalgia (860). Myoadenylate deaminase (AMPD) is the enzyme responsible for converting adenosine 5 monophosphate to inosine monophosphate, and is thus an important regulator of muscle energy metabolism (861, 862). The skeletal muscle specific isoform of AMPD is encoded by the *AMPD1* gene, located on chromosome 1, and is 26,454 base pairs in length, consisting of 16 coding exons (863) (Figure 6.2). The main cause of AMPD deficiency in Caucasians is a nonsense mutation rs17602729 (Q12X, 34C>T) in the second exon of the *AMPD1* gene, resulting in a premature stop codon at codon 12 and thus a severely truncated enzyme (864). Approximately 2% of the general Caucasian population is homozygous for the stop codon and nearly 20% heterozygous for the Q12X mutation (864-867). Although most heterozygotes are generally asymptomatic, some show a mild clinical phenotype, and AMPD activity is reduced even in heterozygotes, with levels typically 38 to 39% of the activity in healthy controls (868). Myoadenylate deaminase deficiency has been associated with several other missense mutations in *AMPD1*, including rs61752479 in exon 3 (P48L, 143C>T) (862), and rs34526199 in exon 7 (K287I, 860A>T) (867). In a case-control study, Vladutiu *et al.* (2006) observed a 3.5 fold increase in homozygotes for myoadenylate deaminase deficiency with severe statin myopathy compared to asymptomatic statin-treated patients (151). However, there was no increase in carrier frequency in symptomatic patients versus control subjects (151). Earlier studies have shown that the *AMPD1* mutation frequency is not increased in patients with neuromuscular disease or exercise intolerance compared to asymptomatic controls (865). On this basis, the increased prevalence of *AMPD1* mutations among patients with statin myopathy suggests that statin treatment may provoke muscle related symptoms in previously asymptomatic patients (857).

6.1.1.3 Carnitine palmitoyl-2 (CPT2) deficiency

CPT2 deficiency is an autosomal recessive disease of lipid metabolism (869), characterised by recurrent myalgia and myoglobinuria precipitated by prolonged exercise,

fasting, cold exposure, stress or infection (870). The *CPT2* gene, located on chromosome 1, encodes the CPT2 enzyme that imports acyl-Co-A into the mitochondrial matrix for fatty acid oxidation, an important energy source for skeletal muscles during exercise (871). Over 40 mutations in the *CPT2* gene have been identified, with the most prevalent being the missense mutation, rs74315294 (S113L, 338C>T) in patients with muscle CPT2 deficiency, which has been found in approximately 60% of mutant alleles (871). It is a rare disorder, with homozygosity for the S113L mutation occurring in one in 300,000 individuals, and a carrier frequency of one in 270 (151). Vladutiu *et al.* (2006) reported a 13-fold higher heterozygous carrier frequency for CPT2 deficiency in a study of 136 patients with statin-associated myopathy than expected in the general population (151). Only one patient, who was homozygous for CPT2 deficiency, was symptomatic prior to initiation of statin therapy, and his symptoms worsened with cerivastatin and continued episodically five years after statin discontinuation (151). In the heterozygotes, symptoms resolved following withdrawal of statin treatment (151). These findings suggest that statins can provoke and worsen symptoms in subjects with asymptomatic or diagnosed CPT2 deficiency (151).

6.1.1.4 Glycogen storage disorders

Myophosphorylase deficiency (McArdle disease) is an autosomal recessive disorder of myophosphorylase, a specific skeletal muscle enzyme that initiates glycogen breakdown (872). McArdle disease, which typically presents in the first two decades of life, is characterised by exercise intolerance, and chronic muscle pain cramps, and in severe cases, rhabdomyolysis and myoglobinuria (872). Myophosphorylase deficiency is caused by mutations in the *PYGM* gene, located on chromosome 11 (873), and at least 85 variants have been identified (874). The most prevalent mutation among Caucasian patients is a nonsense mutation in exon one, rs116987552 (R50X, 148C>T), with allele frequencies as high as 80% (874-876). Homozygosity for McArdle disease occurs in one in 100,000 individuals, with a carrier frequency of one in 170 (877). Most heterozygotes for myophosphorylase deficiency are asymptomatic. To date, four patients with myophosphorylase deficiency (151, 152, 878, 879) and five heterozygotes (151) have been reported with statin myopathy. All heterozygotes were asymptomatic prior to initiation of statin therapy (151). Vladutiu and colleagues showed that the number of carriers for McArdle disease was increased by 20-fold compared to expected population frequencies (151). In most patients, symptoms were reversed within two years of statin discontinuation, suggesting that statins were a causative factor of the myopathic symptoms (151).

Alpha-glucosidase deficiency (Pompe disease/glycogen storage disease type II) is an autosomal recessive disorder of the glycogen-degrading lysosomal enzyme, acid alpha-glucosidase (GAA) (880). Clinically, Pompe disease presents as a wide spectrum of phenotypes ranging from the severe rapidly-progressive infantile form to the slowly progressive relatively mild late-onset form (880). Alpha-glucosidase deficiency is caused by mutations in the *GAA* gene, located on chromosome 17, and to date at least 197 pathogenic mutations have been identified (881). There have been at least two case reports of Pompe disease in previously asymptomatic patients who presented with severe muscle cramps and myalgia after initiation of simvastatin therapy (152, 153).

6.1.2 Statin pharmacokinetic risk factors

Statin related adverse muscle effects occur more frequently with higher doses of the drug and with factors that increase their blood concentrations (882), although drug levels do not completely predict the risk for statin myopathy (883). Plasma levels of statins are affected by both their extensive first-pass uptake in the liver and their rate of catabolism (884). Thus, genetic variants involved in hepatic uptake and statin catabolism that result in increased statin concentrations have been associated with myopathy (143-148).

6.1.2.1 Solute carrier organic anion transporter family member 1B1 (*SLCO1B1*) polymorphisms

There has been a large amount of interest in polymorphisms of the solute carrier organic anion transporter family member 1B1 (*SLCO1B1*) gene. *SLCO1B1* is 112,593 base pairs in length, consisting of 16 exons located on chromosome 12 (885) (Figure 6.3). The encoded polypeptide, organic anion-transporting polypeptide C, (OATP1B1), is known to regulate the hepatic uptake of statins (886). Many statins, including simvastatin (887), atorvastatin, rosuvastatin (886), pravastatin (888), lovastatin (889) and pitavastatin (890) are transported by this mechanism, whereas fluvastatin is not (891). Two non-synonymous SNPs, rs4149056 (512T>C) and rs2306286 (388A>G), affect statin transport by OAT1B1 (892). These polymorphisms occur alone or in combination with each other in three common haplotypes: *SLCO1B1**1b (388A>G), *5 (512T>C) and *15 (388A>G and 512T>C) (893). The effects on transporter function are dependent on the individual combination haplotypes (894). The *SLCO1B1**1b variant is associated with increased OATP1B1 activity and uptake of statins into the liver, and lower plasma statin concentrations (895). On the contrary, the *SLCO1B1**5

and *SLCO1B1**15 variants are associated with reduced liver transporter activity (896) and increased plasma levels of statins (886, 887, 897-901).

Recent studies have demonstrated a potential role of *SLCO1B1* polymorphisms in statin-related myopathy. In a small study, Morimoto *et al.* (2005) (902) observed a significantly higher frequency of the *SLCO1B1**15 variant in patients with pravastatin and atorvastatin related myopathy in comparison to controls with normal creatine kinase (CK) levels on statin therapy. Investigators of the SEARCH (Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine) study performed a genome-wide association scan using 300,000 markers in 85 patients with definite or incipient myopathy who had CK evidence of rhabdomyolysis and 90 controls treated with 80mg simvastatin daily (144). There was a strong association between the risk of myopathy and the non-coding *SLCO1B1* SNP, rs4363657, with an odds ratio for myopathy of 4.3 for one C allele and 17.4 for two C alleles (144). The rs4363657 SNP was shown to be in strong linkage disequilibrium ($r^2=0.97$) with the non-synonymous *SLCO1B1* SNP, rs4149056 (*SLCO1B1**5) (144). The odds ratio for myopathy in patients with the *SLCO1B1**5 variant was 4.5 for one and 16.9 for two C alleles (144). This association was replicated in a cohort of approximately 20,000 patients from the HPS (Heart Protection Study) (68), where 23 cases of myopathy following 40mg simvastatin therapy were significantly associated with *SLCO1B1**5 (144). These findings were confirmed in more recent trials, including the STRENGTH (Statin Response Examined by Genetic Haplotype Markers) study (145), where a significant association was observed between the *SLCO1B1**5 variant and the risk of myalgia, following treatment with simvastatin and atorvastatin but not pravastatin. There was however, a report of pravastatin-induced myopathy in a Japanese patient with a 1628T>G polymorphism in the *SLCO1B1* gene (903). In the GO-DARTS (Genetics of Diabetes Audit and Research in Tayside) study, the *SLCO1B1**5 variant was associated with a higher odds ratios for statin intolerance (2.05, $P=0.043$) and a lower LDL-cholesterol response to statin therapy ($P=0.01$) (904).

6.1.2.2 Cytochrome P enzyme system (CYP) polymorphisms

The CYP enzyme system has a key role in the phase 1 metabolism of statins and has more than 30 known isoenzymes (905). CYP3A4 and CYP2D6 catalyse the majority of CYP-mediated drug metabolism (905). CYP3A4 is the main pathway by which simvastatin, atorvastatin and lovastatin undergo metabolism, whereas fluvastatin and rosuvastatin are primarily metabolised via CYP2C9 (157). Genetic variants of *CYP3A4/5*, *CYP2D6* and

CYP2C9 can affect their rates of metabolism, and in some cases have been associated with an increased risk of statin myopathy, albeit inconsistently. *CYP2D6* plays an important role in the metabolism of simvastatin (906). More than 50 variants of the *CYP2D6* gene, have been described (907), resulting in the classification of patients with normal activity (extensive metabolisers), low or absent activity (poor metabolisers), or high activity (ultra rapid metabolisers) (908, 909). There is marked ethnic variability in the frequency of allelic variants of *CYP2D6* (910). The prevalence of *CYP2D6* poor metabolisers is 5 to 10% in Caucasians, and is less in other ethnic groups such as Black populations (2%), and Asians (<1%) (910). The most common mutant alleles of *CYP2D6* in the Caucasian population are *CYP2D6*3*, *CYP2D6*4* and *CYP2D6*5* (911). Poor metabolisers would be expected to have higher plasma statin levels and thus a higher risk of statin intolerance (146). Mulder *et al.* (2001) (146), demonstrated that among 88 patients treated with simvastatin, 34 (38.6%) had one or more mutated alleles. Homozygotes were shown to have decreased enzyme activity and the highest incidence of myalgia (146). Frudakis *et al.* (2007) reported a significant association between patients with the *CYP2D6*4* polymorphism, the most common non-functioning variant, and atorvastatin-induced muscle effects (147). On the contrary, the prevalence of *CYP2D6* polymorphisms did not differ among 18 patients with statin-related myopathy defined by standard criteria and 12 patients without myopathy in a further study (912). Atorvastatin is also metabolised in part by *CYP3A5* (913). Among patients treated with atorvastatin, homozygotes for the *CYP3A5*3* allele were found to have higher CK levels than heterozygotes, implying more muscle damage in those patients with two copies of the mutant allele (148).

6.1.3 Study hypothesis

Increased knowledge of genetic risk factors associated with statin-induced musculoskeletal side effects may not only provide insight into the mechanism of statin myopathy, but also lead to improved compliance and reduced morbidity through individualised risk assessment and treatment. On this basis, the first hypothesis for this study was that genetic susceptibility to underlying metabolic myopathies and altered statin pharmacokinetics are determinants of statin intolerance. The second hypothesis was that interactions between significant variants are associated with an increased the risk of statin related myopathy. Accordingly, the frequency of previously reported susceptibility polymorphisms or mutations in the *COQ2*, *AMPD1*, *CYPT2*, *PYGM*, *SLCO1B1* and *CYP2D6* genes were compared between cases with a history of statin-induced myopathy and statin

tolerant controls from Christchurch Hospital lipid clinic in order to confirm whether these variants predict an increased risk of statin intolerance. In this study, the more prevalent mutations/polymorphisms of these candidate genes were tested. Genetic analysis was also undertaken in a second independent cohort of cases and controls from a Canadian lipid clinic and data from the two study cohorts were reviewed in a meta-analysis.

Table 6.1 Genetic risk factors associated with myopathic outcomes during statin therapy

Gene (statin)	Polymorphism/Mutation	Outcome
Metabolic muscle disease genes		
<i>COQ2</i> (multiple)	<i>COQ2</i> polymorphisms	Associated with statin intolerance
<i>AMPD1</i> (multiple)	Q12X, P48L	Rhabdomyolysis in homozygotes only
<i>CPT2</i> (multiple)	S113L	Rhabdomyolysis in heterozygotes and homozygotes
<i>PYGM</i> (multiple)	R50X	Rhabdomyolysis in heterozygotes
Statin pharmacokinetics		
<i>SLC01B1</i> (simvastatin)	SNP in intron 11	Mild myopathy with elevated serum CK
<i>SLC01B1</i> (multiple)	V174A (T511C)	Myopathy; increased plasma statin levels, except fluvastatin
<i>CYP2D6</i> (simvastatin)	<i>CYP2D6</i> *4	Atorvastatin and simvastatin-induced myopathy
<i>CYP2D6</i> (fluvastatin)	<i>CYP2D6</i> *3,*5	High incidence of drug intolerance in homozygotes
<i>CYP3A5</i> (lovastatin, pravastatin, simvastatin and atorvastatin)	<i>CYP3A5</i> *3,*5	Elevated plasma simvastatin; homozygotes for <i>CYP3A</i> *5 at greater risk for muscle damage
<i>ABCB1</i> (atorvastatin, simvastatin)	Various	Elevated plasma statin levels
Other		
<i>APOE</i> (multiple)	E4	Reduced compliance in E4 carriers
<i>AGTR1</i> (multiple)	SNP in intron 3	Variations in serum CK during therapy
<i>NOS3</i> (multiple)	D298E	Variations in serum CK during therapy
<i>HTR3B</i> and <i>HTR7</i> (multiple)	One SNP/gene	Individual variations in pain perception
<i>SLC6A4</i>	Serotonin transporter	Down regulated by statin therapy leading to myopathy

Adapted from Vladuti *et al.* (2008) (914). *COQ2*, para-hydroxybenzoate--polyprenyltransferase; *AMPD1*, adenosine monophosphate deaminase 1; *CPT2*, carnitine palmitoyl-2; *PYGM*, myophosphorylase; *SCL01B1*, solute carrier organic anion transporter family member 1B1; *CYP2D6*, cytochrome P450, family 2, subfamily D, polypeptide 6; *CYP3A5*, cytochrome P450, family 3, subfamily A, polypeptide 5; *AGTR1*, angiotensin II receptor type 1; *NOS3*, nitric oxide synthase 3; *HTR3B*, 5-hydroxytryptamine (serotonin) receptor 3B; *HTR7*, 5-hydroxytryptamine (serotonin) receptor 7B; *SLC6A4*, solute carrier family 6, member 4.

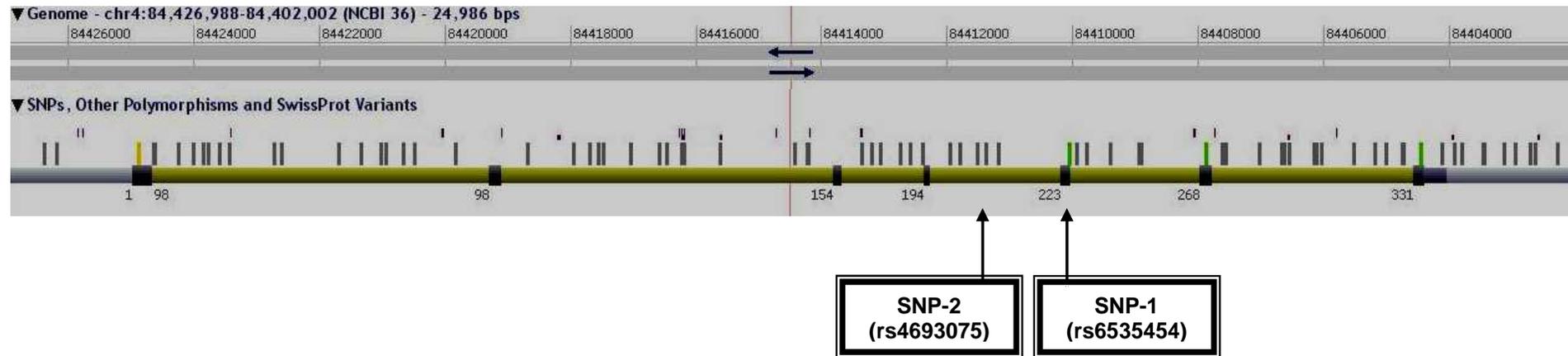


Figure 6.1 The coenzyme Q2 gene (*COQ2*), showing its position on chromosome 4 (above), and SNPs, other polymorphisms and SwissProt variants (below). The 7 exons are shown in dark grey. The first amino acid encoded by each exon is indicated below. Gray vertical lines indicate non-coding SNPs, green lines indicate synonymous coding SNPs, yellow lines indicate non-synonymous coding SNPs, and purple lines indicate non-SNP polymorphisms. The synonymous polymorphism, SNP-1 (rs6535454) in exon 5, and the non-coding polymorphism, SNP-2 (rs4693075) in intron 4 that were evaluated in this study are indicated by boxed arrows (Alamut™, version 1.5)

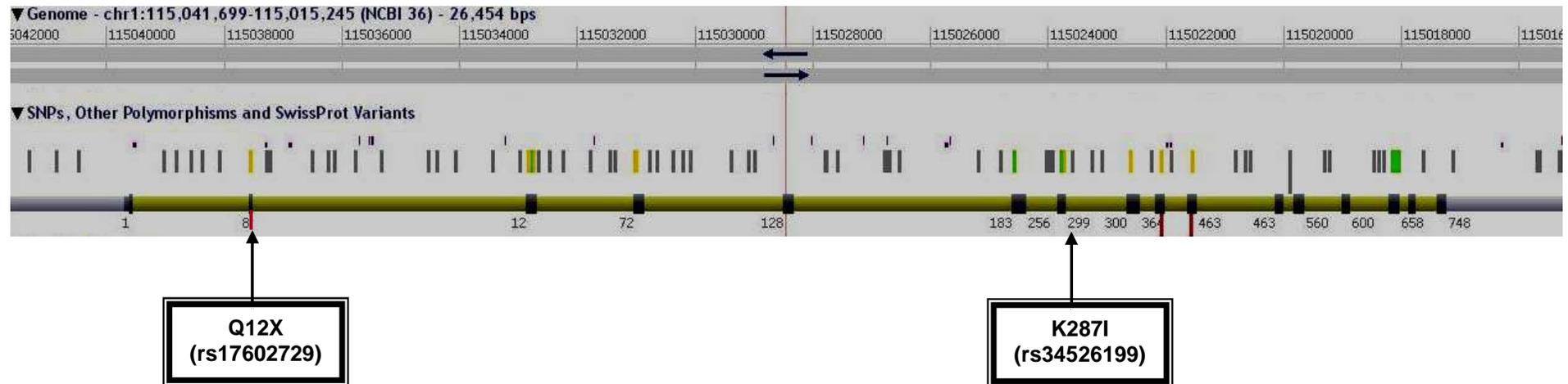


Figure 6.2 The adenosine monophosphate deaminase 1 (isoform M) gene (*AMPD1*), showing its position on chromosome 1 (above), and SNPs, other polymorphisms and SwissProt variants (below). The 16 exons are shown in dark grey. The first amino acid encoded by each exon is indicated below. Gray vertical lines indicate non-coding SNPs, green lines indicate synonymous coding SNPs, yellow lines indicate non-synonymous coding SNPs, red lines indicate pathogenic variants, and purple lines indicate non-SNP polymorphisms. The two non-synonymous mutations evaluated in this study: Q12X (rs17602729) encoded by exon two and K287I (rs34526199) encoded by exon 7 are indicated by boxed arrows (Alamut™, version 1.5)

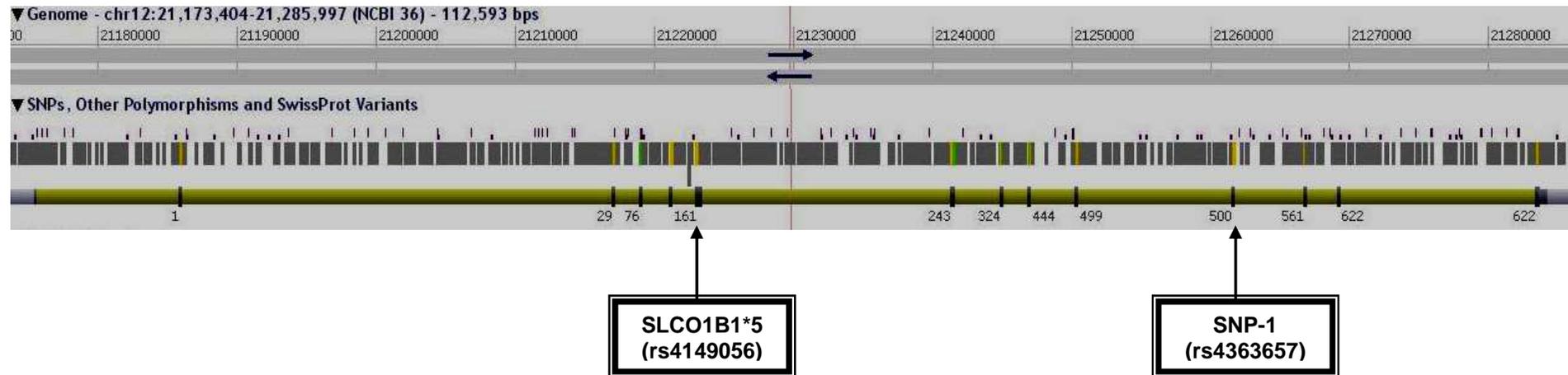


Figure 6.3 The solute carrier organic anion transporter family member 1B1 gene (*SLCO1B1*), showing its position on chromosome 12 (above), and SNPs, other polymorphisms and SwissProt variants (below). The 15 exons are shown in dark grey. The first amino acid encoded by each exon is indicated below. Gray vertical lines indicate non-coding SNPs, green lines indicate synonymous coding SNPs, yellow lines indicate non-synonymous coding SNPs, and purple lines indicate non-SNP polymorphisms. The non-synonymous polymorphism, *SLCO1B1**5 (rs4149056) in exon 5, and the non-coding polymorphism, SNP-1 (rs4363657) in intron 11 that were evaluated in this study are indicated by boxed arrows (Alamut™, version 1.5)

6.2 Methods

6.2.1 Subjects and study design

6.2.1.1 Christchurch subjects

One hundred and twenty eight patients from a single lipid clinic population at Christchurch Hospital were studied. Sixty-seven statin-intolerant patients, defined as having symptomatic muscle weakness, tenderness and/or pain on statin therapy and again on re-challenge, or using two different statins and 61 statin tolerant controls from the lipid clinic, who were taking at least 80mg of simvastatin or atorvastatin for ≥ 3 months with no reported myalgia symptoms were studied. The study protocol was approved by the Upper South B Regional Ethics Committee, and written informed consent was obtained from all participants.

6.2.1.2 Canadian subjects

A second lipid clinic population was studied independently at the University of Western Ontario and Vascular Biology Research Group, Robarts Research Institute, Canada. The case and control patient populations are detailed in the report by Oh *et al.* (2007) (205). 291 patients who were taking statin monotherapy were included. 133 patients (mean age 57.1 ± 12.1 years, 36.6% female) were statin-intolerant with myopathy, defined as having symptomatic muscle weakness, tenderness and/or pain and one of the following; medically advised discontinuation of statin medication on at least two occasions; serum CK elevated to >3 -fold the upper limit of normal (ULN) while on a statin on at least one occasion; or medically diagnosed rhabdomyolysis. 158 clinic patient controls (mean age 55.9 ± 11.3 years, 35.5% female), matched for sex and age, and taking at least 10 mg of atorvastatin or rosuvastatin or 20mg of other statins for ≥ 1 year with no reported symptoms and normal serum CK concentration were evaluated (205).

6.2.2 Genotyping

Genotype analysis of the samples from the Christchurch lipid clinic population was undertaken by Howard Potter at the Molecular Pathology Laboratory, Canterbury Health Laboratories in Christchurch. Genomic DNA was isolated from peripheral blood leukocytes of patients and controls by standard methods (915). DNA was diluted to 10ng/ μ l in water for

polymerase chain reaction (PCR) amplification. Primer pairs were designed to amplify exons 2 and 7 of the *AMPD1*¹ gene, exons 1 and 5 of the *PYGM* gene and exon 3 of the *CPTII* gene based on the reference sequence accession numbers NM_000036.1, NM_005609.2 and NM_000098.2, respectively. Further primer pairs were designed to amplify exon 5 and intron 4 of the *COQ2* gene and exon 6 and intron 11 of the *SLCO1B1* gene based on accession numbers NM_015697.6 and NM_006446.3. The *CYP2D6**4 allele was analysed using an allele-specific PCR as previously described (917). PCR fragments were sequenced on an Applied Biosystems 3130xl genetic analyser using Big-Dye Terminator v3.1 Cycle Sequencing chemistry. Sequence data obtained was used to determine the presence of the *AMPD1* gene mutations Q12X and K287I (rs17602729 and rs34526199), *PYGM* gene mutations R50X and G205S (rs116987552 and rs119103251), the *CPTII* gene mutation SII3L (rs74315294), the *COQ2* SNPs (rs6535454 and rs4693075), the *SLCO1B1* polymorphisms (rs436357 and rs4149056) and the *CYP2D6**4 polymorphism in the statin intolerant cases. The control subjects were genotyped for the polymorphisms identified in the statin intolerant subjects.

6.2.3 Statistical analysis

All statistical analyses were performed using SPSS Base version 17.0 (SPSS, Inc., Chicago, Illinois). Tests for Hardy-Weinberg equilibrium were performed separately for each polymorphism using an online calculator (<http://www.oege.org/software/hwe-mr-calc.shtml>). Pairwise linkage disequilibrium (non-random association) (r^2) between alleles was determined using SHEsis (<http://analysis.bio-x.cn/myAnalysis.php>). Two-site haplotypes were constructed using two platforms; PHASE (v 2.1), for maximal likelihood analysis (918, 919), and SHEsis using a Full-Precise-Iteration algorithm (920, 921). PHASE-predicted haplotypes were collapsed into three groups based on the presence or absence of haplotype 1, and thus participants had one of three possible diploid summary haplotypes: 1/1, 1/X or X/X, where X refers to any non-1 haplotype. Dominant and recessive genetic association models of statin intolerance with genotypes or PHASE-predicted haplotypes, and single haplotype associations were evaluated using chi-square analysis or Fisher's exact test as appropriate. For dominant and recessive models, genotypes were tested for the rare allele and PHASE-predicted haplotypes were tested for the non-1 haplotype alleles. Associations were summarised as odds

¹ All genes discussed are named according to the HUGO Gene Nomenclature Committee (HGNC) nomenclature 916. Seal RL, Gordon SM, Lush MJ, Wright MW, Bruford EA. genenames.org: the HGNC resources in 2011. *Nucleic Acids Res.* 2011 Jan;39(Database issue):D514-9..

ratios with 95% confidence intervals. The odds ratio was calculated as the odds of the presence of a genotype or haplotype among cases divided by the odds of the presence of the genotype or haplotype among controls. Pooled odds ratios were calculated with the Mantel-Haenszel method using RevMan 5.0 (922), which also tested the homogeneity of the association across the two cohorts. Statistical significance was inferred when $P < 0.05$.

6.3 Results

6.3.1 Genetic descriptors of cohorts

The allele and genotype frequencies for *COQ2*, *AMPD1*, *SCLO1B1* and *CYP2D6* polymorphisms from both the Christchurch and Canadian lipid clinic cohorts of cases and controls are shown in Tables 6.2 – 6.5, respectively. The haplotype frequencies for *COQ2*, *AMPD1* and *SCLO1B1* in the both cohorts predicted using PHASE and SHEsis are shown in Tables 6.2 – 6.4, respectively. The most common haplotype for each gene was designated as haplotype 1. Haplotypes were designated 1-4 as follows: *COQ2* haplotypes GC, GG, AC, and AG; *AMPD1* haplotypes CA, CT, TA, and TT; and *SLCO1B1* haplotypes TT, TC, CT, and CC.

6.3.1.1 Genetic descriptors of the Christchurch lipid clinic cohort

The control genotype frequencies for *COQ2*, *AMPD1*, *SCLO1B1* and *CYP2D6* did not deviate from those expected from the Hardy Weinberg distribution ($p > 0.05$). The pairwise linkage disequilibrium correlation coefficient between *COQ2* alleles of SNP-1 and SNP-2 was 0.84 ($P < 0.0001$), and between *SLCO1B1* alleles of *5 and SNP-1 was 0.95 ($p < 0.0001$), indicating strong linkage disequilibrium. The pairwise linkage disequilibrium correlation coefficient between *AMPD1* alleles of Q12X and K287I was 0.00 ($P = 0.99$), indicating linkage equilibrium. Since no mutations were identified in the *CPT2* or *PYGM* genes of the Christchurch lipid clinic cases, they were not tested in the statin tolerant control group or Canadian lipid clinic cohort.

6.3.1.2 Genetic descriptors of the Canadian lipid clinic cohort

The control genotype frequencies for *COQ2*, *AMPD1*, *SCLO1B1* and *CYP2D6* did not deviate from those expected from the Hardy Weinberg distribution ($P > 0.05$). The pairwise

linkage disequilibrium correlation coefficient between *COQ2* alleles of SNP-1 and SNP-2 was 0.65 ($P < 0.0001$), and between *SLCO1B1* alleles of *5 and SNP-1 was 0.80 ($P < 0.0001$), indicating strong linkage disequilibrium. The pairwise linkage disequilibrium correlation coefficient between *AMPDI* alleles of Q12X and K287I was 0.00 ($P = 0.71$), indicating linkage equilibrium.

6.3.2 Genetic associations with statin-intolerance

The results of the genetic association analysis using dominant and recessive models for rare alleles for the genotypes, and non-1 haplotypes are shown in Table 6.6. Single haplotype associations with statin intolerance for haplotypes predicted using SHEsis and PHASE are presented in Table 6.7 and Table 6.8, respectively. Forest plots of comparison for genetic association with statin intolerance are shown in Appendices 12-14.

Under recessive models, the *COQ2* SNP-1 and SNP-2 genotypes and *COQ2* non-1 haplotype all showed significant associations with statin intolerance for the Canadian cohort and pooled cohort, and a trend was observed in the Christchurch cohort. The pooled odds ratios (OR) and 95% confidence intervals for increased risk of statin-induced myopathy among the homozygotes for the rare alleles were 2.34 (1.06 – 5.13), $P = 0.03$; 2.37 (1.28 – 4.42), $P = 0.006$; 2.48 (1.34 – 4.61), $P = 0.004$ for SNP-1 and SNP-2 genotypes and the *COQ2* non-1 haplotype, respectively. Dominant models for the *COQ2* SNP-1 and SNP-2 genotypes and the *COQ2* haplotype were not associated with statin intolerance in either pooled or individual cohorts. In the pooled cohort, the *COQ2* wild-type haplotype 1 (SHEsis) was associated with a lower risk of statin intolerance (OR = 0.73 (0.55 – 0.98), $P = 0.03$), and a similar trend was observed for the PHASE-predicted haplotype 1 ($P = 0.06$).

The SHEsis-predicted *AMPDI* haplotype 3 was significantly associated with an increased odds of statin intolerance (2.12 (1.04 – 4.35), $P < 0.05$) in the Christchurch cohort. Overall, the *AMPDI* haplotype 3 was associated with a trend for increased risk of statin-induced myopathy (OR=1.51 (0.88 – 2.59), $P = 0.13$ for SHEsis, and OR=1.45 (0.97 – 2.17), $P = 0.07$ for PHASE). There was also a trend for increased risk of statin intolerance among the heterozygotes for the rare allele of the *AMPDI* Q12X genotype, with OR=1.49 (0.96 – 2.33), $P = 0.08$ in the pooled cohort. This trend almost reached significance when all cases were incorporated, with an OR=1.55 (1.00 – 2.41), $P = 0.05$. Homozygotes for rare alleles of the

AMPD1 Q12X and K287I genotypes, and the *AMPD1* non-1 haplotype were not associated with statin intolerance in either individual or pooled cohorts.

The *SLCO1B1**5 and SNP-1 genotypes, and *SLCO1B1* non-1 haplotype were not associated with statin intolerance in the individual or pooled cohorts, and there were no single associations between *SLCO1B1* haplotypes and statin-induced myopathy. The *CYP2D6**4 genotype did not predict increased risk of statin intolerance in these cohorts.

6.3.3 Interaction of *COQ2* and *AMPD1* variants on statin intolerance

The interaction of the *COQ2* SNP-2 and *AMPD1* Q12X rare alleles on the risk of statin intolerance for the pooled cohort is presented in Table 6.9. Forest plots of comparison for these interactions are shown in Appendix 15. The cumulative frequency of *COQ2* SNP-2 and *AMPD1* Q12X rare alleles was associated with an increased risk of statin myopathy compared to individuals without variant alleles. OR = 1.64 (1.08 – 2.51), $P=0.02$ for 0 vs 1 or more rare allele; 2.04 (1.21, 3.45), $P=0.008$ for 0 vs 2 or more variant alleles; and 3.06 (0.97, 9.69), $P=0.06$ for 0 vs 3 or more variant alleles.

Table 6.2 *COQ2* genotype, allele and haplotype frequencies

Parameter		Christchurch Cohort		Canadian Cohort		
		Cases (n=67)	Controls (n=61)	Cases (n=133)	Controls (n=158)	
<i>COQ2</i> SNP-1 (rs6535454)	G/G	32 (47.8)	31 (50.8)	65 (48.9)	85 (53.8)	
	G/A	30 (44.8)	27 (44.3)	53 (39.8)	66 (41.8)	
	A/A	5 (7.5)	3 (4.9)	15 (11.3)	7 (4.4)	
	G	94 (70.1)	89 (73.0)	183 (68.8)	236 (74.7)	
	A	40 (29.9)	33 (27.0)	83 (31.2)	80 (25.3)	
<i>COQ2</i> SNP-2 (rs4693075)	C/C	23 (34.3)	27 (44.3)	55 (41.4)	69 (43.7)	
	C/G	34 (50.7)	30 (49.2)	55 (41.4)	76 (48.1)	
	G/G	10 (14.9)	4 (6.6)	23 (17.3)	13 (8.2)	
	C	80 (59.7)	84 (68.9)	165 (62.0)	214 (67.7)	
	G	54 (40.3)	38 (31.1)	101 (38.0)	102 (32.3)	
<i>COQ2</i> haplotypes (PHASE)	1,1	26 (38.8)	27 (44.3)	54 (40.6)	67 (42.4)	
	1,2	8 (11.9)	4 (6.6)	10 (7.5)	17 (10.8)	
	1,3	0	0	0	2 (1.3)	
	1,4	24 (35.8)	26 (42.6)	44 (33.1)	59 (37.3)	
	2,2	1 (1.5)	0	1 (0.8)	1 (0.6)	
	2,4	3 (4.5)	1 (1.6)	9 (6.8)	5 (3.2)	
	3,3	0	0	1 (0.8)	0	
	3,4	0	0	1 (0.8)	0	
	4,4	5 (7.5)	3 (4.9)	13 (9.8)	7 (4.4)	
		1	62.7	68.9	60.9	67.1
		2	9.7	4.1	7.9	7.6
		3	0	0	1.1	0.6
		4	27.6	27.0	30.1	24.7
<i>COQ2</i> haplotypes (SHEsis)	1	59.7	68.9	60.8	67.0	
	2	10.4	4.1	8.0	7.7	
	3	0	0	1.2	0.7	
	4	29.9	27.0	30.0	24.6	

Table 6.3 *AMPDI* genotype, allele and haplotype frequencies

Parameter		Christchurch Cohort		Canadian Cohort	
		Cases (n=67)	Controls (n=61)	Cases (n=133)	Controls (n=158)
<i>AMPDI</i> Q12X (rs17602729)	C/C	47 (67.1)	55 (78.6)	94 (72.3)	113 (77.9)
	C/T	21 (30.0)	15 (21.4)	34 (26.2)	30 (20.7)
	T/T	2 (2.9)	0	2 (1.5)	2 (1.4)
	C	82.1	89.3	85.4	88.3
	T	17.9	10.7	14.6	11.7
<i>AMPDI</i> K287I (rs34526199)	A/A	67 (95.7)	65 (92.9)	126 (96.9)	136 (93.8)
	A/T	3 (4.3)	5 (7.1)	4 (3.1)	9 (6.2)
	T/T	0	0	0	0
	A	97.9	96.4	98.5	96.9
	T	21.0	3.6	1.5	3.1
<i>AMPDI</i> haplotypes (PHASE)	1,1	44 (62.9)	52 (74.3)	92 (70.8)	105 (72.4)
	1,2	3 (4.3)	3 (4.3)	2 (1.5)	8 (5.5)
	1,3	21 (30.0)	13 (18.6)	32 (24.6)	29 (20.0)
	2,3	0	2 (2.9)	2 (1.5)	1 (0.7)
	3,3	2 (2.9)	0	2 (1.5)	2 (1.4)
	1	80.0	85.7	83.9	85.2
	2	2.1	3.6	1.5	3.1
	3	17.9	10.7	14.6	11.7
<i>AMPDI</i> haplotypes (SHEsis)	1	80.0	86.9	84.5	85.2
	2	2.1	2.4	0.9	3.1
	3	17.9	9.6	14.0	11.7
	4	0	0	0.6	0.0

Table 6.4 *SLCO1B1* genotype, allele and haplotype frequencies

Parameter		Christchurch Cohort		Canadian Cohort	
		Cases (n=67)	Controls (n=61)	Cases (n=133)	Controls (n=158)
<i>SLCO1B1</i> *5 (rs4149056)	T/T	51 (76.1)	41 (67.2)	92 (73.0)	97 (67.4)
	T/C	16 (23.9)	19 (31.1)	32 (25.4)	42 (29.2)
	C/C	0	1 (1.6)	2 (1.6)	5 (3.5)
	T	88.1	82.8	216 (85.7)	236 (81.9)
	C	11.9	17.2	36 (14.3)	52 (18.1)
<i>SLCO1B1</i> SNP-1 (rs4363657)	T/T	48 (71.6)	40 (65.6)	91 (72.2)	97 (67.4)
	T/C	19 (28.4)	20 (32.8)	35 (27.8)	46 (31.9)
	C/C	0	1 (1.6)	0	1 (0.7)
	T	85.8	82.0	217 (86.1)	240 (83.3)
	C	14.2	18.0	35 (13.9)	48 (16.7)
<i>SLCO1B1</i> haplotypes (PHASE)	1,1	48 (71.6)	40 (65.6)	90 (71.4)	94 (65.3)
	1,2	3 (4.5)	1 (1.6)	2 (1.6)	3 (2.1)
	1,3	0	0	1 (0.8)	2 (1.4)
	1,4	16 (23.9)	19 (31.1)	31 (24.6)	40 (27.8)
	3,3	0	0	0	1 (0.7)
	3,4	0	0	2 (1.6)	3 (2.1)
	4,4	0	1 (1.6)	0	1 (0.7)
	1	85.8	82.0	84.9	80.9
	2	2.2	0.8	0.8	1.0
	3	0	0	1.2	2.4
4	11.9	17.2	13.1	15.6	
<i>SLCO1B1</i> haplotypes (SHEsis)	1	85.8	82.0	84.9	80.9
	2	2.2	0.8	0.8	1.1
	3	0	0	1.2	2.5
	4	11.9	17.2	13.1	15.6

Table 6.5 *CYP2D6* genotype and allele frequencies

Parameter	Christchurch Cohort		Canadian Cohort		
	Cases (n=67)	Controls (n=61)	Cases (n=133)	Controls (n=158)	
<i>CYP2D6</i> *4 (rs3892097)	*1*1	48 (71.6)	42 (68.9)	86 (65.2)	102 (68.5)
	*1*4	19 (28.4)	16 (26.2)	37 (28.0)	37 (24.8)
	*4*4	0	3 (4.9)	9 (6.8)	10 (6.7)
	*1	85.8	82.0	209 (79.2)	241 (80.9)
	*4	14.2	18.0	55 (20.8)	57 (19.1)

Table 6.6 Dominant and recessive genetic models of association with statin-intolerance

Parameter		Christchurch Cohort OR (95% CI)†	Canadian Cohort OR (95% CI)†	Pooled Cohorts OR (95% CI)†	Overall Effect <i>P</i> -value‡	Heterogeneity <i>P</i> -value‡
<i>COQ2</i> SNP-1	A-dominant	1.13 (0.56 – 2.26)	1.22 (0.77 – 1.93)	1.19 (0.81 – 1.75)	0.37	0.86
	A-recessive	1.56 (0.36 – 6.82)	2.74 (1.08 – 6.94)*	2.34 (1.06 – 5.13)	0.03	0.53
<i>COQ2</i> SNP-2	G-dominant	1.52 (0.74 – 3.10)	1.10 (0.69 – 1.75)	1.21 (0.82 – 1.79)	0.34	0.46
	G-recessive	2.50 (0.74 – 8.44)	2.33 (1.13 – 4.81)*	2.37 (1.28 – 4.42)	0.006	0.92
<i>COQ2</i> haplotype§	non-1 dominant	1.25 (0.62 – 2.53)	1.08 (0.67 – 1.72)	1.13 (0.76 – 1.67)	0.55	0.73
	non-1 recessive	2.21 (0.64 – 7.59)	2.58 (1.26 – 5.28)*	2.48 (1.34 – 4.61)	0.004	0.83
<i>AMPD1</i> Q12X	T-dominant	1.79 (0.84 – 3.83)	1.35 (0.78 – 2.34)	1.49 (0.96 – 2.33)	0.08	0.55
	T-recessive	5.15 (0.24 – 109.15)	1.12 (0.16 – 8.05)	1.75 (0.33 – 9.20)	0.51	0.41
<i>AMPD1</i> K287I	T-dominant	0.58 (0.13 – 2.54)	0.48 (0.14 – 1.60)	0.52 (0.20 – 1.32)	0.17	0.84
	T-recessive	-	-	-	-	-
<i>AMPD1</i> haplotype§	non-1 dominant	1.71 (0.83 – 3.52)	1.08 (0.64 – 1.83)	1.27 (0.83 – 1.94)	0.27	0.32
	non-1 recessive	1.00 (0.14 – 7.31)	1.50 (0.33 – 6.84)	1.29 (0.39 – 4.32)	0.68	0.75
<i>SLCO1B1</i> *5	C-dominant	0.64 (0.30 – 1.40)	0.79 (0.47 – 1.34)	0.74 (0.48 – 1.15)	0.18	0.66
	C-recessive	0.30 (0.01 – 7.47)	0.38 (0.02 – 9.37)	0.34 (0.03 – 3.26)	0.35	0.92
<i>SLCO1B1</i> SNP-1	C-dominant	0.75 (0.36 – 1.59)	0.76 (0.45 – 1.29)	0.74 (0.49 – 1.17)	0.21	0.98
	C-recessive	0.30 (0.01 – 7.47)	0.45 (0.09 – 2.35)	0.41 (0.09 – 1.80)	0.24	0.83
<i>SLCO1B1</i> haplotype§	non-1 dominant	0.75 (0.36 – 1.59)	0.75 (0.45 – 1.26)	0.75 (0.49 – 1.15)	0.19	1.00
	non-1 recessive	0.30 (0.01 – 7.47)	0.45 (0.09 – 2.35)	0.41 (0.09 – 1.80)	0.24	0.83
<i>CYP2D6</i> *4	*4-dominant	0.88 (0.41 – 1.87)	1.16 (0.71 – 1.91)	1.07 (0.70 – 1.62)	0.76	0.54
	*4-recessive	0.12 (0.01 – 2.45)	1.02 (0.40 – 2.58)	0.57 (0.09 – 3.71)	0.56	0.18

†Chi-square or Fisher's exact test, ‡Pooled Mantel-Haenszel chi-square test. § PHASE predicted haplotypes. OR (95% CI), odds ratio (95% confidence interval). **P*<0.05.

Table 6.7 Single haplotype associations with statin-intolerance (SHEsis -predicted haplotypes)

Parameter		Christchurch Cohort OR (95% CI) [†]	Canadian Cohort OR (95% CI) [†]	Pooled Cohorts OR (95% CI) [†]	Overall Effect <i>P</i> -value [‡]	Heterogeneity <i>P</i> -value [‡]
<i>COQ2</i> haplotype	1	0.67 (0.40 – 1.12)	0.76 (0.54 – 1.07)	0.73 (0.55 – 0.98)	0.03	0.68
	2	2.73 (0.95 – 7.82)	1.04 (0.57 – 1.92)	1.53 (0.61 – 3.85)	0.37	0.12
	3	-	1.79 (0.30 – 10.80)	1.79 (0.30 – 10.80)	0.52	-
	4	1.15 (0.67 – 1.98)	1.31 (0.91 – 1.89)	1.26 (0.93 – 1.71)	0.14	0.69
<i>AMPDI</i> haplotype	1	0.59 (0.31 – 1.13)	0.96 (0.60 – 1.53)	0.79 (0.50 – 1.26)	0.33	0.23
	2	1.00 (0.20 – 5.04)	0.24 (0.05 – 1.13)	0.48 (0.12 – 1.95)	0.31	0.21
	3	2.12 (1.04 – 4.35)*	1.21 (0.73 – 2.00)	1.51 (0.88 – 2.59)	0.13	0.21
	4	0.20 (0.01 – 4.14)	5.62 (0.27 – 117.58)	1.05 (0.04 – 28.08)	0.98	0.13
<i>SLCO1B1</i> haplotype	1	1.33 (0.68 – 2.60)	1.33 (0.84 – 2.09)	1.33 (0.91 – 1.94)	0.14	1.00
	2	2.77 (0.28 – 27.00)	0.76 (0.13 – 4.59)	1.25 (0.30 – 5.12)	0.76	0.38
	3	-	0.48 (0.12 – 1.89)	0.48 (0.12 – 1.89)	0.30	-
	4	0.65 (0.32 – 1.32)	0.81 (0.50 – 1.32)	0.76 (0.51 – 1.13)	0.17	0.61

[†]Chi-square or Fisher's exact test, [‡]Pooled Mantel-Haenszel chi-square test.

OR (95% CI), odds ratio (95% confidence interval). **P*<0.05.

Table 6.8 Single haplotype associations with statin-intolerance (PHASE-predicted haplotypes)

Parameter		Christchurch Cohort OR (95% CI) [†]	Canadian Cohort OR (95% CI) [†]	Pooled Cohorts OR (95% CI) [†]	Overall Effect <i>P</i> -value [‡]	Heterogeneity <i>P</i> -value [‡]
<i>COQ2</i> haplotype	1	0.76 (0.45 – 1.28)	0.76 (0.54 – 1.07)	0.76 (0.57 – 1.01)	0.06	0.99
	2	2.51 (0.87 – 7.27)	1.04 (0.57 – 1.92)	1.45 (0.63 – 3.34)	0.38	0.16
	3	-	1.79 (0.30 – 10.80)	1.79 (0.30 – 10.80)	0.52	-
	4	1.03 (0.59 – 1.78)	1.31 (0.91 – 1.89)	1.22 (0.90 – 1.65)	0.20	0.47
<i>AMPDI</i> haplotype	1	0.67 (0.36 – 1.25)	0.90 (0.57 – 1.44)	0.81 (0.56 – 1.18)	0.27	0.45
	2	0.59 (0.14 – 2.52)	0.49 (0.15 – 1.60)	0.53 (0.21 – 1.32)	0.17	0.84
	3	1.81 (0.91 – 3.61)	1.29 (0.78 – 2.12)	1.45 (0.97 – 2.17)	0.07	0.43
	4	-	-	-	-	-
<i>SLCO1B1</i> haplotype	1	1.33 (0.68 – 2.60)	1.33 (0.84 – 2.09)	1.33 (0.91 – 1.94)	0.14	1.00
	2	2.77 (0.28 – 27.00)	0.76 (0.13 – 4.59)	1.25 (0.30 – 5.12)	0.76	0.38
	3	-	0.48 (0.12 – 1.89)	0.48 (0.12 – 1.89)	0.30	-
	4	0.65 (0.32 – 1.32)	0.81 (0.50 – 1.32)	0.76 (0.51 – 1.13)	0.17	0.61

[†]Chi-square or Fisher's exact test, [‡]Pooled Mantel-Haenszel chi-square test.

OR (95% CI), odds ratio (95% confidence interval). **P*<0.05.

Table 6.9 Interaction of *COQ2* SNP-2 and *AMPDI* Q12X variants on statin intolerance in the pooled cohort

Number of variant alleles	Cases (n=200)	Controls (n=216)	OR (95% CI) [†]	Overall effect <i>P</i> -value [‡]	Heterogeneity <i>P</i> -value [‡]
0	51	78	1.00	-	-
1 or more	149	138	1.64 (1.08, 2.51)	0.02	0.46
2 or more	60	45	2.04 (1.21, 3.45)	0.008	0.35
3 or more	10	5	3.06 (0.97, 9.69)	0.06	1.00

[†]Chi-square or Fisher's exact test, [‡]Pooled Mantel-Haenszel chi-square test.

OR (95% CI), odds ratio (95% confidence interval).

6.4 Discussion

6.4.1 Influence of *COQ2* polymorphisms on statin intolerance

This investigation confirmed that genetic variation in *COQ2* is significantly associated with statin intolerance. Homozygotes for the rare alleles of SNP-1, SNP-2 and the non-1 haplotype had an increased odds ratio of statin myopathy. There was also a significant association between the wild-type haplotype and a reduced risk of statin myopathy. These results concur with the study by Oh and colleagues (205). Our findings are also consistent with interim results from the STRENGTH study (145), where the SNP-2 variant was significantly associated with intolerance to rosuvastatin (OR 2.6, 95% CI, 1.7 – 4.4, $P < 0.001$) (923). Furthermore, an association between the *COQ2* polymorphism and the *SLCO1B1**5 polymorphism was seen in atorvastatin-treated patients presenting with both muscular symptoms and CK increase (OR 3.1, 95% CI 1.9 – 6.4, $P < 0.001$) (923). A pathogenic basis for the CoQ₁₀ pathway in statin-related myopathy is supported by studies showing reduced CoQ₁₀ levels in muscle biopsies from patients on high-dose simvastatin (475), and CoQ₁₀ muscle deficiency in patients with severe statin-induced myopathies (151). In addition, primary CoQ₁₀ deficiency, resulting from *COQ2* gene mutations (459, 460, 463), is dramatically improved by high-dose CoQ₁₀ administration. Taken together, these findings suggest that statin intolerance may result from variation in *COQ2* and the CoQ₁₀ biosynthetic pathway. Further evidence supporting an association between genetic variation in *COQ2* and statin-induced myalgia, was recently documented in a large case-control study by Ruano *et al.* (2011), who evaluated a number of candidate genes in lipid clinic outpatients undergoing statin therapy (924).

6.4.2 Influence of metabolic myopathy mutations on statin intolerance

AMPDI haplotype 3 was associated with significantly increased odds of statin intolerance in the Christchurch cohort. To our knowledge this is the first study to report an increase in mild statin-induced myopathy in association with *AMPDI*. An association was also observed between heterozygotes for the *AMPDI* Q12X mutation and an increased risk of statin intolerance in two independent cohorts and in the pooled cohort. Although the *AMPDI* K287I mutation is relatively common in the population at 5.1% (925), the frequency of heterozygotes did not differ between patients with myalgia and control subjects. This study

supports the findings by Vladitui *et al.* (2006) (151), who demonstrated a 3.25 fold increased frequency of homozygotes for *AMPD1* deficiency in patients with severe myopathy associated with lipid lowering therapies, but no increase in the carrier frequency. Homozygotes were found in 7 of 100 (6.5%) cases versus 2% of normal controls and 1.7 % in asymptomatic treated controls (151). All patients with *AMPD1* mutations (Q12X, P48L and K287I) had significantly elevated CK levels, with 80% of patients displaying CK levels ≥ 10 times ULN. Since the patients in the present study exhibited milder muscle symptoms, this may explain why no increased frequency of homozygotes for *AMPD1* deficiency was observed. These findings indicate that statin therapy can trigger mild muscle symptoms in previously asymptomatic patients with the *AMPD* Q12X mutation.

The common mutations which cause *CPT2* deficiency and myophosphorylase deficiency were not observed in any of the statin intolerant cases in the Christchurch cohort, and were therefore not investigated further. In contrast, Vladitui *et al.* (2006) found that the carrier frequency for myophosphorylase deficiency and *CPT2* deficiency was increased 20-fold and 13-fold, respectively, over the general population (151). Patients with molecular diagnoses had abnormal CK elevations and 50% had CK levels ≥ 10 ULN. The finding of two homozygous mutations in their study, one for myophosphorylase deficiency and the other for *CPT2* deficiency was noteworthy since homozygotes are rare in the general population (151), and probably reflects a higher frequency of more severe metabolic myopathies in association with severe statin related myopathy. The absence of these mutations in the present study probably reflects the milder myopathy in these patients. These findings indicate that mutations causing *CPT2* deficiency and McArdle disease may play a more important role in patients who develop more severe myopathy in response to statin treatment.

6.4.3 Influence of *SLCO1B1* polymorphisms on statin intolerance

In this present study, the lack of association between *SLCO1B1* polymorphisms rs4149056 (*SLCO1B1**5) and rs4363657 (SNP-1) and statin intolerance, suggests that the efficacy of statin transport by OAT1B1 does not have an important influence on the development of mild myopathy. Similarly, Hermann *et al.* (2006) (926) did not observe any difference in the frequency of *SLCO1B1* variants in patients with atorvastatin-related myopathy versus untreated controls.

In contrast, several trials, including genome-wide studies have reported significant associations between *SLCO1B1* gene variants and severe statin myopathy (143-145, 896, 904). In a small study comprising ten patients with myopathy, diagnosed as abnormal CK elevation or severe muscle complaints, and 26 patients without myopathy on treatment, Morimoto *et al.* (2005) (902) found a significant association between the presence of the *SLCO1B1**15 allele and pravastatin or atorvastatin-induced myopathy. More recently, the SEARCH study provided strong evidence of a link between genetic variants in *SLCO1B1* and severe simvastatin-induced myopathy (144). This was revealed through a genome-wide association study involving 300,000 candidate genes in 85 patients with prior myocardial infarction, and definite or incipient myopathy and 90 controls taking 80mg simvastatin daily (144). A significant association was observed between the risk of myopathy and the *SLCO1B1* SNP in intron 11 (rs4363657), with an odds ratio for statin intolerance of 4.3 and 17.4 for one or two C alleles (144). This SNP was in strong linkage disequilibrium with the *SLCO1B1**5 SNP (144). Homozygotes for the *SLCO1B1**5 variant displayed a 4.5-fold increase in the incidence of myopathy, whereas the homozygotes had a 16.5-fold increase (144). This association with the *SLCO1B1**5 was replicated in a cohort of patients from the HPS study (68), where there were 23 cases of definite or incipient myopathy on 40mg simvastatin in comparison with nine cases on placebo (144). The relative risk of myopathy was 2.6 per copy of the C allele (144); lower than the SEARCH cohort, which probably reflects the lower statin dose used.

SLCO1B1 variants have also been related to milder muscle complaints in patients on statin therapy. In the STRENGTH study a significant association was observed between the *SLCO1B1**5 variant and the risk of myalgia in patients discontinuing the trial for any adverse effect (145). A gene-dose effect was also observed in patients with none, one or two alleles having a 19%, 27% and 50% trend to reporting an adverse event, respectively (145). Voora *et al.* (2009) (145) also found a non-significant increase in myopathy among carriers of the *SLCO1B1* alleles in patients on atorvastatin, but not in patients on pravastatin. In the GO-DARTS study, a total of 4,196 patients with type 2 diabetes mellitus receiving statins, were genotyped, to establish whether the *SLCO1B1**5 is also associated with general statin intolerance (904). Intolerance was defined by serum biochemistry and also by discontinuation, switching, or reduction in dose of the prescribed statin drug (904). Homozygosity for the *SLCO1B1**5 variant showed a 2.05-fold increase in statin intolerance, and also a reduced LDL-cholesterol response to statin therapy (904).

The lack of association of *SLCO1B1* polymorphisms with mild myopathy in patients from the present study suggests that these variants maybe more relevant in predicting severe statin-induced myopathy, as suggested in the SEARCH and HPS studies (144), particularly in patients who take high doses of statin therapy or concurrently receiving drugs that inhibit statin metabolism. However, as the *SLCO1B1**5 variant is also more frequent in subjects with mild statin-related myopathies, as reported in the STRENGTH and GO-DART studies, it is also possible that the present study was limited by small sample size.

6.4.4 Influence of *CYP2D6* polymorphisms on statin intolerance

There was no association between the *CYP2D6**4 polymorphism and statin-related myopathy in the two independent case-control cohorts or the pooled data. There have been inconsistent reports of an association between genetic variation in *CYP2D6* and statin intolerance (146, 147, 912). Similar to the current study, Zuccaro and colleagues, found no difference in the prevalence of *CYP2D6**4 polymorphisms between 18 patients with fluvastatin or simvastatin-induced myopathy and 12 patients without myopathy (912). Adverse events were defined as muscle pain, with or without CK elevation, requiring dose reduction or withdrawal from therapy (912). The investigators did, however, observe a significant association between increased statin efficacy and *CYP2D6**1/*4 and *CYP2D6**4/*4 poor metaboliser status (912), indicating that the *CYP2D6**4 polymorphism does influence the cholesterolaemic response to pravastatin and fluvastatin therapy.

In contrast, two studies have reported an increased incidence of statin intolerance in patients with variants in *CYP2D6*, including the *4 allele (146, 147). Mulder *et al.* (2001) (146) showed that among 88 simvastatin-treated patients, 34 (38.6%) had one or more mutated *CYP2D6* alleles. Simvastatin treatment was discontinued for any one of the following reasons: cholesterol levels remaining above 5.0 mmol/L, elevated liver function tests, drug-related gastro-intestinal complaints, or myopathy characterised by myalgia and persistent CK elevations more than 3 times ULN. The highest incidence of statin intolerance was found in the patients with homozygous mutant *CYP2D6* variants (80%), compared with heterozygous patients (46%) and the homozygous wildtype patients (17%) (146). Only four patients however, discontinued simvastatin therapy because of myopathy (146). Frudakis *et al.* (2007) reported a significant association between 75 patients with the *CYP2D6**4 polymorphism and atorvastatin-induced muscle effects, compared with 188 controls (147), providing the strongest evidence to date for the *CYP2D6**4 variant in predicting statin related myopathy.

Adverse events were defined as muscular effects that manifested with treatment initiation and resolved upon discontinuation of the statin. Fifty percent of patients with atorvastatin related muscle side effects carried the *CYP2D6**4 variant, compared to 28% of the atorvastatin tolerant controls (147). The odds ratio for atorvastatin-related myopathy was 2.5 ($P=0.001$) (147). This effect was independent of concurrent medications, and demographic, clinical or population-structure differences between cases and controls (147). In a further cohort of 169 patients, Frudakis *et al.* (2007) (147) observed a trend for an increased frequency of the *CYP2D6**4 allele among 61 patients with simvastatin-induced muscle events (50%) compared with controls (36%), (OR 1.7 ($P=0.067$)).

Several factors may reduce the importance of CYP variation in statin myopathy, including the fact that the pathway is probably most important in the presence of concomitant medications, which may inhibit statin metabolism and increase levels. Statins are also metabolised by UGT1A1 dependent glucuronidation, independently of the CYP enzyme system. In particular the *UGT1A1**28 variant may affect statin response (927). This variant occurs in 39% of Caucasians (928). Given the inconsistent results to date, further studies are required to confirm whether the *CYP2D6**4 polymorphism is an important determinant of statin-related musculoskeletal side effects.

6.4.5 Interaction of *COQ2* and *AMPD1* variants on statin intolerance

The cumulative frequency of *COQ2* SNP-2 and *AMPD1* Q12X variant alleles was associated with increased odds for statin myopathy compared with those patients without these variant alleles. This is the first study to report this interaction. These results indicate that genetic predisposition to statin related myopathy may reflect a combination of rare mutations and common genetic polymorphisms. In a recent study, Puccetti and colleagues showed that the association between the *COQ2* and *SLCO1B1**5 polymorphisms recurred significantly in patients presenting with both muscular symptoms and CK increase in atorvastatin-treated patients (923). These findings also suggest that a combination of genetic variants involved in muscle metabolism and/or statin pharmacokinetics may increase susceptibility to statin-related muscular side effects. Furthermore, individuals with genetic variation for metabolic muscle diseases may represent a subgroup of the statin-treated population for whom CoQ₁₀ supplementation may be more likely to confer a clinical benefit.

6.4.6 Study limitations

As with many association studies, this study is limited by the small sample size. In the Christchurch cohort, CK levels at baseline and on treatment and the use of other medications that may affect muscle function were not documented. As well, patients were not excluded if they were on concomitant lipid lowering therapy. Both patient cohorts had relatively mild myopathy compared to those enrolled in previous studies, which may explain the lack of an association between statin intolerance and some of the genetic variants investigated. A further limitation is the absence of a direct functional consequence of the *COQ2* variants studied; it is possible that the *COQ2* SNPs studied may not directly determine the association. Also, only some of the known mutations were screened for in the genes studied, and it may have been more informative to include all of the known mutations, particularly in the *AMPD1* gene, such as P48L, which has been associated with an increased risk of statin related myopathy and is relatively common.

6.4.7 Significance

As statin therapy is often limited by muscle related side effects, prompting recent FDA guidance on use of higher doses, approaches to prevent statin intolerance are needed. In addition to the many clinical factors that contribute to statin-induced myopathy (age, gender, renal and liver function, concomitant medications) there is increasing evidence that genetic risk factors play an important role. This data suggests that *COQ2* and *AMPD1* variants and their interaction may precipitate myalgia in patients with previously asymptomatic inherited myopathies. These results have important implications for clinical practice, since the majority of statin intolerant patients have mild muscle symptoms. Improved detection of relevant risk alleles may allow individualised lipid management, and increase compliance with lipid modifying therapy by reducing morbidity from statin based myopathic side effects (914).

6.4.8 Conclusions

The present study confirmed that genetic variation in *COQ2* is significantly associated with the increased risk of statin-induced myopathy in the pooled data from two independent cohorts of statin intolerant patients. Homozygotes for the rare alleles of *COQ2* SNP-1, SNP-2 and the non-1 haplotype had an increased odds ratio of statin myopathy. There was also a significant association between *AMPD1* haplotype 3 and statin intolerance in the Christchurch cohort, and a similar trend when both cohorts were pooled. A significant gene-gene

interaction between the *COQ2* SNP-2 and *AMPD1* Q12X was observed, with the cumulative frequency of affected alleles associated with increased odds for the risk of statin myopathy. *CYP2D6**4 and *SLCO1B1* polymorphisms were not associated with an increased risk of statin intolerance, suggesting that these genetic variants may be less relevant in predicting mild statin myopathy. The findings indicate that genomic variants of *COQ2* and *AMPD1* may have an important role in determining increased risk of statin musculoskeletal side effects and further studies are required.

7 Relationship between Plasma Coenzyme Q₁₀ levels and Arterial Stiffness in Patients with Phenotypic or Genotypic Familial Hypercholesterolemia on Long-term Statin Therapy

7.1 Background

Familial hypercholesterolemia (FH) is a monogenic co-dominant disorder, characterised by a defect in the synthesis or function of the low-density lipoprotein receptor (LDLR) and markedly increased LDL-cholesterol levels that predispose patients to severe premature cardiovascular disease (929, 930). The prevalence of heterozygous FH is at least 1 in 500 worldwide (929), with a higher frequency estimated in certain populations such as Afrikaners in South Africa and French Canadians due to founder effects (931, 932). Untreated FH confers a more than 50% cumulative risk of fatal or non-fatal coronary heart disease by age 50 years in men and at least 30% in women aged 60 years (933-935). Patients require aggressive LDL-cholesterol lowering, generally with high doses of statins as the first choice of pharmacotherapy, to improve their cardiovascular risk status (936). Statins inhibit synthesis of mevalonate, a precursor of CoQ₁₀, an endogenous lipophilic antioxidant in its reduced form that acts as a primary scavenger of free radicals, thereby protecting LDL against oxidative damage (335) (Chapter 1, section 1.3.3.3.1.2). It has been demonstrated that statin therapy lowers plasma CoQ₁₀ levels (186-203, 758, 858, 937), which may reflect fewer LDL-cholesterol carriers or a reduction in CoQ₁₀ synthesis (188, 194, 197, 858). The magnitude of CoQ₁₀ depletion is dose-dependent (188), and reversible on discontinuation of statin therapy (191).

There is evidence that heterozygous FH patients may be more susceptible to statin-induced reductions of CoQ₁₀, but the extent of statin-induced CoQ₁₀ depletion has not been well documented (197, 938). Human *et al.* (1997) (197) showed that plasma CoQ₁₀ levels were compromised in FH patients after short-term statin therapy, as indicated by a decrease in the ratio of CoQ₁₀ to total cholesterol, which was not observed in statin-treated LDLR mutation negative patients. In contrast, a smaller trial by Mabuchi *et al.* (2004) (939) found no

influence of statin therapy on plasma CoQ₁₀ concentrations in FH patients, but a reduction of CoQ₁₀ levels in the LDL-cholesterol fraction. More recently, Kawashiri *et al.* (2008) (858) reported statin-dependent effects on CoQ₁₀ levels, with atorvastatin lowering the ratio of CoQ₁₀ to LDL-cholesterol in FH patients, whereas no change was observed with pitavastatin. Furthermore, Lankin *et al.* (2003) (940) found that plasma LDL from patients with FH was more oxidisable in comparison to LDL from patients with other types of hypercholesterolaemia. A decrease in CoQ₁₀, particularly in the LDL fraction in FH patients, could affect its susceptibility to oxidative modification and might thus influence the atherosclerotic process (187).

Arterial stiffness is increasingly recognised as an independent risk factor for cardiovascular disease (941, 942). The vascular endothelium is a key regulator of the elastic properties of large vessels (943, 944). FH is strongly associated with endothelial dysfunction and reduced nitric oxide bioavailability (21, 36, 569, 945-947), a key contributor to increased vascular tone. Studies have demonstrated increased arterial stiffness by a variety of techniques in asymptomatic untreated patients with FH, (692, 948-952), although not consistently (953, 954). The augmentation index (AIx) represents the degree to which central systolic pressure is influenced by wave reflection within the arterial tree (707, 955-958), and thus provides a composite measure of wave reflection and the stiffness of the large conduit arteries (707, 955-958). AIx is measured either directly by carotid tonometry or estimated using a transfer function from radial artery tonometry, and has been shown to be an independent predictor of cardiovascular risk (959). In a recent study, local and systemic arterial stiffness was found to be increased in asymptomatic children with FH, as evidenced by an increased carotid AIx (692), suggesting that hypercholesterolaemia plays a key role in arterial mechanical impairment early in childhood (692). Statin therapy has been associated with improvements in arterial stiffness in FH patients (960).

7.1.1 Study hypotheses

The first hypothesis for this study was that statin-treated FH patients with identified LDL receptor mutations are more likely to have lower plasma CoQ₁₀ levels in comparison to gender matched LDL receptor mutation negative FH patients on equivalent statin doses. The second hypothesis was that lower CoQ₁₀ levels will be associated with a greater degree of arterial stiffness as assessed by pulse wave analysis. Accordingly, plasma CoQ₁₀, lipid profiles and the augmentation index were measured in a case-control study to determine

whether plasma CoQ₁₀ concentrations are lower in LDLR mutation positive FH patients than LDLR mutation negative patients on long-term statin therapy and whether plasma CoQ₁₀ concentrations are determinants of increased arterial stiffness. Since LDL-cholesterol levels of statin-treated FH subjects approximate those of the unaffected population, levels of CoQ₁₀ and arterial stiffness were also compared between treated FH and untreated healthy controls.

7.2 Methods

7.2.1 Subjects

Thirty heterozygous FH patients with identified LDLR mutations (LDLRmut+) and 30 LDLR mutation negative patients with clinical FH (LDLRmut-), matched for gender, and statin duration and dose were recruited from the Lipid Disorders and Cardiovascular Prevention Outpatient Clinic at Christchurch Hospital and private outpatient clinics in Christchurch, New Zealand. All patients were aged ≥ 18 years, and stabilised on statin therapy (≥ 20 mg/day atorvastatin or equivalent), with no history of unstable angina pectoris, acute myocardial infarction, cerebral vascular accident or intervention within the prior six months to enrolment. Patients underwent mutation screening of the LDLR and apolipoprotein B genes on the basis of clinically defined FH or pre-treatment total cholesterol levels ≥ 8 mmol/L (961), and were excluded with the presence of apolipoprotein B mutations. LDLR genetic mutations of FH patients enrolled in the LDLRmut+ group are shown in Table 7.1. There were 28 independent probands in the LDLRmut+ cohort. Apart from the negative LDLR mutation status, LDLRmut- patients had cholesterol levels and other clinical features consistent with the FH condition. A further 30 healthy controls, matched for gender, and with no existing vascular disease, type 2 diabetes mellitus or treatment with endothelial influencing medications were evaluated. FH and control subjects were excluded from the study if they had an LDL-cholesterol >4.5 mmol/L, triglycerides >2.5 mmol/L, significantly deranged liver function (alanine aminotransferase >3 x upper level of normal), significant renal impairment (creatinine >150 μ mol/L), congestive heart failure, atrial fibrillation or other significant co-morbidities, warfarin treatment, or supplementation with CoQ₁₀ or unknown vitamins and antioxidants. The study size (n=90) was based on the reported normal plasma CoQ₁₀ levels (328), with a mean of 0.94 μ mol/L and standard deviation of 0.32 μ mol/L. Assuming a 25% or more difference in CoQ₁₀ levels between controls and cases (i.e. a difference from 0.94 to

<0.70 $\mu\text{mol/L}$), it was calculated that 30 subjects were required in each group to provide the study with an 80% statistical power at a two tailed probability level <0.05. The study protocol was approved by the Upper South B Regional Ethics Committee, and written informed consent was obtained from all participants.

Table 7.1 LDL receptor mutations in LDLR mutation positive patients

Age (yrs)	Sex	LDLR Gene Mutation	Exon
57	F	G-2R	1
47	M	W23X	2
40	M	R60C	3
63	F	W66X	3
33	M	D69G	3
50	M	E80K	3
57	M	E80K	3
37	F	313+1G>A	intron 3
48	M	313+1G>A	intron 3
60	F	313+1G>A	intron 3
48	F	D147N	4a
56	F	D147N	4a
41	M	442-504del	4a
57	M	582delTAGCCCC	4b
33	M	657delC	4b
48	M	FsG198 delC (657 delC)	4b
51	M	D206E FH Afrikaner-1	4b
54	M	D206E FH Afrikaner-1	4b
62	M	E219K	5
51	M	G322S	7
53	M	T392M	9
54	F	1206-1209delCT	9
63	M	1206-1209delCT	9
65	M	R419G	9
54	F	G457R	10a
37	M	D452N	10
49	M	G544A	11
60	F	G544A	11
26	M	P664L	14
62	F	ex15-18del	15-18

7.2.2 Study design

This was an observational case-control study. Control subjects provided a fasting blood test for measurement of plasma lipids, renal and liver function to assess eligibility. Eligible control subjects and patients then attended a study visit to enable collection of data on age, ethnicity, past and current medical disorders, smoking history, daily alcohol consumption and physical activity. Height, weight and waist were measured and body mass index (BMI) calculated. The percentage body fat was determined by a bioimpedance method using a body fat analyser (Tanita Corp., Tokyo, Japan). Fasting blood samples were collected for the measurement of plasma levels of CoQ₁₀, lipoproteins, haematocrit, white blood cells, plasma glucose, electrolytes, renal and liver function, high sensitivity C-reactive protein (hs-CRP) and asymmetric dimethylarginine (ADMA). Systemic arterial stiffness was assessed by pulse wave analysis. Assessments were made after a 12-hour overnight fast. FH scores were calculated using the Dutch criteria (962).

7.2.3 Pulse wave analysis

Arterial stiffness was assessed by pulse wave analysis using applanation tonometry of the radial artery with the SphygmoCor version 7.1 software (AtCor Medical, Sydney, Australia) according to Wilkinson *et al.* (675), as described in detail in Chapter 2, section 2.5.5.1. Briefly, after 5 minutes lying in the recumbent position, blood pressure was measured in duplicate in the non-dominant arm using a validated oscillometric sphygmomanometer (HEM-705CP; Omron Corporation, Japan). A high-fidelity micromanometer (SPC-301; Millar Instruments, TX, USA) was used to obtain recordings of peripheral pressure waveforms by applying gentle pressure over the non-dominant radial artery. Data was collected directly into a laptop and recordings were assessed visually to ensure that the best possible recording was obtained and with minimisation of movement related artefacts. After 20 sequential waveforms were acquired, an averaged peripheral waveform was generated. A corresponding central waveform was then derived from a validated transfer function, and from this the AIx, ascending aortic pressure, and heart rate, were determined using the integral software. AIx was defined as the difference between the first and second systolic peaks of the central arterial waveform (augmented pressure (AP)), expressed as a percentage of the pulse pressure (PP), ($AIx = AP / PP \times 100\%$). Since the AIx is influenced by heart rate (688), an index normalised for a heart rate at 75 bpm (AIx@HR75) is reported. Blood pressure and pulse wave analysis measurements were repeated after a two

minute interval. All assessments were made by a single observer, in duplicate and mean values were calculated for analysis. In our vascular laboratory, we observed that between-day intra-class correlation coefficients for pulse wave analysis parameters are 0.92 for AIx@HR75, 0.91 for AIx, 0.90 for AP and AP@HR75, and 0.89 for T_r .

7.2.4 Biochemical parameters

Total plasma CoQ₁₀ was measured by reverse phase high performance liquid chromatography (HPLC) with electrochemical detection using a previously described method (408). Plasma total cholesterol, triglycerides, and HDL-cholesterol were determined by an enzymatic colorimetric method (Architect c8000 analyser, Abbott Laboratories, Abbott Park, Illinois, U.S.A.). LDL-cholesterol was calculated from the Friedewald equation. Pre-statin lipid levels of FH patients were obtained from clinic records. Lipoprotein (a), apolipoprotein A1, apolipoprotein B concentrations and hs-CRP levels were determined by rate nephelometry (Siemens BN II System). Plasma ADMA and L-arginine were measured using HPLC with fluorescence detection according to the method of Teerlink *et al.* (2002) (963). Plasma glucose, urea, creatinine and liver function (Architect c8000 analyser) and a full blood count (Coulter Electronics, Luton, UK) were also measured. Biochemical analyses were performed by Canterbury Health Laboratories, an ISO15189 accredited (human) pathology laboratory, with coefficients of variation between 3 – 5%.

7.2.5 Statistical analysis

All statistical analyses were performed using SPSS Base version 17.0 (SPSS, Inc., Chicago, Illinois). Data are expressed as mean (SEM). The differences in continuous biochemical and clinical parameters among the groups were tested using analysis of variance (ANOVA), analysis of covariance (ANCOVA), and Fisher's least significant difference test as appropriate. Categorical variables were compared between groups using Chi-square or Fisher's exact tests. Correlation analysis was performed using Pearson's correlation coefficient. Variables not normally distributed were log_e transformed prior to analysis. Statistical significance was inferred when $P < 0.05$.

7.3 Results

7.3.1 Clinical characteristics of statin-treated FH patients and controls

Table 7.2 summarises the clinical characteristics of the statin-treated FH patients and the healthy controls. The groups were matched for gender, and the FH cohorts were matched for statin duration and current statin dose, but were not identical for other medications. The LDLRmut+ cohort exhibited higher Dutch scores ($p<0.001$), even allowing for presence of a mutation ($p<0.001$), and also had a greater prevalence of an early family history of coronary heart disease compared to the LDLRmut- cohort ($p=0.02$). There was however, a lower prevalence of coronary heart disease and diagnosed hypertension in the LDLRmut+ group.

7.3.2 Haemodynamics of statin-treated FH patients and controls

Peripheral and central haemodynamic parameters are presented in Table 7.3. After correction for age, mean brachial systolic and diastolic blood pressure and mean arterial pressure were significantly higher in the LDLRmut- group in comparison to the healthy control group ($P<0.05$), but similar between FH cohorts. Correspondingly, mean central arterial parameters corrected for age, including aortic systolic and diastolic blood pressure were also significantly higher in the LDLRmut- group compared to the healthy control group ($P<0.05$). Heart rate was comparable between the groups. After correction for age and blood pressure, the augmented pressure standardised to a heart rate of 75 bpm ($AP@HR75^{adj}$) was significantly higher in the LDLRmut- compared to healthy controls ($P=0.03$), and there was also a trend for increased $AP@HR75^{adj}$ in the LDLRmut+ group compared to controls ($P=0.09$). The AIX, corrected for age and blood pressure and standardised to a heart rate of 75 bpm ($AIX@HR75^{adj}$) was significantly elevated in the LDLRmut- patients in comparison to the controls ($P=0.04$), and there was a trend for increased $AIX@HR75^{adj}$ in the LDLRmut+ patients compared to controls ($P=0.09$).

7.3.3 Effect of long-term statin treatment on lipid levels of FH patients

Total cholesterol, LDL-cholesterol and the total cholesterol to HDL-cholesterol ratio were significantly higher in the FH cohorts prior to initiation of statin therapy compared to

levels in untreated controls ($P<0.001$). Baseline LDL-cholesterol levels were higher in the LDLRmut+ cohort compared to the LDLRmut- cohort ($P=0.004$), whereas baseline triglycerides were significantly higher in the LDLRmut- cohort versus the LDLRmut+ cohort ($P=0.02$) (Table 7.4). HDL-cholesterol concentrations were similar between FH and control groups. Long-term statin treatment was associated with significant reductions in concentrations of total cholesterol, LDL-cholesterol, and the total cholesterol to HDL-cholesterol ratio in both FH groups ($P<0.001$) (Table 7.4). Triglycerides were also significantly reduced in LDLRmut- patients ($P<0.001$) and reduced in LDLRmut+ patients after statin therapy ($P<0.05$). HDL-cholesterol levels were unchanged in the LDLRmut- group, and there was a small, non-significant reduction in the LDLRmut+ group ($P<0.05$). The changes in lipid profiles associated with long term statin therapy were not significantly different between the FH patient groups (Table 7.4).

Table 7.2 Clinical characteristics of statin-treated FH patients and controls

Parameter	LDLRmut+ (n=30)	LDLRmut- (n=30)	Controls (n=30)	<i>P</i> -value† (LDLRmut+ vs LDLRmut-)	<i>P</i> -value† (LDLRmut+ vs controls)	<i>P</i> -value† (LDLRmut+ vs controls)
Age, years	51 ± 2	54 ± 2	49 ± 2	0.12	0.63	<0.05
Male, n (%)	20 (67%)	20 (67%)	20 (67%)	1.00	1.00	1.00
BMI, kg/m ²	27.5 ± 0.7	28.1 ± 0.8	24.6 ± 0.6	0.54	0.005	0.001
Waist circumference, cm	93.8 ± 2.1	95.0 ± 2.2	86.6 ± 2.0	0.68	0.02	0.006
Previous smoker, n (%)	9 (30%)	12 (40%)	3 (10%)	0.42	0.07	0.01
Alcohol intake, units per week	6 ± 1	7 ± 1	5 ± 1	0.25	0.73	0.14
Diabetes, n (%)	1 (3%)	1 (3%)	-	1.00	-	-
Diagnosed hypertension, n (%)	7 (23%)	12 (40%)	-	0.17	-	-
Diagnosed CHD, n (%)	9 (30%)	15 (50%)	-	0.11	-	-
Family history of premature CHD, n (%)	27 (90%)	18 (60%)	-	0.02	-	-
Dutch score	20 ± 1	8 ± 1	-	<0.001	-	-
Dutch score minus LDLR gene mutation	12 ± 1	8 ± 1	-	<0.001	-	-
Equivalent atorvastatin dose, mg/day	47.3 ± 4.2	47.7 ± 4.3	-	0.96	-	-
Years on statin treatment	9.6 ± 1.1	7.8 ± 1.0	-	0.24	-	-
Years on current statin dose	3.8 ± 0.5	3.9 ± 0.7	-	0.89	-	-
Ezetimibe, n (%)	21 (70.0%)	14 (46.7%)	-	0.07	-	-
Fibrate, n (%)	2 (6.7%)	2 (6.7%)	-	1.00	-	-
Nicotinic acid, n (%)	3 (10.0%)	2 (6.7%)	-	1.00	-	-
Questran, n (%)	1 (3.3%)	0 (0%)	-	1.00	-	-
Aspirin, n (%)	11 (36.7%)	19 (63.3%)	-	0.04	-	-

Table 7.2 Clinical characteristics of statin-treated FH patients and controls (continued)

Parameter	LDLRmut+ (n=30)	LDLRmut- (n=30)	Controls (n=30)	<i>P</i> -value† (LDLRmut+ vs LDLRmut-)	<i>P</i> -value† (LDLRmut+ vs controls)	<i>P</i> -value† (LDLRmut+ vs controls)
Antihypertensive therapy, n (%)	10 (33.3%)	17 (56.7%)	-	0.07	-	-
ACE inhibitor, n (%)	5 (16.7%)	7 (23.3%)	-	0.52	-	-
Beta blocker, n (%)	5 (16.7%)	12 (40.0%)	-	0.05	-	-

Values are mean \pm SEM or number (percentage).

†One-way ANOVA, chi-square or Fisher's exact test for comparison of between-group differences.

CHD, coronary heart disease.

Table 7.3 Peripheral and central haemodynamics of statin-treated FH patients and controls

Parameter†	LDLRmut+ (n=30)	LDLRmut- (n=30)	Controls (n=30)	<i>P</i> -value‡ (LDLRmut+ vs LDLRmut-)	<i>P</i> -value‡ (LDLRmut+ vs controls)	<i>P</i> -value‡ (LDLRmut- vs controls)
Peripheral						
Brachial SBP, mmHg	128 ± 3	134 ± 3	124 ± 3	0.24	0.27	0.02
Brachial DBP, mmHg	77 ± 2	81 ± 2	75 ± 2	0.10	0.59	0.02
Brachial MAP, mmHg	95 ± 2	101 ± 2	93 ± 2	0.09	0.47	0.007
Heart rate, bpm	60 ± 2	63 ± 2	59 ± 2	0.15	0.65	0.10
Central						
Aortic SBP, mmHg	118 ± 3	125 ± 3	114 ± 3	0.17	0.21	0.006
Aortic DBP, mmHg	78 ± 2	83 ± 2	76 ± 2	0.09	0.61	0.01
AP@HR75 ^{adj} , mmHg	8 ± 1	9 ± 1	6 ± 1	0.35	0.09	0.03
AIx@HR75 ^{adj} , %	19.5 ± 1.6	21.2 ± 1.6	16.1 ± 1.6	0.34	0.09	0.04

Values are mean ± SEM.

† All parameters adjusted for age.

‡ One-way ANOVA for comparison of between-group differences.

SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; AP@HR75^{adj}, augmented pressure standardised to a heart rate of 75bpm and corrected for age and brachial SBP and DBP; AIxHR75^{adj}, augmentation index standardised to a heart rate of 75bpm and corrected for age and brachial SBP and DBP.

Table 7.4 Pre-treatment lipid levels and changes following long-term statin treatment in FH patients

Parameter	Baseline LDLRmut+ (n=30)	Baseline LDLRmut- (n=30)	<i>P</i> -value†	ΔLDLRmut+	ΔLDLRmut-	<i>P</i> -value‡
Total cholesterol, mmol/L	9.9 ± 0.3	9.1 ± 0.3	0.04	-4.8 ± 0.3**	-4.2 ± 0.2**	0.17
LDL cholesterol, mmol/L	7.8 ± 0.3	6.8 ± 0.2	0.004	-4.3 ± 0.3**	-3.8 ± 0.2**	0.19
Triglycerides, mmol/L	1.7 ± 0.2	2.5 ± 0.3	0.02	-0.6 ± 0.2*	-1.0 ± 0.2**	0.18
HDL cholesterol, mmol/L	1.30 ± 0.08	1.40 ± 0.08	0.36	-0.12 ± 0.05*	-0.06 ± 0.06	0.51
Total cholesterol/HDL-cholesterol ratio	8.4 ± 0.5	7.1 ± 0.4	0.03	-3.9 ± 0.5**	-3.3 ± 0.4**	0.30

Values are mean ± SEM.

†One-way ANOVA for comparison of between-group differences at baseline.

‡Independent t-test for comparison of between-group changes.

P*<0.05, *P*<0.001 for comparison of within-group changes from baseline using paired t-tests.

7.3.4 Biochemical parameters of study populations

Biochemical parameters of the study groups are summarised in Table 7.5. Plasma CoQ₁₀ concentrations (Figure 7.1) and CoQ₁₀ adjusted for total cholesterol and LDL-cholesterol (Figure 7.2) were similar between statin-treated FH patient cohorts and untreated healthy controls. Lipid levels were also comparable between statin-treated FH cohorts and untreated controls, except that the statin-treated LDLRmut+ group had higher mean LDL-cholesterol levels compared to the LDLRmut- group ($P=0.005$), and a higher total cholesterol to HDL-cholesterol ratio than either the LDLRmut- group ($P=0.02$) or control group ($P=0.03$). HDL-cholesterol levels were significantly higher in the controls in comparison to the treated LDLRmut+ group ($P=0.007$). Apolipoprotein B and the apolipoprotein B ratio to apolipoprotein A-I were elevated in both treated FH patient groups compared to healthy controls, but only significantly in the LDLRmut+ group ($P<0.05$). Lipoprotein (a) levels were higher in LDLRmut- patients than LDLRmut+ patients ($P=0.03$) and controls ($P=0.008$), and higher in the LDLRmut+ patients than controls ($P<0.001$). Plasma ADMA levels were significantly increased in the LDLRmut+ group compared to the LDLRmut- group ($P=0.003$) and untreated control group ($P=0.006$), whereas the L-arginine to ADMA ratio did not differ between the three groups. Plasma glucose concentrations and serum levels of hs-CRP were similar between the three groups. Furthermore, no clinically significant derangements were observed in plasma markers of renal and liver function or haematological indices between the groups (data not shown). However, the estimated glomerular filtration rate was lower in the LDLRmut- cohort compared to controls (86 ± 3 mL/min/1.73 m² vs 78 ± 2 mL/min/1.73 m², $p=0.02$), and GGT levels were 1.8 – 2.4 fold higher in the LDLRmut- group compared to the LDLRmut+ and control groups ($P<0.01$). There were no other statistically significant differences in liver function tests. Neutrophils were higher in the LDLRmut- cohort in comparison to the control cohort (3.6 μ L vs 3.0 μ L, $P<0.05$).

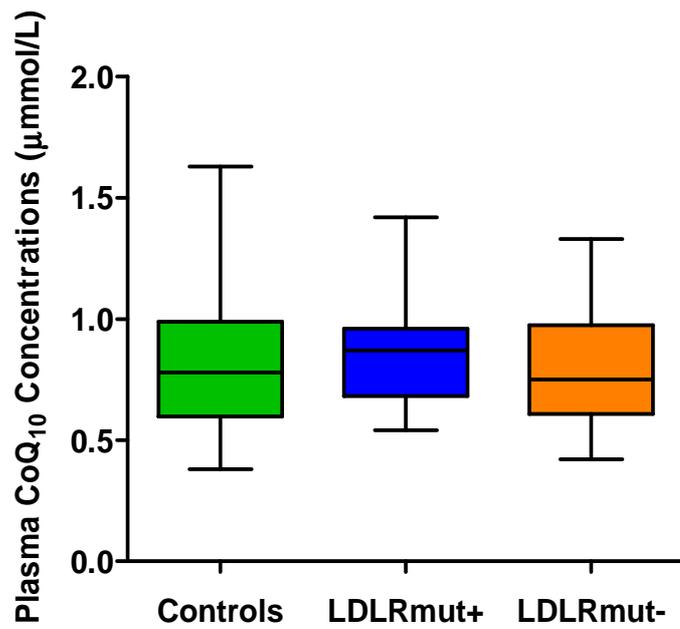


Figure 7.1 Box and whiskers plots for the plasma CoQ₁₀ concentrations for controls and statin-treated FH patients with (LDLRmut+) and without identified LDLR mutations (LDLRmut-)

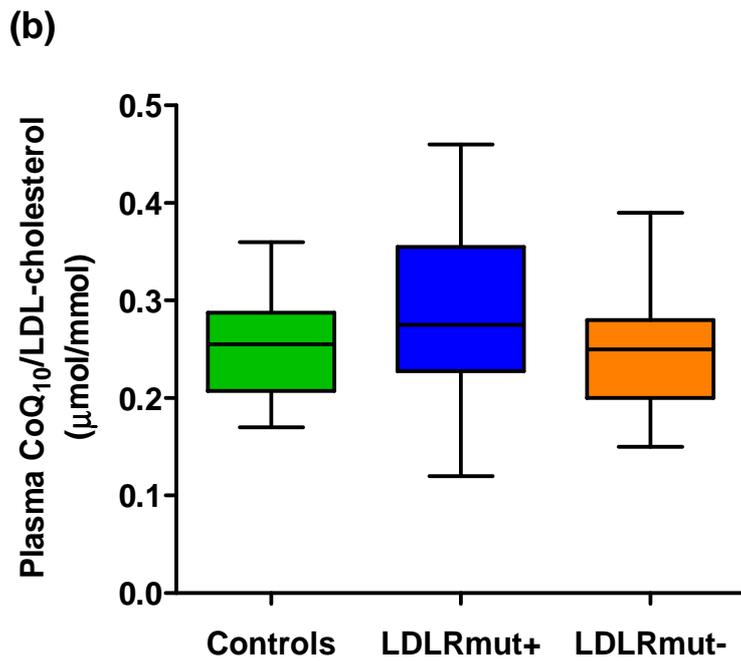
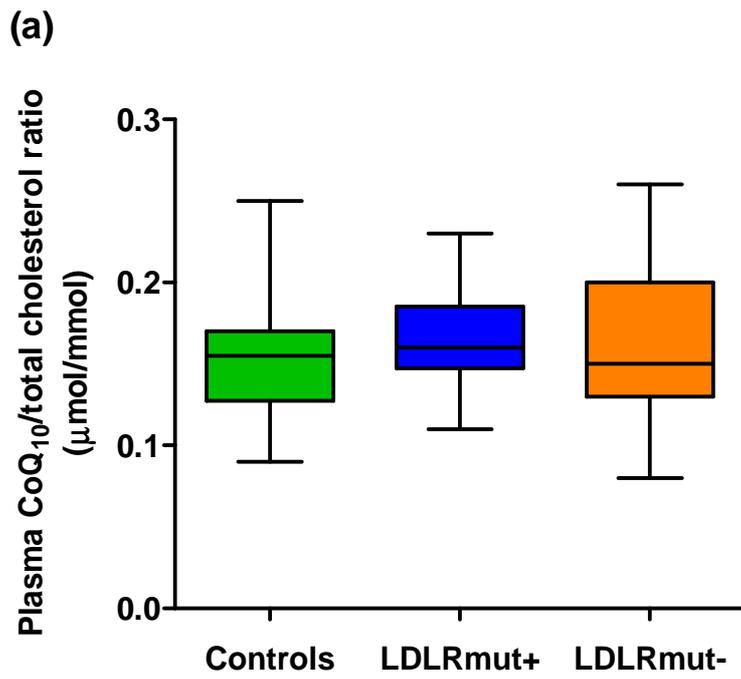


Figure 7.2 Box and whiskers plots for the (a) CoQ₁₀/total cholesterol ratio, and (b) CoQ₁₀/LDL-cholesterol ratio for controls and statin-treated FH patients with (LDLRmut+) and those without identified LDLR mutations (LDLRmut-)

Table 7.5 Biochemical parameters of statin-treated FH patients and controls

Parameter	LDLRmut+ (n=30)	LDLRmut- (n=30)	Controls (n=30)	<i>P</i> -value‡ (LDLRmut+ vs LDLRmut-)	<i>P</i> -value‡ (LDLRmut+ vs controls)	<i>P</i> -value‡ (LDLRmut- vs controls)
CoQ ₁₀ , μmol/L	0.86 ± 0.04	0.81 ± 0.05	0.81 ± 0.05	0.51	0.52	0.98
CoQ ₁₀ /total cholesterol, μmol/mmol	0.17 ± 0.01	0.17 ± 0.01	0.16 ± 0.01	0.77	0.23	0.36
CoQ ₁₀ /LDL cholesterol, μmol/mmol	0.25 ± 0.01	0.28 ± 0.02	0.25 ± 0.01	0.07	0.93	0.06
Total cholesterol, mmol/L	5.1 ± 0.1	4.9 ± 0.1	5.2 ± 0.2	0.38	0.69	0.21
LDL cholesterol, mmol/L	3.4 ± 0.1	2.9 ± 0.1	3.3 ± 0.1	0.005	0.32	0.07
Triglycerides, mmol/L	1.1 ± 0.9	1.4 ± 0.1	1.2 ± 0.1	0.08	0.92	0.09
HDL cholesterol, mmol/L	1.18 ± 0.05	1.34 ± 0.55	1.42 ± 1.07	0.06	0.007	0.37
Total cholesterol/HDL cholesterol ratio	4.5 ± 0.2	3.8 ± 0.2	3.9 ± 0.2	0.02	0.03	0.89
ApoA-1, g/L	1.35 ± 0.05	1.43 ± 0.04	1.47 ± 0.05	0.31	0.10	0.52
ApoB, g/L	0.99 ± 0.03	0.96 ± 0.05	0.85 ± 0.42	0.56	0.02	0.07
ApoB/ApoA-1 ratio	0.77 ± 0.04	0.70 ± 0.04	0.60 ± 0.03	0.24	0.004	0.08
Lipoprotein (a), mg/L†	205 (131, 322)	438 (243, 789)	80 (51, 125)	0.03	0.008	<0.001
Glucose, mmol/L	4.7 ± 0.1	5.0 ± 0.1	4.8 ± 0.08	0.11	0.92	0.14
Hs-CRP, mmol/L	1.32 ± 0.30	1.38 ± 0.25	1.24 ± 0.26	0.87	0.84	0.72
ADMA, μmol/L	0.66 ± 0.04	0.53 ± 0.02	0.54 ± 0.02	0.003	0.006	0.82
L-arginine/ADMA ratio	126.4 ± 7.8	122.8 ± 6.9	134.3 ± 8.1	0.74	0.47	0.29

Values are mean ± SEM or †log transformed mean (95% CI).

‡One-way ANOVA for comparison of between-group differences.

CoQ₁₀, coenzyme Q₁₀; ApoA-1, apolipoprotein A-1; ApoB, apolipoprotein B; hs-CRP, high sensitivity C-reactive protein; ADMA, asymmetric arginine.

7.3.5 Correlation analyses

Plasma CoQ₁₀ levels were significantly correlated with total cholesterol ($r=0.58$, $P<0.001$), LDL-cholesterol ($r=0.53$, $P<0.001$), triglycerides ($r=0.42$, $P=0.004$), and apolipoprotein B ($r=0.58$, $P<0.001$) in FH patients and controls. Plasma CoQ₁₀ concentrations were not associated with age, weight, BMI or waist measurements. The CoQ₁₀ to total cholesterol ratio was positively correlated with weight ($r=0.29$, $P=0.006$) and waist circumference ($r=0.29$, $P=0.005$), as was the CoQ₁₀ to LDL-cholesterol ratio ($r=0.26$, $P=0.01$ and $r=0.29$, $P=0.006$, respectively). AIx@HR75 unadjusted was significantly correlated with age ($r=0.63$, $P<0.001$), systolic blood pressure ($r=0.34$, $P=0.001$) and diastolic blood pressure ($r=0.33$, $P=0.002$). There was significant negative association between AIx@HR75^{adj} and plasma CoQ₁₀ levels in FH patients ($r=-0.33$, $P=0.01$) (Table 7.6). AIx@HR75^{adj} was also negatively correlated with the CoQ₁₀ to total cholesterol ratio ($r=-0.42$, $P=0.001$) and CoQ₁₀ to LDL-cholesterol ratio ($r=-0.31$, $P=0.02$) within FH cohorts (Table 7.6). There was no association between AIx@HR75^{adj} and CoQ₁₀ in controls (Table 7.6). AIx@HR75^{adj} was not associated with the following lipid parameters: total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, apolipoprotein A-1 or B ($P>0.05$). A positive association was observed between the AIx@HR75^{adj} and ADMA in the LDLRmut- group ($r=0.42$, $P=0.02$), but not within the LDLRmut+ or control groups. Plasma ADMA levels were also significantly correlated with hs-CRP levels in both cases and controls ($r=0.26$, $P=0.01$). There was a significant relationship between the L-arginine/ADMA ratio and HDL-cholesterol in statin-treated LDLRmut+ patients ($r=0.43$, $P=0.02$), but this was not observed in either LDLRmut- patients or controls.

Table 7.6 Correlation coefficients for CoQ₁₀ versus AIx@HR75^{adj}

	AIx@HR75 ^{adj} (%)	
	FH cohorts† (n=60)	Controls (n=30)
CoQ ₁₀ , μmol/L	<i>r</i> =-0.33, <i>P</i> =0.01	<i>r</i> =-0.05, <i>P</i> =0.83
CoQ ₁₀ /total cholesterol ratio, μmol/mmol	<i>r</i> =-0.42, <i>P</i> =0.001	<i>r</i> =-0.06, <i>P</i> =0.78
CoQ ₁₀ /LDL-cholesterol ratio, μmol/mmol	<i>r</i> =-0.31, <i>P</i> =0.02	<i>r</i> =-0.03, <i>P</i> =0.87

†LDLRmut+ and LDLRmut- groups.

AIxHR75^{adj}, augmentation index standardised to a heart rate of 75bpm and corrected for age and brachial systolic and diastolic blood pressure.

7.4 Discussion

7.4.1 Plasma CoQ₁₀ levels in FH patients on long-term statin therapy

The present study demonstrated that plasma CoQ₁₀ levels in FH patients with identified LDLR mutations are similar to both LDLRmut- patients on equivalent doses of long-term statin therapy and untreated controls. These findings indicate that plasma CoQ₁₀ levels are not compromised by high-dose long term statin treatment in phenotypic or genotypic FH patients, which do not support the conclusions from two prior short-term studies (197, 858). The ratio of CoQ₁₀ to LDL-cholesterol was also comparable between the statin-treated FH groups and the controls, implying that the antioxidant capacity of LDL-cholesterol is not affected in FH patients following chronic statin dosing. This study supports the findings of Elmberger *et al.* (1991) (187), where a reduction in plasma CoQ₁₀ levels was observed in proportion to the reduction in LDL-cholesterol levels after short-term pravastatin treatment. Furthermore, there was no change in the ratio of CoQ₁₀ to LDL-cholesterol following statin therapy. In another trial of heterozygous FH patients, plasma CoQ₁₀ concentrations remained unchanged after 24 weeks of statin therapy, although there was a 50% reduction in the LDL compartment of CoQ₁₀ (939). The sample size of their study was small, with only seven patients and may therefore not represent the FH patient response to statins. However, a larger trial that examined the long-term effects of pravastatin therapy on plasma CoQ₁₀ levels and cardiovascular outcomes in a nested case-control design of patients with existing cardiovascular disease from the LIPID study concurs with the current findings (964). Investigators observed no alteration in the cholesterylester-adjusted CoQ₁₀ concentrations in pravastatin-treated patients compared to the placebo group, despite a significant reduction in plasma CoQ₁₀ levels (964).

In contrast to the present findings, Human *et al.* (1997) (197), investigated the effect of 14 weeks of simvastatin (10 mg/day or 20 mg/day) on plasma CoQ₁₀ concentrations in heterozygous FH and non-FH patients and observed a significant decline in CoQ₁₀ in both groups. FH patients experienced a significant reduction in the CoQ₁₀ to total cholesterol ratio, whereas there was no change in the non-FH group (197), however their study was limited by the short-term duration of statin treatment (197). More recently, Kawashiri *et al.* (2008) (858), reported statin-dependent effects on CoQ₁₀ levels, with atorvastatin lowering both CoQ₁₀

levels and the ratio of CoQ₁₀ to LDL-cholesterol in heterozygous FH patients, whereas there were no changes with pitavastatin, despite comparable reductions in lipid levels after eight weeks treatment. The investigators suggest that pitavastatin may inhibit HMG-CoA reductase to a lesser extent than atorvastatin (858), implying that the depletion of plasma CoQ₁₀ may not be a direct result of LDL-cholesterol reductions. Certainly other cholesterol lowering agents such as the selective cholesterol absorption inhibitor, ezetimibe, do not significantly alter CoQ₁₀ levels despite significant LDL-cholesterol reductions (201). It is also possible that the relatively short treatment period of eight weeks may not reflect the long-term effects of statin therapy on CoQ₁₀.

7.4.2 Plasma lipoproteins in FH patients on long-term statin therapy

Cholesterol-lowering therapy can prevent or delay the onset of cardiovascular disease in patients with FH (965, 966). More aggressive statin treatment has been shown to further decrease cardiovascular risk (967, 968), resulting in the development of stringent treatment targets for patients with FH (969, 970). In the current study of FH patients, chronic high dose statin therapy in combination with other cholesterol modifying agents was associated with significant reductions in LDL-cholesterol levels after a mean of 8 years therapy; comparable in effect to that reported in other studies with FH subjects (947, 968, 971, 972). However, despite aggressive statin treatment, only 7% of LDLRmut- patients, and no LDLRmut+ patients achieved LDL-cholesterol levels below 2.0mmol/L, as per NZ guidelines (970). This is consistent with other studies (973, 974).

7.4.3 Arterial stiffness in FH patients on long-term statin therapy

Despite long term high dose statin therapy, FH patients' exhibit increased systemic arterial stiffness compared to untreated controls with similar lipid levels. This may reflect long-term exposure to high levels of LDL-cholesterol, although there was no correlation with AIx@HR75^{adj}, as has been shown previously in hypercholesterolaemic patients (975). These findings concur with prior studies, where increased arterial stiffness has been demonstrated in asymptomatic untreated patients with FH (692, 948-952), although reductions have been observed following statin treatment (960). Smilde *et al.* (960) found that 12 months of treatment with simvastatin or atorvastatin 40-80 mg/day resulted in a significant decrease in carotid and femoral arterial wall stiffness in 45 patients with FH. The investigators deduced

that an LDL-cholesterol reduction of 44.8% was necessary to induce significant decreases in arterial distensibility and compliance (960). The majority of studies investigating patients with polygenic hypercholesterolemia have also demonstrated improvements in arterial elasticity with statin therapy (976-979). These beneficial effects exerted by statins may result from a fall in plasma cholesterol, an alteration in the lipid profile, or a pleiotropic effect of statins (955).

The inclusion of patients with stable coronary artery disease in the current study may account for the higher $\text{AIx@HR75}^{\text{adj}}$ values seen in statin-treated FH patients versus controls, since atherosclerosis per se is linked to increased aortic and carotid stiffness and arterial remodelling (955, 980, 981). This may also explain the higher $\text{AIx@HR75}^{\text{adj}}$ observed in statin-treated LDLRmut- patients, as there was a trend for a higher prevalence of coronary heart disease and diagnosed hypertension in these patients. Waist circumference and lipoprotein (a) concentrations were also higher in the two FH cohorts compared to controls, which may have contributed to the increased $\text{AIx@HR75}^{\text{adj}}$ found in these patients, given that both visceral adiposity and lipoprotein (a) levels are independent risk factors for arterial stiffness (982, 983). The percentage of ex-smokers was higher in the FH cohorts compared to controls and may have contributed to the higher $\text{AIx@HR75}^{\text{adj}}$ in these patient groups, since both smoking and duration of smoking cessation has been related to an increase in arterial stiffness (984). Hs-CRP concentrations in treated FH patients were equivalent to untreated controls, which may reflect a reduction in plasma hs-CRP concentrations with statin therapy, as reported in a number of statin trials, largely independently of LDL-cholesterol levels (63, 985, 986). Although elevated hs-CRP concentrations have also been previously associated with reduced arterial elasticity (987, 988), there was no relationship with $\text{AIx@HR75}^{\text{adj}}$.

Inhibition of endogenous nitric oxide production has been shown to lead to increased arterial stiffness (989). Plasma concentrations of the endogenous nitric oxide synthase inhibitor, ADMA, were raised in the LDLRmut+ cohort, and there was a significant association between $\text{AIx@HR75}^{\text{adj}}$ and ADMA in the LDLRmut- group. Previous studies have reported a negative relationship between the AIx and flow-mediated dilation of the brachial artery (FMD), a non-invasive measure of endothelial function that predicts and precedes overt cardiovascular disease (676, 990). FMD has also been shown to be an independent predictor of AIx, suggesting that the AIx may reflect endothelial function as well as arterial stiffness (990). AIx has also been associated with carotid intima media thickness, demonstrating a significant relationship between arterial stiffness and overt cardiovascular

disease (991). We also observed a significant relationship between ADMA and hs-CRP levels, as previously documented in patients with type 2 diabetes (992, 993) and renal disease (994), suggesting a link between elevated circulating ADMA concentrations and inflammation.

7.4.4 Relationship between plasma CoQ₁₀ and arterial stiffness

To my knowledge, this is the first study to report a negative association between plasma CoQ₁₀ concentrations and systemic arterial stiffness in FH patients. CoQ₁₀ has a high index of individuality, where individuals have values that are tightly distributed around different homeostatic means (442). Thus plasma CoQ₁₀ levels may fall significantly but still remain within the normal range. Pre-statin CoQ₁₀ may have been higher in the FH cohorts but this could not be demonstrated. However, given the negative association, it could be speculated that higher CoQ₁₀ concentrations may be protective, and that CoQ₁₀ supplementation is warranted in these patients. An intervention trial would be required to test this hypothesis. Studies have revealed that CoQ₁₀ administration can restore plasma levels in patients receiving statin therapy (477, 560, 995), and increase the CoQ₁₀H₂ content of LDL, and lead to a decrease in their peroxidisability (359). CoQ₁₀ improves endothelial function in patients with coronary artery disease (368), advanced heart failure (522) and type 2 diabetes (369), including combined therapy with statins (371) and fenofibrate (487), via decreases in oxidative stress within the arterial wall and reductions in vascular tone (363).

7.4.5 Study limitations

The absence of baseline assessment prior to initiation of statin therapy precludes any interpretation of the effects of long-term statin therapy on changes in arterial stiffness or CoQ₁₀ levels in FH patients. Although 53% of the LDLRmut- patients had clinically definite or probable FH based on Dutch scores, some may have had other metabolic conditions such as familial combined hyperlipidaemia. Mutations in other genes, including the proprotein convertase subtilisin kexin 9 (*PCSK9*) gene that encodes PCSK9, an important regulator of LDLR (996), were not screened in all FH patients. Since gain-of-function mutations in the *PCSK9* gene causes autosomal dominant hypercholesterolaemia (997), the possibility that these mutations may have been contributing to elevated cholesterol levels cannot be excluded. Treatment regimes were managed independently of the present trial and thus, although FH patients were matched for current statin dose, the use of other therapies was not identical

(Table 7.2). It is possible that the higher $\text{AIx@HR75}^{\text{adj}}$ values observed in the FH groups may have reflected the inclusion of a greater number of patients with clinical coronary artery disease, since atherosclerosis *per se* is linked to increased arterial stiffness, and may explain the higher values in the LDLRmut- group, although corrections were made for systolic and diastolic blood pressure in addition to age. The observational design of the study limited the ability to establish any causal relationship between plasma CoQ₁₀ levels and the $\text{AIx@HR75}^{\text{adj}}$. Furthermore, it may have been more informative to examine the relationship between plasma CoQ₁₀ levels and other clinical measures of vascular function, in addition to the AIx, such as pulse wave velocity and flow-mediated dilation of the brachial artery, where previous studies have reported impaired vascular function in FH patients (692, 946, 998).

7.4.6 Significance

Some evidence suggests that FH patients may be more susceptible to CoQ₁₀ reductions following short-term statin treatment compared to treated patients without identified mutations, which may indicate a role for CoQ₁₀ supplementation. CoQ₁₀ concentrations are not compromised in LDLRmut+ patients following long-term statin therapy, since CoQ₁₀ levels and the ratio of CoQ₁₀ to LDL-cholesterol were equivalent to LDLR- patients and to untreated controls. This study confirmed previous reports that the majority of FH patients are not meeting defined lipid targets, despite combination therapy with high statin doses and other lipid lowering agents (970). In addition, this study revealed an increased systemic arterial stiffness in statin-treated FH patients compared to healthy controls, despite similar lipid levels, and an association between low plasma CoQ₁₀ levels and increased arterial stiffness in FH patients. Taken together, these findings highlight the importance of additional therapeutic strategies to further reduce the risk of premature cardiovascular disease in this high risk patient population.

7.4.7 Conclusions

This study demonstrated that chronic, high-dose, long-term statin treatment in FH patients with and without identified LDLR mutations did not lead to subnormal plasma CoQ₁₀ concentrations, although systemic arterial stiffness was higher in statin-treated FH patients compared to controls despite similar lipid profiles. ADMA levels were higher in LDLR mutation positive patients. Plasma CoQ₁₀ concentrations were negatively associated with arterial stiffness within FH patients, but not controls. Prospective trials would be required to

show whether CoQ₁₀ supplementation can ameliorate arterial stiffness in statin-treated subjects with FH.

8 Effect of Atorvastatin on Plasma Asymmetric Dimethylarginine Levels in Patients with Non-Ischaemic Systolic Heart Failure

8.1 Background

Abnormal endothelium-dependent regulation of the peripheral circulation is a key feature in chronic heart failure (CHF) (25, 26, 999-1002), and contributes to increased peripheral vascular resistance and reduced cardiac output (1003). The endothelial L-arginine-nitric oxide (NO) pathway has been shown to be defective in patients with heart failure (25, 26, 999-1002), with the degree of endothelial dysfunction being related to the severity of disease (1004). Furthermore, endothelial dysfunction is an independent adverse prognostic factor for cardiac death and hospitalisation in patients with CHF (93, 94).

Endothelial dysfunction itself results from heart failure, irrespective of the presence of ischaemic heart disease (999), and has been recognised as a potential treatment target (1004). Improvement in tissue perfusion is an important goal in patients with heart failure, in terms of both the peripheral and coronary circulation (1004). Although not indicated for CHF, statin therapy may result in clinical benefits through its endothelium enhancing properties and other pleiotropic effects that include upregulation of endothelial NO synthase (eNOS) expression and function (93, 94, 96), alleviation of oxidative stress (99-101), and reduced inflammation (102, 103). Retrospective analyses of the Scandinavian Simvastatin Survival Study (4S study) demonstrated that simvastatin decreased the risk for progression to heart failure in patients following myocardial infarction (1005). Statin treatment has been associated with improved survival of patients with heart failure in a number of cohort studies (1006-1008), and several small prospective trials have shown that statins ameliorate endothelium-dependent function in non-ischaemic heart failure, through lipid-independent pathways (758, 1009).

Systemic accumulation of asymmetric dimethylarginine (ADMA), an endogenous eNOS inhibitor has been implicated in the pathogenesis of heart failure (1006). ADMA is derived from the proteolysis of cellular proteins containing arginine residues (1010), and is

metabolised via the activity of dimethylarginine dimethylaminohydrolase (DDAH) to citrulline and dimethylamine. In healthy volunteers, intravenous infusion of low-dose ADMA increases systemic blood pressure and impairs cardiac output (1011). Indeed, in patients with CHF, circulating levels of ADMA were found to be elevated two to three fold compared with healthy controls (1012-1014), and plasma concentrations were positively correlated with disease severity (1012). Elevated levels of ADMA have been associated with reduced NO synthesis as assessed by impaired endothelium-dependent vasodilation in patients with CHF (1015), suggesting that ADMA may represent a novel marker of endothelial dysfunction in CHF. Decreased DDAH activity or expression resulting from increased oxidative stress and inflammation is believed to be the mechanism responsible for the increase in ADMA concentrations and subsequent ADMA mediated impairment of eNOS (13, 346, 1016). The L-arginine to ADMA ratio has also been proposed as a potential marker of endothelial function, with reductions seen in a number of cardiovascular disease states, including hypertrophic cardiomyopathy (1017). Thus ADMA may represent a therapeutic target for amelioration of vascular dysfunction in CHF.

8.1.1 Study hypothesis

The present chapter is an extension of a study that was originally designed to investigate the effects of short-term atorvastatin treatment on endothelial and cardiac function, and neurohormonal activation in non-ischaemic heart failure patients (1018). In that study, an improvement in endothelium-dependent vasodilation and neurohormonal status was observed. The study was undertaken jointly with Strey *et al.* (2006) (1018), and is not included in this thesis, apart from the endothelial function and lipid data which are reiterated in this chapter. Given the potential non-lipid mediated benefits of statins in CHF, it was hypothesised that restoration of endothelial function in patients with CHF may be mediated in part, through reductions in ADMA. Accordingly, the effect of six weeks atorvastatin treatment on plasma ADMA levels was examined in patients with stable, non-ischaemic, left ventricular dysfunction (562).

8.2 Methods

8.2.1 Subjects

Twenty four patients were recruited from the Cardiology Department outpatient clinic at Christchurch Hospital. All had symptomatic heart failure (New York Heart Association Functional Class II or III), and reduced left ventricular ejection fraction (<40%) on echocardiography. Four patients had type 2 diabetes mellitus and eight had hypertension. No patient had atrial fibrillation, a prior acute coronary event or revascularisation, stenotic cardiac valve disease, impairment of liver, renal (glomerular filtration rate <100 ml/min) or pulmonary function or was on lipid-modifying medications. However, four patients had some features of atherosclerotic disease on angiogram, but with no symptoms of myocardial ischaemia. Patients were receiving furosemide (n=18), ACE inhibitor (n=18) or angiotensin II receptor antagonist (n=4) with or without digoxin (n=2), a beta-adrenergic blocker (n=13), spironolactone (n=4) and aspirin (n=14). Fourteen patients used aspirin throughout.

8.2.2 Study design

All patients received 40 mg of atorvastatin or a matching placebo once daily in the evening for six weeks in a randomised, placebo-controlled, cross-over study, with a two-week washout period between treatments (Figure 8.1). A computer generated block randomisation list was prepared in advance and made available to the study pharmacist. Patients maintained their standard anti-failure therapies for the duration of the study period and the prior three months. Assessments were performed at baseline, and at the end of both treatment periods. Patients underwent testing at 0730 hours, after an overnight fast, in a quiet, air-conditioned room with a stable temperature of 22⁰C for the following: brachial ultrasound, blood sample collection and forearm plethysmography. The study protocol was approved by the Christchurch Ethics Committee (NZ), and written informed consent was obtained from all participants.

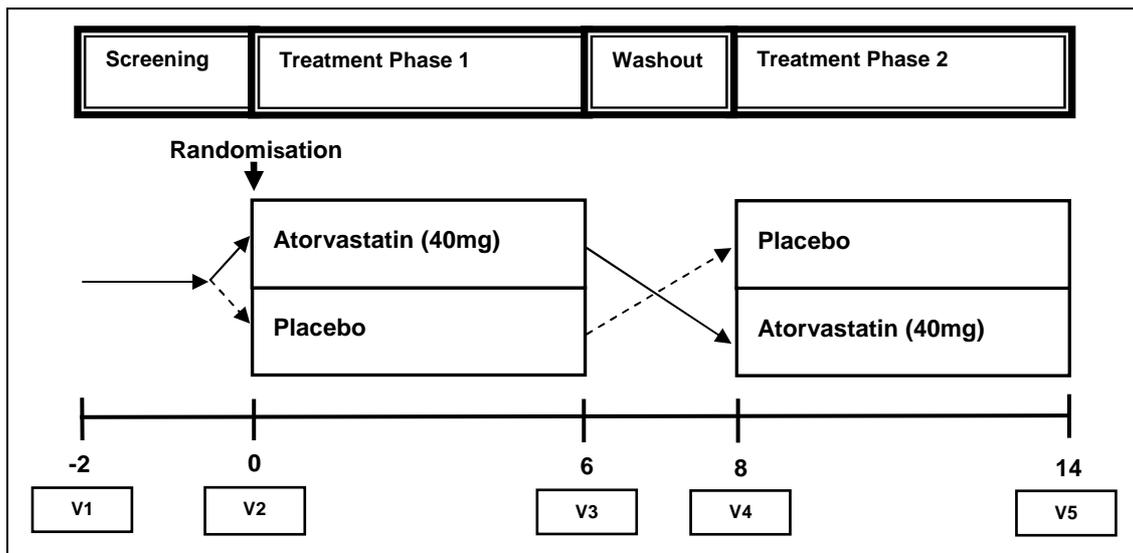


Figure 8.1 Summary outline of the six-week randomised, double-blind, placebo-controlled, cross-over study of atorvastatin treatment in patients with non-ischaemic heart failure

8.2.3 Assessment of flow-mediated dilation of the brachial artery

Conduit artery function was assessed by brachial ultrasound based on the protocol described by Celermajer *et al.* (1992) (569), which is discussed in detail in Chapter 2, section 2.2.6. In brief, the participant stayed in a recumbent position for 15 minutes prior to commencement of the measurements, with the non-dominant arm placed in a specially designed cradle and the ultrasound transducer fixed in position with a stereotactic clamp. The brachial artery was imaged in the longitudinal plane at baseline, after five minutes of forearm ischaemia to assess endothelial-dependent flow-mediated dilation (FMD), after a second resting baseline and then four minutes after administration of 800 µg of sublingual glyceryl trinitrate to assess endothelial-independent vasodilation (EID). Both these procedures were performed using a fully digitised ultrasound system (Logiq 700 Expert Series, GE Medical Systems) with a high-resolution, broadspectrum transducer (6 – 13 MHz, LA39, GE Medical Systems). As previously discussed, recordings were only made when well defined; double-line patterns were observed proximally and distally throughout the entire region of measurement. Anatomical landmarks and direct measurements were used to minimise positional variations between study visits. Scans were recorded on super VHS tapes and digitised (Pinnacle DV500 Plus, Pinnacle Systems). All recordings were by the same investigator and the frame analyses were completed prior to the unblinding of the study

medication allocation. Diastolic frames were identified by gating simultaneously recorded Doppler curves and then measured frame-by-frame using specially developed edge-detection software. Vessel diameter was defined as the distance between the distal and proximal luminal–intimal interface. FMD and EID were expressed as the percentage increase in vessel diameter from baseline $[(\text{maximum diameter after ischaemia or glyceryl trinitrate administration} - \text{baseline diameter}) / \text{baseline diameter}] \times 100$. The coefficient of variation was 3% for measurement of baseline diameters, 22% for FMD and 11% for EID.

8.2.4 Assessment of forearm blood flow

Resistance vessel function assessment by invasive venous occlusion plethysmography was based on the protocol described by Watts *et al.* (1996) (1019), and detailed methodological aspects are reviewed in Chapter 2, section 2.3. Briefly, the brachial artery in the non-dominant arm was cannulated with a 27 g needle for intra-arterial infusions of 0.9 % saline for 30 minutes (baseline), acetylcholine at 7.5, 15 and 30 $\mu\text{g}/\text{minute}$ each for three minutes, sodium nitroprusside at 1.5, 3, 10 $\mu\text{g}/\text{minute}$ each for three minutes and N^{G} -monomethyl-L-arginine (L-NMMA) (Clinalfa AG, Australia) at 4.0 units/minute alone and co-infused with acetylcholine at the above concentrations for three minutes each. Blood flow measurements were made after the intra-arterial infusion of 0.9 % saline for 30 minutes (baseline) then again after each infusion rate of the above vasoactive compounds. There was a break of 15 minutes between different infusions during which the saline infusion continued. Control blood flow measurements were made in the dominant (non-infused) arm. Forearm distension was achieved by inflating and deflating brachial cuffs to supra-venous pressure levels (45 mmHg) with a rapid cuff inflator for five and ten seconds, respectively. Ten inflation/deflation cycles were recorded for each stage of the protocol and averaged. Forearm blood flow was expressed as the area under the curve (AUC) according to the trapezoid rule, with specially developed software. Blood flow data was expressed as the ratio between AUC in the infused and the non-infused arm to correct for any systemic changes. The intra-observer (between-weeks) CV for this method was 19% in the resting state and 17% post five minutes of ischaemia in healthy subjects.

8.2.5 Biochemical parameters

All biochemical analyses were performed by Canterbury Health Laboratories, an ISO15189 accredited (human) pathology laboratory. Plasma ADMA and L-arginine were

measured by high performance liquid chromatography (HPLC) with fluorescence detection using the method of Teerlink *et al.* (2004) (963). Plasma total cholesterol, triglycerides, and HDL-cholesterol were determined by an enzymatic colorimetric method (Aeroset analyser Model LN, Abbott Laboratories, Illinois IL, USA). LDL-cholesterol was calculated from the Friedewald equation. Plasma glucose, urea, creatinine and liver function were also measured (Aeroset analyser Model LN).

8.2.6 Statistical analysis

The study size (n=24) was based on the assumption, from reported effects of statins on endothelial function under other clinical circumstances (1020, 1021), that statin therapy would increase endothelial-dependent blood flow by 30% with a standard deviation of 20% at $P<0.05$ with a power of 80%. Furthermore, based on reported effects of statin treatment on ADMA concentrations (1022), it was calculated that 24 patients in a cross-over design would provide sufficient power, at $P<0.05$, to detect a clinically significant effect of statin therapy on reductions in ADMA levels.

All statistical analyses were performed using SPSS Base version 17.0 (SPSS, Inc., Chicago, Illinois). Data are expressed as mean (SEM). The differences between placebo and statin phases were tested using paired t-tests or the non-parametric Wilcoxon signed-rank test, as appropriate. Correlation analysis was performed using Pearson's correlation coefficient. Statistical significance was inferred when $P<0.05$.

8.3 Results

Six weeks of atorvastatin therapy was well tolerated. One patient with longstanding, slowly progressive myotonic dystrophy developed myalgia, but these symptoms were tolerable and the participant chose to complete the study. One participant was withdrawn from the study after randomisation due to worsening heart failure and a total of 23 patients completed the study (Figure 8.2). Levels of plasma creatine kinase and markers of liver function did not increase significantly in any of the participants (data not shown). The overall compliance to atorvastatin treatment was 98% and compliance with placebo administration was 97%.

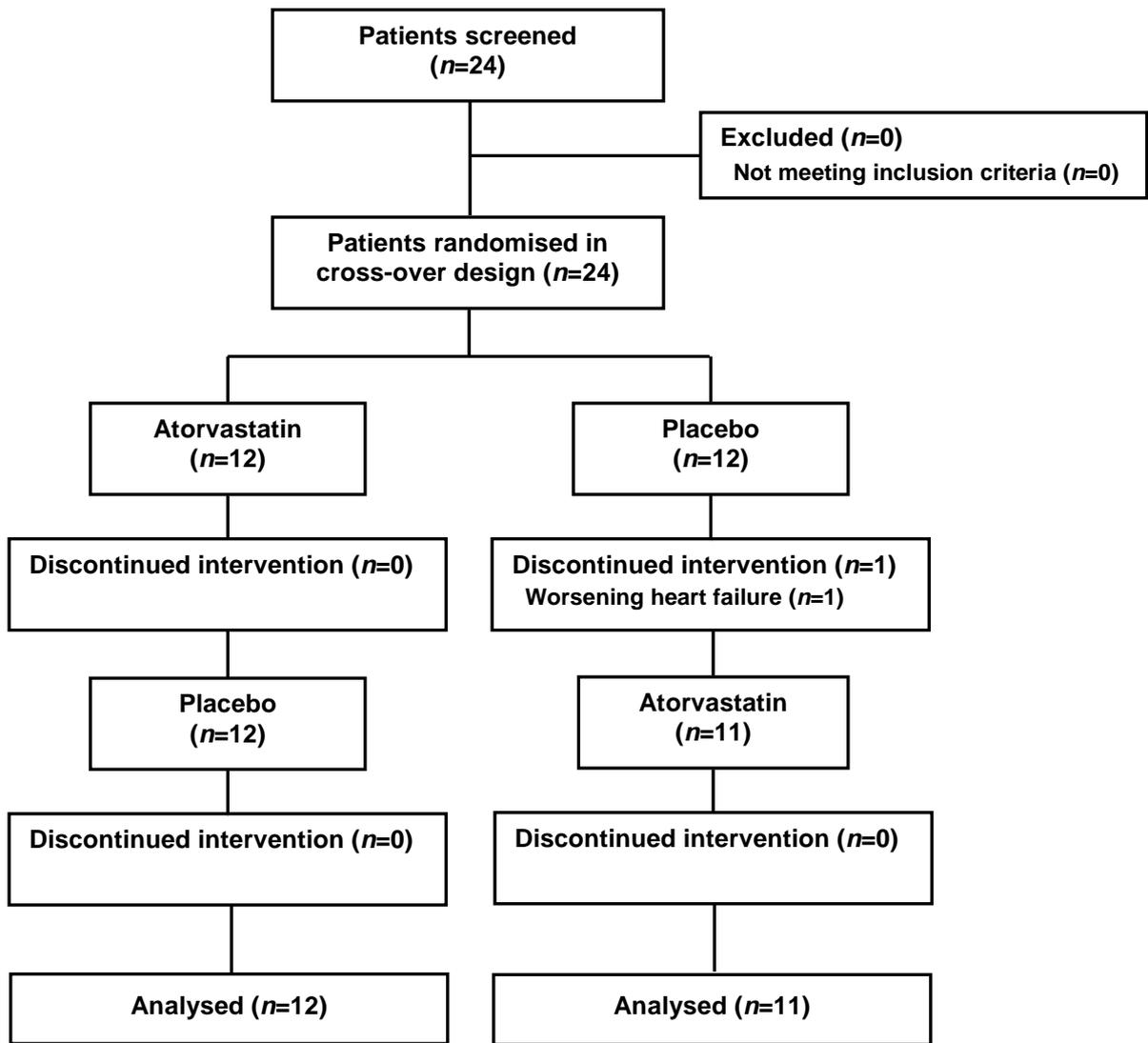


Figure 8.2 The flow of participants through the study

8.3.1 Lipid levels

Baseline lipid profiles were total cholesterol 5.3 ± 0.2 mmol/L (mean \pm SEM); LDL-cholesterol 3.3 ± 0.2 mmol/L; triglycerides 1.6 ± 0.1 mmol/L and HDL-cholesterol 1.22 ± 0.08 mmol/L. Table 8.1 summarises the effect of six-weeks of statin therapy on lipoprotein profiles. Atorvastatin treatment was associated with reductions in total cholesterol (37%), LDL-cholesterol (50%), and triglycerides (26%) in comparison to placebo ($P < 0.001$ for all). HDL-cholesterol levels remained unchanged.

8.3.2 Endothelial function by brachial ultrasound and forearm plethysmography

Conduit vessel function after administration of placebo and atorvastatin is shown in Figure 8.3 and Figure 8.4. There was no change in baseline brachial diameter after placebo or statin treatment ($P=NS$). Statin treatment improved endothelium-dependent dilation (FMD) by 1.3 ± 0.6 % compared to placebo administration ($P<0.05$), but not endothelium-independent dilation (EID). Table 8.2 summarises forearm resistance vessel function during the infusion of acetylcholine, sodium nitroprusside and L-NMMA alone or in combination with acetylcholine after statin and placebo administration. Endothelium-dependent vasodilation of the forearm resistance vessels was significantly greater following statin therapy than after placebo administration during the infusion of the second highest dose of acetylcholine. Improvements in resistance vessel function observed during acetylcholine infusion were eliminated during co-infusion with the NO antagonist L-NMMA. Basal forearm blood flow and endothelium-independent vasodilation of forearm resistance vessels during intra-arterial infusion of sodium nitroprusside was similar between placebo and statin treatment.

8.3.3 ADMA and L-Arginine/ADMA ratio

There was no change in the concentrations of ADMA ($P=0.25$) or the L-Arginine to ADMA ratio ($P=0.29$) after six weeks of treatment with atorvastatin in comparison to placebo, as shown in Figure 8.5 and Figure 8.6, respectively.

Table 8.1 Effect of six weeks of atorvastatin treatment on lipoprotein levels in 23 patients with non-ischaemic heart failure

	Placebo	Atorvastatin	% Reduction	<i>P</i> -value†
Total cholesterol, mmol/L	5.5 ± 0.2	3.5 ± 0.2	36.6 ± 1.8	<0.001
LDL-cholesterol, mmol/L	3.6 ± 0.2	1.8 ± 0.1	50.4 ± 2.1	<0.001
HDL-cholesterol, mmol/L	1.17 ± 0.07	1.18 ± 0.07	-2.1 ± 2.5	0.55
Triglycerides, mmol/L	1.7 ± 0.2	1.2 ± 0.1	26.3 ± 3.6	<0.001

Values are mean ± SEM.

†Paired t-test for comparison of between-group changes.

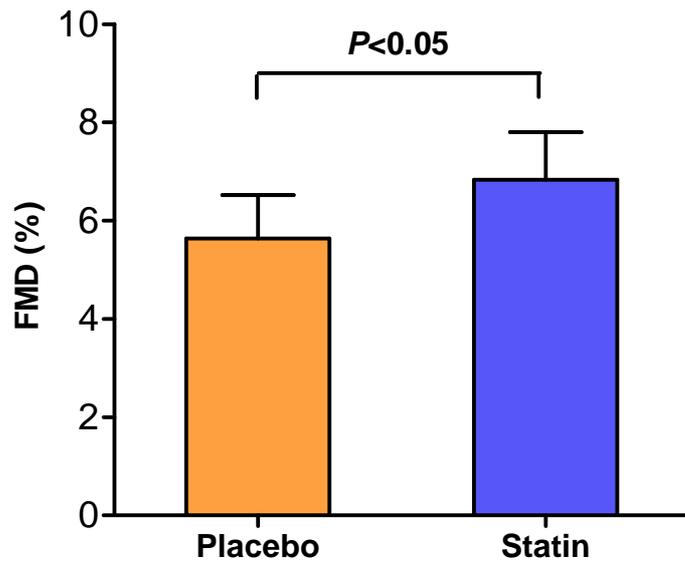


Figure 8.3 Flow-mediated dilation (FMD) of the brachial artery following placebo and six weeks of statin administration

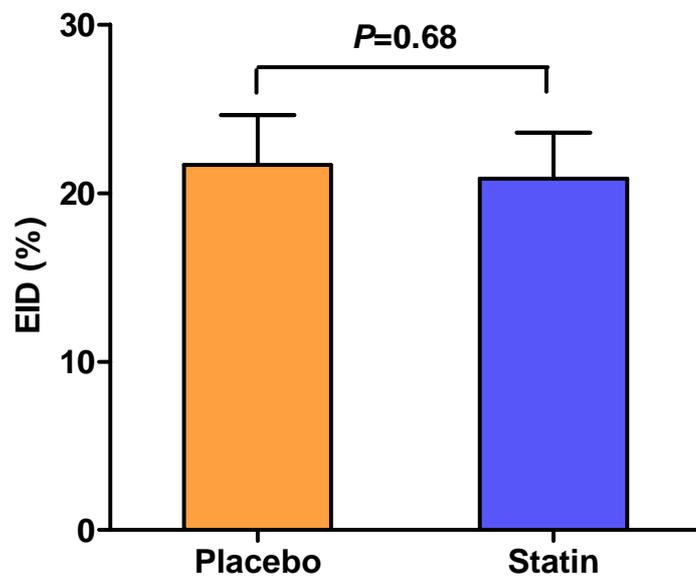


Figure 8.4 Endothelium-independent dilation (EID) of the brachial artery following placebo and six weeks of statin administration

Table 8.2 Effect of six weeks of atorvastatin treatment on resistance vessel function

AUC Ratio	Placebo	Atorvastatin	<i>P</i> -value†
ACh 7.5	2.45 ± 0.58	2.76 ± 0.71	0.66
ACh 15	2.67 ± 0.48	3.72 ± 0.65	0.015
ACh 30	3.68 ± 0.53	4.59 ± 0.63	0.07
SNP 1.5	2.38 ± 0.22	3.00 ± 0.30	0.06
SNP 3	3.43 ± 0.38	3.69 ± 0.35	0.48
SNP 10	5.62 ± 0.69	6.25 ± 0.64	0.29
L-NMMA	0.83 ± 0.11	0.99 ± 0.10	0.19
ACh 7.5 + L-NMMA	2.28 ± 0.51	2.29 ± 0.51	0.99
ACh 15 + L-NMMA	2.66 ± 0.44	2.77 ± 0.59	0.78
ACh 30 + L-NMMA	3.62 ± 0.67	3.97 ± 0.90	0.69

Values are mean ± SEM.

†Independent t-test for comparison of between-group changes.

AUC, area under the curve for the effect of the following intra-arterial infusions on forearm blood flow: ACh, acetylcholine (7.5 - 30 µg/min); SNP, sodium nitroprusside (1.5 - 10 µg/min); L-NMMA, NG-monomethyl L-Arginine.

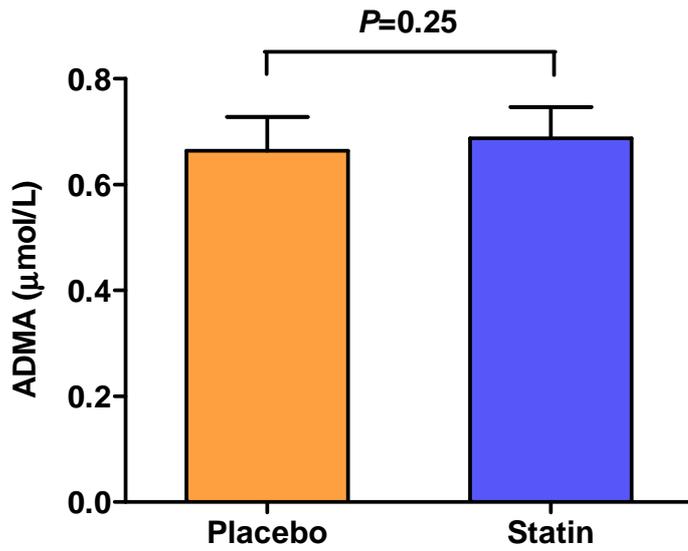


Figure 8.5 Plasma concentrations of asymmetric dimethylarginine (ADMA) following placebo and after six weeks of atorvastatin administration

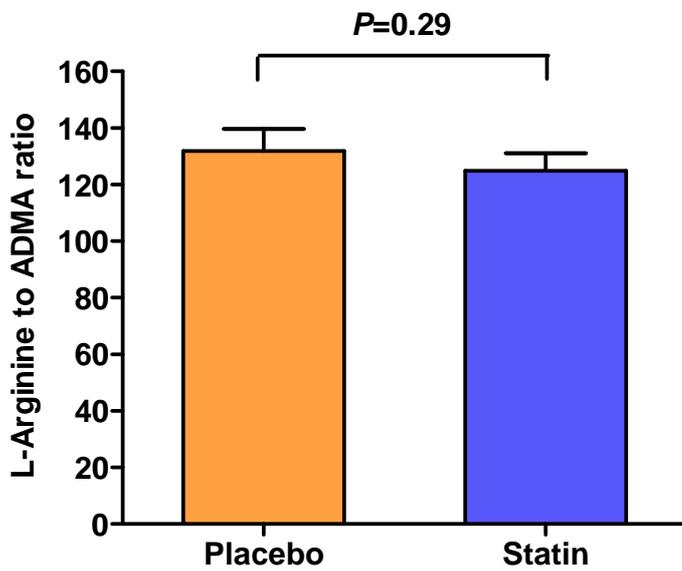


Figure 8.6 Plasma concentrations of the L-arginine to ADMA ratio following placebo and after six weeks of atorvastatin administration

8.3.4 Correlation analysis

At baseline there was a trend for ADMA to negatively correlate with FMD and resistance vessel function at increasing ACh dosages (Table 8.3). Conversely, the L-Arginine to ADMA ratio was not associated with endothelial-dependent function (Table 8.3), and neither ADMA nor the L-arginine to ADMA ratio were correlated with LDL-cholesterol ($r=-0.14$, $P=0.52$ for ADMA and $r=0.13$, $P=0.56$ for the L-Arginine to ADMA ratio) or endothelium-independent vasodilation. Statin-induced LDL-cholesterol reductions were independent of improvements in FMD ($r=-0.27$, $P=0.26$) and resistance vessel function ($r=-0.94$, $P=0.71$ for ACh at 15 $\mu\text{g}/\text{min}$ and $r=-0.42$, $P=0.08$ for ACh at 30 $\mu\text{g}/\text{min}$). Furthermore, endothelial-dependent vascular function was not associated with either ADMA or the L-arginine to ADMA ratio in response to six weeks of statin therapy.

Table 8.3 Correlation coefficients for ADMA and the L-Arginine/ADMA ratio versus endothelium-dependent resistance and conduit function at baseline

	ADMA		L-Arginine/ADMA ratio	
	<i>r</i>	<i>P</i> -value	<i>r</i>	<i>P</i> -value
FMD	-0.31	0.18	0.20	0.41
AUC ACh 7.5	-0.17	0.49	0.19	0.46
AUC ACh 15	-0.41	0.12	0.27	0.32
AUC ACh 30	-0.42	0.09	0.27	0.29

AUC area under the curve for the effect of the intra-arterial infusions of acetylcholine on forearm blood flow; ACh, acetylcholine (7.5 - 30 $\mu\text{g}/\text{min}$); FMD, flow-mediated dilation.

8.4 Discussion

8.4.1 Effect of statin therapy on endothelial function

In this study, six weeks of atorvastatin treatment in patients with non-ischaemic heart failure was associated with improved endothelium-dependent vasodilation in resistance and conduit vessels (1018). This finding of improved macrovascular function is consistent with a study by Node *et al.* (2003) (1009) who reported an increase in FMD from 8 % to 13 % ($P<0.01$) for 14 weeks of simvastatin 10 mg daily in addition to standard therapy in

patients with idiopathic dilated cardiomyopathy. The less pronounced FMD improvement in the study described here may be due to a shorter treatment period, younger patient group and lower baseline FMD, suggesting more profound endothelial dysfunction in these patients that may be less amenable to treatment. More recent studies have confirmed the amelioration of conduit vessel function following statin therapy in heart failure patients with and without ischaemic aetiologies (1023-1025). In one trial, 12 weeks of atorvastatin therapy significantly improved FMD by 1.4% in patients with mild to moderate heart failure, secondary to non-ischaemic dilated cardiomyopathy (1023). In a further 12-week trial, FMD was increased by 163% following 40 mg of rosuvastatin in patients with CHF as a result of ischaemic heart disease or dilated cardiomyopathy (1024). Improvement of endothelial function in conduit arteries may translate into a reduction in the progression of ischaemic CHF (1004), as endothelial function in the peripheral macrovasculature closely mirrors the functional state of the endothelium in the coronary circulation (618, 1026).

Treating endothelial dysfunction in resistance vessels of patients with CHF may increase exercise capacity and improve peripheral perfusion (1004). This study demonstrated that statin-induced improvement in resistance vessels is dependent on endothelial NO, since an increase in blood flow was found only during NO-stimulating acetylcholine infusion and reversed during co-infusion of the NO inhibitor, L-NMMA. A comparable study by Laufs *et al.* (2004) (1027) did not observe statin-induced improvements of resistance vessel function in patients with non-ischaemic dilated cardiomyopathy after 20 weeks of cerivastatin treatment. Tousoulis *et al.* (2005) (1028) were also unable to demonstrate an effect of four weeks of atorvastatin therapy on the maximum hyperaemic forearm blood flow in patients with ischaemic and non-ischaemic heart failure. However, in those two studies the vascular response was measured by non-invasive plethysmography post ischaemia, which has a low specificity for endothelial function (658).

8.4.2 Pleiotropic effects of statin therapy

In the current study, amelioration of endothelial function with atorvastatin therapy was largely independent of LDL-cholesterol. The study subjects did not have significant coronary artery disease and their plasma LDL-cholesterol concentrations were below the population average. Furthermore, neither baseline LDL-cholesterol levels nor LDL-cholesterol reductions determined the degree of endothelial improvement. Cholesterol independent or pleiotropic statin effects have long been postulated (1029), recently gaining

more widespread recognition (1030-1032). Pleiotropic effects of statins contribute to the reversal of important pathogenic mechanisms leading to endothelial dysfunction in heart failure, including decreased vasodilating NO and increased vasoconstricting endothelin-1 (103). Statins stabilise and up-regulate eNOS, independent of LDL-cholesterol reduction, thereby increasing endothelial NO production (93, 94, 96), and decreasing endothelin-1 expression in endothelial cells (1033). In the present study a significant reduction in endothelin-1 levels were seen after atorvastatin treatment (1018). Increased oxidative stress is also an important feature of endothelial dysfunction and is associated with worsening heart failure (1034). Statins may alleviate oxidative stress by inhibiting NADPH oxidase activity, which leads to a reduction in free radical generation (99-101), and reduced activation of nuclear factor kappa beta (NFkB) that controls oxidative stress and inflammatory signalling pathways (104). A recent study reported a decrease in oxidised LDL and lipid peroxidation with 12 weeks of rosuvastatin therapy, in addition to improving FMD and ejection fraction (1024), supporting an antioxidant role of statin therapy in CHF. Statins may also ameliorate endothelial dysfunction by reducing pro-inflammatory cytokines (102, 103) that play a significant role in the development and progression of CHF (1035). Although two large outcome trials failed to show an effect of TNF ∞ antagonism on improving heart failure (1036), small clinical studies have demonstrated beneficial effects of statins on pro-inflammatory cytokines, as well as cardiac function (1008, 1037).

Evidence of pleiotropic statin-induced endothelial benefits in CHF has been derived from several clinical trials (1009, 1023, 1025, 1038). Landmesser *et al.* (2005) (1038) showed improvements in flow-mediated dilation in patients with CHF on statin therapy, but not with ezetimibe, despite almost identical LDL-cholesterol reductions. This was confirmed in a more recent study by Gounari *et al.* (2010) (1025) where enhanced endothelial function was observed with rosuvastatin but not with ezetimibe in patients with ischaemic heart failure in spite of comparable LDL-cholesterol reductions. In statin trials of non-ischaemic heart failure, LDL-cholesterol was not a predictor of statin-induced improvement in endothelial function (1009, 1018), and in a later trial, atorvastatin augmented FMD in patients with normal serum cholesterol levels (1023), providing additional evidence for pleiotropic effects.

8.4.3 ADMA as a marker of endothelial dysfunction

Recent publications have suggested that ADMA can be considered as a marker of endothelial dysfunction (1039, 1040). Elevated ADMA levels have been reported in patient groups with endothelial dysfunction and increased cardiovascular risk, including hypercholesterolaemia (1039), peripheral artery disease (1041), hypertension (1042), chronic renal failure (1043), type 2 diabetes mellitus (1044), as well as CHF (1012-1014). An earlier trial in hypercholesterolaemic patients showed plasma ADMA concentrations correlated with the degree of endothelial dysfunction as assessed by flow-mediated dilation of the brachial artery (1039). Prospective clinical trials have shown that high ADMA levels are associated with increased incidence of cardiovascular events and mortality in high and medium risk populations such as patients undergoing haemodialysis (1045), after acute myocardial infarction (1046), and in patients with stable coronary artery disease (1047, 1048), and peripheral artery disease (1049). There is evidence that increased free radical generation and inflammation observed in CHF, may impair DDAH activity or expression and lead to subsequent ADMA accumulation (13, 346, 1016). In the present study, a trend was observed for a negative correlation between ADMA concentrations and both macro and micro-vascular endothelial function in patients with non-ischaemic heart failure, supporting the notion that ADMA is a novel marker of endothelial dysfunction in CHF. This is consistent with a study by Hornig *et al.* (1998) (1015) that demonstrated a significant negative correlation between ADMA and endothelium-dependent vasodilation in CHF. Furthermore, in recent studies, ADMA has been found to be an independent predictor of disease progression and long term cardiovascular outcomes in patients with CHF (1050, 1051). The ratio of L-arginine to ADMA has also been proposed as a potential marker of endothelial function, with reductions seen in a number of cardiovascular disease states, including hypertrophic cardiomyopathy (1017). There was however, no trend for a positive association between the L-arginine to ADMA ratio and endothelial vasodilator responses, suggesting the L-arginine to ADMA ratio may not represent a marker of endothelial function in patients with non-ischaemic dilated cardiomyopathy.

8.4.4 Effects of statin therapy on ADMA levels

ADMA has emerged as a therapeutic target for improving endothelial function, potentially in patients with CHF. Since the non-lipid mediated benefits of statins include upregulation of eNOS and NO function (93, 94, 96), reduced oxidative stress (100, 101), and inflammation (102, 103), it was speculated that atorvastatin would lower ADMA

concentrations and thereby facilitate restoration of endothelial function in CHF. This is the first study to investigate the effects of short-term statin therapy on ADMA levels in patients with non-ischaemic heart failure. However, no changes were observed in either plasma ADMA levels or in the L-arginine to ADMA ratio following six weeks of statin treatment despite improvement in macro and microvascular endothelial function. These findings suggest that statin therapy restores endothelium-dependent function by alternative mechanisms to ADMA in this patient population.

Statin therapy has been variably linked to modifications in ADMA levels in other high cardiovascular risk patients, including those with hypercholesterolaemia, chronic kidney disease and the metabolic syndrome. The majority of studies however, have demonstrated that low or high dose statin treatment for four weeks to six months does not influence ADMA levels, despite improved lipid profiles (1052-1059), which is consistent with the present findings. One explanation for the apparent lack of effect of statin therapy on ADMA observed in these trials was that treatment periods were insufficient to affect ADMA metabolism (1053, 1056). It is also possible that plasma ADMA levels may not accurately reflect endothelial ADMA levels (1054). To date, only three studies have shown benefits of statin therapy on ADMA levels in hypercholesterolaemic patients (1022, 1060, 1061). Lu *et al.* (2004) (1022) found a significant correlation between the rosuvastatin-induced improvement in flow-mediated dilation and reduction in ADMA levels after six weeks treatment in patients with hypercholesterolaemia ($P<0.017$). Fluvastatin therapy (80 mg/day) in patients with the metabolic syndrome resulted in a significant decrease in ADMA after six weeks (1060), and a more recent study found 80 mg simvastatin, but not 40 mg simvastatin lowered ADMA levels after one month of treatment (1062). However, although not significant, baseline ADMA levels were higher in the statin-treated groups compared to placebo administration in two of those trials (1022, 1060), thus the reductions in ADMA levels after therapy may simply reflect a regression towards the mean.

Interestingly, ADMA has been shown to modulate the effect of statin therapy on endothelial function (1063). This was first reported by Janatiunen *et al.* (2003) (1064), who investigated the contribution of plasma ADMA concentrations to the response of pravastatin therapy on myocardial blood flow in young adults with mild hypercholesterolaemia. They found that low baseline plasma ADMA concentrations, below the median of 0.35 $\mu\text{mol/L}$, predicted a significant improvement of adenosine-induced blood flow after statin intervention ($p=0.004$), but this effect was not observed in

patients with severely elevated ADMA (1064). Boger *et al.* (2007) (1057) demonstrated that simvastatin does not enhance endothelium-dependent vasodilation in hypercholesterolaemic patients with elevated ADMA, whereas it does in patients with low ADMA levels. A more recent study confirmed that ADMA determines the extent of improvement in endothelial function with 80 mg/day simvastatin in hypercholesterolaemic patients (1059). Furthermore, baseline ADMA concentrations were inversely correlated with the changes in adenosine-stimulated blood flow following pravastatin (1064), and simvastatin-induced changes in flow mediated dilation (1057, 1059). These outcomes suggest that low ADMA levels may allow statins to upregulate eNOS, thereby enhancing NO production and augmenting endothelial function in subjects with low concentrations of ADMA. By contrast, in patients with high concentrations of ADMA, statin-induced upregulation of eNOS may not result in enhanced NO-mediated vascular function because eNOS is competitively blocked by ADMA (1063, 1064). Increased ADMA levels may thus account for the failure of some trials to show improved vascular function with statin therapy. Since patients in the current study had relatively normal levels of ADMA, this may explain why there was a beneficial effect of atorvastatin therapy on endothelial function. Furthermore, administration of L-arginine in combination with simvastatin improved endothelium-dependent vascular function in hypercholesterolaemic patients with high ADMA levels, but had no further effect in subjects with low ADMA (1057). This implies that excess L-arginine competitively displaces ADMA from NOS, thereby reducing its inhibitory effect and restoring NOS activity (1057, 1063). This finding provides further support for the hypothesis that ADMA is an important modulator of the pleiotropic effects of statins. Combined L-arginine and statin treatment may have clinical relevance in CHF patients with high ADMA levels and abnormal vascular function.

8.4.5 Study limitations

In the absence of baseline data prior to both the treatment periods, it is not possible to exclude a time effect on the findings. However, the short term, cross-over design of the study did minimise such potential time effects. The treatment order had no effect on results (i.e. there was no significant difference when comparing the results of the participants who were treated with the active drug first versus those who were first treated with the placebo, (data not shown)). Although patients were selected with non-ischaemic cardiomyopathy, it was impossible to entirely exclude a potential ischaemic component in eight participants. This study allows only limited insights into the underlying mechanisms of statin associated improvements and the findings relate specifically to patients with systolic heart failure.

The numbers may have been too limited in order to establish any significant associations between ADMA and endothelium-dependent vasodilation.

8.4.6 Significance

The findings from this study were the first to show that patients with non-ischaemic heart failure have greater endothelium-dependent vasodilation in resistance vessels during statin therapy compared with the placebo group. Beneficial effects were detectable after only six weeks, which may translate into increased exercise capacity and improved peripheral perfusion when statins are given for longer (1004). Statin-induced amelioration of endothelial function was largely independent of LDL-cholesterol reductions, but these benefits were not mediated by a decrease in ADMA levels, suggesting that alternative pleiotropic mechanisms were involved. The study further strengthened the notion of using statins in heart failure regardless of its aetiology and in the absence of hyperlipidaemia. Large observational studies (1065), post hoc analyses of randomised controlled trials (838, 1006, 1040, 1066-1068), and small prospective studies (1008, 1009, 1023-1025, 1037, 1069, 1070) have suggested that statins could have beneficial effects in CHF, in relation to reduced mortality and hospitalisations, and enhanced ejection fraction and endothelial function.

However, two recently published, large, prospective randomised controlled trials did not find any significant clinical benefits of statins on primary outcomes in heart failure patients (80, 81). The Controlled Rosuvastatin Multinational Trial in Heart Failure (CORONA) investigators failed to show a reduction in major vascular events in older patients with moderate to severe systolic heart failure of ischaemic etiology (80). Similarly the Effect of Rosuvastatin in Patients with CHF (GISSI-HF) trial found no effect of rosuvastatin (10 mg/day) with respect to time to death or admission to hospital for cardiovascular reasons in patients with symptomatic heart failure of both ischaemic and non-ischaemic cause (81). Several explanations for this apparent discrepancy have been proposed. Investigators of the CORONA study suggested that their patients may have had vascular disease that was too advanced to modify and that statins may have a different effect in patients with mild CHF (80). Alternatively, the median follow-up of 32.8 months may not have been sufficiently long enough to observe beneficial effects of treatment (80). A retrospective analysis of the CORONA study showed in patients with a CRP ≥ 2 mg/L, all-cause death and hospitalisations were significantly reduced with rosuvastatin compared with placebo, suggesting that statins may be more beneficial in this subgroup (1071). It is

possible that the survival benefit of statins may result from the prevention of coronary artery disease, and following the onset of irreversible organ failure initiation of statin therapy may be futile (1072). The therapeutic effects of statins may not be a class effect, and rosuvastatin in particular may not provide benefit in heart failure patients (1072). This is supported by post-hoc analyses of a recent meta-analysis of statin trials in patients with ischaemic heart failure that showed heterogeneity among different statins (1073). The authors demonstrated that randomisation to atorvastatin significantly decreased all-cause mortality and hospitalisation for worsening heart failure, and randomisation to atorvastatin and simvastatin led to a significant improvement in left ventricular ejection fraction, whereas these benefits were not seen in patients randomised to rosuvastatin (1073).

Furthermore, lipoprotein reductions could negate statin specific, pleiotropic benefits in CHF (1074), since low cholesterol levels have been implicated as an independent predictor of poor prognosis in CHF which are potentially exacerbated by statin therapy (1075). Statin-induced CoQ₁₀ depletion observed in patients with non-ischaemic heart failure (758), may also be a contributory factor, potentially explaining why statin intervention has not improved outcomes in CHF (1076). Myocardial depletion of CoQ₁₀ has been demonstrated in heart failure and inversely correlated with severity (81). Furthermore, plasma levels of CoQ₁₀ were an independent predictor of survival in 200 CHF patients (504), and meta-analyses of CoQ₁₀ supplementation in CHF have reported significant improvements in stroke volume, ejection fraction, cardiac output, cardiac index, and end diastolic volume index (507, 508). However, the cause-effect relationship of these observations has not been determined and in the present study the beneficial effects of statin treatment on endothelium-dependent vasodilation in CHF were strongly associated with reductions in CoQ₁₀, indicating that statin-induced reduction of CoQ₁₀ levels may limit the maximum favourable effects of atorvastatin on the microcirculation or may simply represent a marker of the degree of pleiotropic effects of statin therapy (758). However, in a recent substudy of CORONA, low CoQ₁₀ levels were shown to be a marker of disease severity, but not an independent prognostic marker of mortality in 1,191 patients with CHF (505). This study had larger number of patients and a higher event rate than the earlier trial by Molyneux *et al.* (2008) (504), allowing for the adjustment of more covariates in the multivariate analyses (505, 506). Rosuvastatin therapy reduced plasma CoQ₁₀ levels, but even in patients with a low baseline CoQ₁₀, statin treatment was not associated with a significantly worse outcome (505). The authors did acknowledge there was limited power to exclude an interaction between CoQ₁₀ levels and the effect of statins

(505). Given these observations and the complex interplay of cholesterol, statin therapy and clinical outcomes in heart failure, one can not rule out the prospect that future trials incorporating a CoQ₁₀ supplementation treatment arm together with statin may confer improved clinical outcomes that CORONA (80) and GISSI-HF (81) did not show (506, 1076).

It is also possible that elevated baseline ADMA concentrations may have limited the beneficial effects of statin therapy on primary outcomes in the large prospective trials. Previous studies have shown that plasma ADMA levels can modulate the effect of statin treatment on myocardial blood flow (1064), as discussed in detail in section 8.4.4. In these trials, hypercholesterolaemic patients with severely elevated ADMA levels or higher ADMA levels but within the normal reference range did not respond to statin intervention as evidenced by a lack of improvement in adenosine-induced blood flow and endothelium-dependent vasodilation, whereas benefits were observed in patients with low ADMA levels (1057, 1059, 1064). Thus, high ADMA levels may explain the failure of statins to reduce cardiovascular events in the CORONA (80) and GISSI-HF (81) trials, and retrospective analysis should be undertaken in order to establish whether this hypothesis is correct. If ADMA concentrations are found to be a determinant of the reduction in primary cardiovascular endpoints in these trials, then future trials in CHF might include L-arginine and statin intervention since this combined treatment regime has been shown to ameliorate FMD in subjects with high ADMA levels (1057). However, based on the findings from the recent large, prospective studies (80, 81) the routine use of statins in patients with CHF due to non-ischaemic causes can not be recommended until further trials have been undertaken.

8.4.7 Conclusions

This study demonstrated that short-term treatment with atorvastatin had no influence on plasma ADMA levels or the L-arginine to ADMA ratio in patients with non-ischaemic heart failure, despite marked improvement in lipid profiles and endothelium-dependent vasodilatory responses of both the micro- and macrovascular circulation. Statin-induced amelioration of endothelial dysfunction was mainly independent of LDL-cholesterol reductions, a finding consistent with comparable studies. A trend for a negative association between ADMA levels and endothelium-dependent vasodilation was observed at baseline in these patients, supporting the notion that ADMA may represent a marker of endothelial dysfunction in heart failure. If elevated ADMA levels are a marker of endothelial dysfunction in non-ischaemic heart failure, the present study indicates that statin-induced

improvements must be mediated via alternative pathways. This study and more recent studies highlight the need for further prospective outcome trials to establish whether there is a role for statin therapy in improving cardiovascular outcomes in patients with CHF.

9 Conclusions and Future Work

Despite the use of currently available therapies, many patients remain at significant cardiovascular risk, creating a need for additional treatment modalities to further improve cardiovascular outcomes. The endothelium is essential for the regulation of vascular tone and the maintenance of vascular homeostasis. Endothelial dysfunction has been shown to independently predict cardiovascular risk in subjects with and without established cardiovascular disease. Consequently, the endothelium has been recognised as an important treatment target. There is evidence to suggest that coenzyme Q₁₀ (CoQ₁₀) may have a potential role in modifying endothelial function and cardiovascular risk. Statins have well proven effects on endothelial function and cardiovascular risk reduction. However, additional approaches are required to reduce or eliminate muscle related side effects of statins, in order to enable improved tolerance to this important lipid-modifying agent.

The work described in this thesis investigated strategies for cardiovascular risk reduction in patients where absolute risk remains high, despite standard therapies. Specifically, this thesis examined the effects of CoQ₁₀ supplementation and/or statin therapy on modifying endothelial dysfunction and cardiovascular risk factors, and attempted to elucidate potential mechanisms of improvement in the vascular endothelium. In addition, this thesis investigated the impact of CoQ₁₀ on statin myopathy, and explored genetic risk factors associated with statin intolerance.

CoQ₁₀ supplementation was shown to have beneficial effects on conduit artery function in simvastatin-treated patients with the metabolic syndrome. Brachial artery endothelial function was significantly improved, without modifying the response to glyceryl trinitrate, after 12 weeks of oral CoQ₁₀ compared to placebo administration. A four-fold increase in plasma CoQ₁₀ concentrations was achieved on supplementation, although this increase was not correlated with improved endothelium-dependent vasodilation. The findings from this study concur with a previous hypothesis that statin-induced reductions in plasma CoQ₁₀ levels may be limiting the maximum beneficial effects of statins on the vasculature, and thus CoQ₁₀ therapy will further augment endothelial function (758). Importantly, this study supports a potential role for CoQ₁₀ in the modification of cardiovascular risk in statin-treated patients.

Conversely, 12 weeks of supplementation with oral CoQ₁₀ did not result in clinically significant reductions in systolic or diastolic 24-hr ambulatory blood pressure or heart rate in patients with the metabolic syndrome and inadequately treated hypertension. However, daytime diastolic blood pressure loads were significantly reduced by supplementation. CoQ₁₀ was well tolerated and was not associated with any clinically relevant changes in safety parameters. Whereas one cannot exclude the possibility that CoQ₁₀ may have clinically useful antihypertensive effects in selected populations, the findings suggest that it cannot currently be recommended in the routine management of patients with the metabolic syndrome whose blood pressure control is inadequate despite regular antihypertensive therapy. This study, however does not rule out the possibility of a smaller hypotensive effect that may still have a benefit at the population level.

Furthermore, CoQ₁₀ supplementation was not associated with improvement in statin tolerance or myalgia, in patients with prior self reported statin-induced muscle complaints, despite achieving significant increases in plasma CoQ₁₀ levels. These findings suggest that CoQ₁₀ may not have an etiological role in statin-induced myalgia. Although the results of this study do not support the routine use of CoQ₁₀ to mitigate myalgia associated with statin therapy, it is possible that CoQ₁₀ supplementation might still be beneficial in specific subpopulations, dependent on risk factors such as age, severity of myalgia, concomitant disease, or genetic susceptibility to statin intolerance.

Genetic variation in *COQ2* was confirmed to be significantly associated with statin intolerance in the pooled results from two independent cohorts of statin intolerant patients. Homozygotes for the rare alleles of *COQ2* SNP-1, SNP-2 and the non-1 haplotype had an increased odds ratio of statin myopathy. There was also a significant association between the *AMPD1* haplotype 3 and statin intolerance in the Christchurch cohort, and a similar trend when both cohorts were pooled. A significant gene-gene interaction between the *COQ2* SNP-2 and *AMPD1* Q12X was observed, with the cumulative frequency of affected alleles associated with increased odds for the risk of statin myopathy. *CYP2D6**4 and *SLCO1B1* polymorphisms were not associated with statin intolerance, indicating that these polymorphic alleles may be less relevant in predicting mild statin-related myopathy, whereas genetic variation in *COQ2* and *AMPD1* and their interaction may have an important role in predicting increased risk of statin-induced myopathy.

Although recent evidence has suggested that CoQ₁₀ levels may be compromised in statin-treated patients with genotypic familial hypercholesterolemia (FH) (197, 938), the results of the case-control study showed that high-dose, long-term statin treatment in FH patients with and without identified LDL receptor (LDLR) mutations does not lead to subnormal plasma CoQ₁₀ concentrations. However, despite statin treatment, systemic arterial stiffness was elevated in FH patients compared to controls with similar lipid levels, indicating residual cardiovascular risk. This study found a significant negative association between systemic arterial stiffness and plasma CoQ₁₀ concentrations in FH patients, but not controls.

Short-term treatment with atorvastatin significantly improved endothelial function but had no influence on plasma asymmetric dimethylarginine (ADMA) levels in patients with non-ischaemic systolic heart failure. Statin-induced improvement of endothelial dysfunction was mainly independent of LDL-cholesterol reduction. A trend was observed for a negative association between ADMA concentrations and endothelial function, suggesting that ADMA may be a marker of endothelial dysfunction in chronic heart failure (CHF). The study showed that statin-induced improvements in endothelial function are mediated by alternative pathways in patients with stable, non-ischaemic, left ventricular dysfunction. Since the completion of this study, large prospective randomised trials have failed to demonstrate any significant clinical benefits of rosuvastatin in heart failure patients of both ischaemic and non-ischaemic etiology on primary outcomes (80, 81). As ADMA can modulate the effect of statin therapy on endothelial function and myocardial blood flow, with limited responses in patients with higher ADMA level (1057, 1059, 1064) (80, 81), it is could be speculated that elevated ADMA concentrations may explain the failure of statins to reduce cardiovascular events. However, the routine use of statins in patients with heart failure due to non-ischaemic causes can not be currently recommended.

Further work is needed to investigate the effects of complementary CoQ₁₀ administration on modifying cardiovascular risk. Large cardiovascular endpoint trials are required to confirm the clinical relevance of the observed improvement in endothelial function with CoQ₁₀ therapy in statin-treated patients. Investigations should include patients who are not receiving ACE inhibitors, since this subgroup showed a trend for more marked improvement in endothelium-dependent vasodilation. Furthermore, since conduit artery function was not completely restored in statin-treated patients, investigations into the synergistic effects of CoQ₁₀ with other therapies that may improve cardiovascular

function are warranted. Studies are required to elucidate the mechanisms by which CoQ₁₀ exerts its vasoprotective effects.

Further randomised controlled trials are required to establish whether CoQ₁₀ has any role as an adjunct or alternative to conventional therapy in hypertensive patients. Since the possibility of a smaller treatment effect cannot be excluded in patients with modest blood pressure elevations further trials are warranted, but would require larger numbers. Such trials could include patients with borderline hypertension or washout of antihypertensive agents, a direct comparison of CoQ₁₀ therapy with other antihypertensive agents, or combination treatment with other therapies such as fenofibrate where interactive effects with CoQ₁₀ have been observed (486), and further exploration of effects on diastolic blood pressure loads. If the blood pressure lowering effect of CoQ₁₀ is verified in short-term randomised controlled trials, longer term studies would be needed to evaluate the impact of CoQ₁₀ on specific clinical endpoints, such as cardiovascular mortality.

The potential benefits of CoQ₁₀ supplementation on vascular dysfunction in patients with FH is another area for investigation. Since plasma CoQ₁₀ levels were negatively correlated with systemic arterial stiffness in statin-treated FH and given the high index of individuality of CoQ₁₀, it could be speculated that despite normal levels, higher CoQ₁₀ levels may be protective. Thus prospective controlled trials are required to examine whether CoQ₁₀ supplementation can ameliorate arterial stiffness and other clinical measures of vascular dysfunction in this high-risk patient population. Considerations for such trials should include standardisation of the dose of lipid lowering therapies, additional clinical measures of vascular function, and the selection of genotypic FH patients with low 'normal' CoQ₁₀ levels.

Further prospective, randomised controlled trials are needed to establish whether there is a role for CoQ₁₀ in reducing or eliminating statin myopathy. Such trials should incorporate patients with more severe and clearly defined myopathy, initiation of CoQ₁₀ prior to statin therapy, a more objective myopathic pain score, susceptible genotypes, and muscle biopsy studies. One such trial is currently underway, to determine whether CoQ₁₀ supplementation can decrease the incidence of muscle pain in patients with previous statin-related myalgia (380). Other high cardiovascular risk populations that may benefit from CoQ₁₀ supplementation include patients with CHF, where meta-analyses have reported significant improvements in cardiac output and ejection fraction (507, 508). The results of

the international, randomised, double-blind multi-center intervention “Q-SYMBIO” study, currently being undertaken to investigate the effects of CoQ₁₀ on mortality and cardiovascular outcomes in patients with CHF, will help to clarify whether there is a role for CoQ₁₀ in this patient population (503).

CoQ₁₀ appears to have good patient tolerability and safety profile, at least in the short term, and longer term studies should incorporate safety measures to confirm this. Although the studies presented in this thesis suggest that CoQ₁₀ has little benefit for treated hypertension in patients with modestly elevated blood pressure or in patients with mild self reported myalgia symptoms, given the good safety profile of CoQ₁₀, it is not unreasonable to recommend this supplement as an adjunct to conventional antihypertensive or statin therapy, pending large scale trials to define its true therapeutic value.

More evidence is needed to support the use of CoQ₁₀ in preventing statin-related myalgia. However, if further CoQ₁₀ supplementation trials fail to demonstrate beneficial effects on statin-induced myopathy, then alternative mechanistic pathways within the cholesterol biosynthetic pathway will need to be explored. Elucidation of the etiology or aetiologies of statin-induced myopathy will allow the assessment of potential treatment strategies to eliminate or reduce muscle related side effects and thereby contribute to improved cardiovascular risk management. There is also increasing evidence that genetic variability is an important risk factor for statin-related myopathy and further case control studies are needed to explore and confirm other candidate genes, in addition to those already identified. In the future, improved detection of relevant risk alleles may allow individualised lipid management through screening for risk alleles prior to statin initiation. However, genotype directed drug studies would be required to assess the clinical feasibility of such an approach.

Additional prospective outcome trials are necessary in order to establish whether statin therapy improves cardiovascular outcomes in patients with CHF of non-ischaemic aetiologies. These trials should focus on co-supplementation with CoQ₁₀, treatment with alternative statins to rosuvastatin, and selection of patients with mild to moderate symptomatic heart failure, and/or increased C-reactive protein concentrations. Furthermore, if plasma ADMA levels are demonstrated to be a determinant of the reduction in primary cardiovascular endpoints in the current CHF outcome trials, then future trials should include L-arginine and statin therapy since this combined treatment

regime has been shown to improve endothelial function in patients with high ADMA levels (1057).

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Appendices

Appendix 1. Sample size table for clinical studies measuring aortic wave reflection indices

Absolute difference	Parallel design			Cross-over design			Parallel design			Cross-over design		
	1	2	3	1	2	3	1	2	3	1	2	3
(%)	AIx						AIx@HR75					
1	1099	1057	1043	174	132	118	922	893	883	127	98	88
2	280	269	265	44	33	30	262	254	251	36	28	25
3	125	120	119	20	15	13	117	113	112	16	12	11
4	71	68	67	11	8	8	66	64	63	9	7	6
5	45	43	43	-	-	-	42	41	40	-	-	-
6	31	30	30	-	-	-	29	28	28	-	-	-
7	23	22	22	-	-	-	22	21	21	-	-	-
8	18	17	17	-	-	-	16	16	16	-	-	-
9	14	13	13	-	-	-	13	13	12	-	-	-
10	11	11	11	-	-	-	11	10	10	-	-	-
(mmHg)	AP						AP@HR75					
1	238	227	223	45	34	30	147	140	138	27	20	18
2	60	57	56	11	9	8	37	35	35	7	5	5
3	26	25	25	-	-	-	16	16	15	-	-	-
4	15	14	14	-	-	-	9	9	9	-	-	-
5	10	9	9	-	-	-	-	-	-	-	-	-

Appendix 1. Sample size table for clinical studies measuring aortic wave reflection indices (continued)

Absolute difference (ms)	Parallel design			Cross-over design		
	1	2	3	1	2	3
	T_r					
1	2996	2534	2379	1152	687	535
2	749	633	595	288	172	134
3	333	282	264	128	77	59
4	187	158	149	72	43	33
5	120	101	95	46	28	21
6	84	71	66	32	19	15
7	61	52	49	24	14	11
8	47	40	37	18	11	8
9	37	31	29	14	9	-
10	30	25	24	12	-	-

Values are sample size estimates for each parameter based on the absolute difference and the number of replicate recordings* for parallel and cross-over study designs assuming an 80% power at a two tailed significance level of $\alpha=0.05$. -, sample size < n=8; AIx, augmentation index; AIx@HR75, augmentation index corrected for a heart rate of 75 bpm; AP, augmentation pressure; T_r, time to reflection.

Appendix 2. Sample size table for clinical studies measuring endothelial function by pulse wave analysis

	Absolute Difference (%)	Parallel design	Cross-over design
AIx@HR75 Δ GTN	1	353	38
	2	63	21
	3	28	9
	4	16	5
	5	10	3
	6	7	-
AIx@HR75 Δ Salbutamol	1	251	85
	2	63	21
	3	28	9
	4	16	5
	5	10	3
	6	7	-

Values are sample size estimates based on the absolute difference for parallel and cross-over study designs assuming an 80% power at a significance level of $\alpha=0.05$ (two tailed). The sample size for parallel design is the number required per group. – indicates sample size <4. AIx@HR75, augmentation index corrected for a heart rate of 75 bpm; Δ GTN, glyceryl trinitrate-mediated change; Δ Salbutamol, salbutamol-mediated change.

Appendix 3. Effect of CoQ₁₀ and placebo on arbitrarily defined daytime and night-time blood pressures and heart rate

	CoQ ₁₀		Placebo		Advantage to CoQ ₁₀ [†]	P-value [‡]
	Baseline	12-weeks	Baseline	12-weeks		
Daytime [§]						
SBP, mmHg	148.8 ± 2.7	148.0 ± 2.4	148.6 ± 2.7	149.8 ± 2.2	2.0 (-2.2, 6.2)	0.35
DBP, mmHg	82.2 ± 1.6	82.2 ± 1.8	81.0 ± 1.7	83.4 ± 1.6*	2.4 (-0.2, 5.0)	0.07
PP, mmHg	66.5 ± 2.0	65.8 ± 1.7	67.5 ± 2.0	66.4 ± 1.7	-0.4 (-3.7, 2.9)	0.79
MAP, mmHg	104.4 ± 1.8	104.1 ± 1.8	103.6 ± 1.9	105.5 ± 1.7	2.3 (-0.6, 5.1)	0.18
HR, bpm	72.2 ± 1.9	72.1 ± 1.8	72.3 ± 2.2	73.8 ± 2.2	1.7 (-1.0, 4.3)	0.21
Night-time [§]						
SBP, mmHg	136.7 ± 3.3	135.1 ± 3.2	135.7 ± 3.0	133.4 ± 3.3	-0.7 (-5.6, 4.3)	0.79
DBP, mmHg	73.6 ± 2.1	72.9 ± 2.0	73.2 ± 1.9	72.1 ± 2.1	-0.4 (-3.8, 3.1)	0.84
PP, mmHg	63.1 ± 1.9	62.2 ± 2.0	62.5 ± 2.0	61.3 ± 2.0	-0.3 (-2.7, 2.1)	0.79
MAP, mmHg	94.6 ± 2.4	93.6 ± 2.3	94.0 ± 2.2	92.5 ± 2.4	-0.5 (-4.3, 3.4)	0.81
HR, bpm	64.8 ± 1.8	64.1 ± 1.7	63.7 ± 1.7	66.0 ± 2.0*	3.0 (0.2, 5.8)	0.04

Values are mean ± SEM.

[†]Difference between mean change after placebo and CoQ₁₀ (95% CI). [‡]ANOVA with repeated measures for comparison of between group changes. [§]Daytime and night-time defined by arbitrarily. **P*<0.05 using paired t-test for within-group changes from baseline.

SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; HR, heart rate.

Appendix 4. Effect of CoQ₁₀ and placebo on participant defined daytime and night-time blood pressures and heart rate

	CoQ ₁₀		Placebo		Advantage to CoQ ₁₀ [†]	P-value [‡]
	Baseline	12-weeks	Baseline	12-weeks		
Daytime [§]						
SBP, mmHg	149.0 ± 2.7	149.0 ± 2.5	148.9 ± 2.7	150.2 ± 2.3	1.3 (-3.0, 5.5)	0.55
DBP, mmHg	82.4 ± 1.7	82.9 ± 1.7	81.5 ± 1.7	83.5 ± 1.6	1.5 (-1.1, 4.1)	0.24
PP, mmHg	66.6 ± 2.0	66.0 ± 1.9	67.5 ± 1.9	66.6 ± 1.8	-0.3 (-3.7, 3.1)	0.87
MAP, mmHg	104.6 ± 1.9	104.9 ± 1.8	104.0 ± 1.9	105.8 ± 1.7	1.4 (-1.4, 4.2)	0.31
HR, bpm	73.1 ± 2.0	72.8 ± 1.8	73.0 ± 2.2	74.3 ± 2.2	1.7 (-1.2, 4.6)	0.25
Night-time [§]						
SBP, mmHg	135.6 ± 3.6	133.4 ± 3.3	133.6 ± 3.2	131.9 ± 3.4	0.4 (-3.7, 4.4)	0.86
DBP, mmHg	73.0 ± 2.1	71.7 ± 2.0	71.4 ± 2.0	71.3 ± 2.1	1.2 (-1.6, 3.9)	0.39
PP, mmHg	62.6 ± 2.1	61.8 ± 1.9	62.2 ± 2.1	60.6 ± 2.0	-0.8 (-3.1, 1.5)	0.49
MAP, mmHg	93.8 ± 2.5	92.3 ± 2.3	92.2 ± 2.3	91.5 ± 2.4	0.9 (-2.1, 3.9)	0.55
HR, bpm	63.1 ± 1.7	62.8 ± 1.7	61.7 ± 1.6	64.7 ± 2.0*	3.3 (0.7, 5.8)	0.01

Values are mean ± SEM.

[†]Difference between mean change after placebo and CoQ₁₀ (95% CI). [‡]ANOVA with repeated measures for comparison of between group changes. [§]Daytime and night-time defined by participant's bed and rise times. **P*<0.05 using paired t-test for within-group changes from baseline. SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; HR, heart rate.

Appendix 5. Effect of CoQ₁₀ and placebo on arbitrarily defined daytime and night-time ambulatory blood pressure loads

	CoQ ₁₀		Placebo		Advantage to CoQ ₁₀ [†]	P-value [‡]
	Baseline	12-weeks	Baseline	12-weeks		
Daytime BP loads [§]						
% SBP >140 mmHg	63.8 ± 5.1	64.5 ± 4.4	64.1 ± 4.5	67.7 ± 4.3	3.0 (-6.2, 12.1)	0.51
% SBP ≥130 mmHg	81.0 ± 3.5	81.5 ± 3.2	80.1 ± 3.2	84.0 ± 3.0	3.4 (-4.6, 11.5)	0.39
% DBP >90 mmHg	30.3 ± 4.5	26.9 ± 4.2	26.3 ± 4.1	31.2 ± 4.6	8.3 (1.9, 14.8)	0.01
% DBP ≥ 85 mmHg	42.9 ± 5.2	40.8 ± 5.0	38.9 ± 4.7	44.8 ± 5.1	8.1 (1.4, 14.8)	0.02
Night-time BP loads [§]						
% SBP >125 mmHg	68.8 ± 5.7	64.8 ± 5.5	66.6 ± 5.6	64.4 ± 5.7	1.8 (-8.1, 11.7)	0.71
% SBP ≥120 mmHg	75.0 ± 4.7	73.2 ± 5.1	74.5 ± 5.0	71.8 ± 5.3	-0.9 (-9.1, 7.3)	0.83
% DBP >75 mmHg	48.0 ± 6.8	44.1 ± 6.3	43.7 ± 5.8	38.3 ± 6.3	-1.4 (-11.3, 8.5)	0.77
% DBP ≥ 70 mmHg	59.9 ± 6.4	55.1 ± 6.2	57.2 ± 5.7	51.9 ± 6.3	-0.5 (-9.7, 8.8)	0.92

Values are mean ± SEM.

[†]Difference between mean change after placebo and CoQ₁₀ (95% CI).

[‡]ANOVA with repeated measures for comparison of between group changes.

[§]Daytime and night-time defined arbitrarily.

SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; HR, heart rate.

Appendix 6. Effect of CoQ₁₀ and placebo on participant defined daytime and night-time ambulatory blood pressure loads

	CoQ ₁₀		Placebo		Advantage to CoQ ₁₀ [†]	P-value [‡]
	Baseline	12-weeks	Baseline	12-weeks		
Daytime BP loads§						
% SBP >140 mmHg	63.7 ± 5.0	66.0 ± 4.4	64.3 ± 4.5	68.1 ± 4.4	1.6 (-7.9, 11.0)	0.74
% SBP ≥130 mmHg	82.1 ± 3.3	82.5 ± 3.3	80.9 ± 3.2	84.8 ± 3.0	3.5 (-4.6, 11.7)	0.38
% DBP >90 mmHg	29.9 ± 4.7	28.3 ± 4.3	26.7 ± 4.1	31.5 ± 4.7	6.5 (0.5, 12.5)	0.03
% DBP ≥ 85 mmHg	42.2 ± 5.3	41.9 ± 5.1	39.6 ± 4.9	44.6 ± 5.2	5.3 (-1.5, 12.0)	0.12
Night-time BP loads§						
% SBP >125 mmHg	65.5 ± 6.4	63.3 ± 5.4	63.1 ± 6.2	61.3 ± 6.3	0.4 (-9.1, 9.8)	0.94
% SBP ≥120 mmHg	71.4 ± 5.6	71.6 ± 5.2	70.2 ± 5.7	68.9 ± 5.8	-1.5 (-10.2, 7.1)	0.72
% DBP >75 mmHg	46.5 ± 7.0	42.2 ± 6.3	41.3 ± 6.2	38.1 ± 6.4	1.2 (-6.8, 9.1)	0.76
% DBP ≥ 70 mmHg	58.1 ± 6.5	52.7 ± 6.4	54.7 ± 6.1	50.6 ± 6.6	1.3 (-6.6, 9.1)	0.75

Values are mean ± SEM.

[†]Difference between mean change after placebo and CoQ₁₀ (95% CI).

[‡]ANOVA with repeated measures for comparison of between group changes.

[§]Daytime and night-time defined by participant's bed and rise times.

SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; HR, heart rate.

Appendix 7. Effect of CoQ₁₀ and placebo on nocturnal fall in blood pressures (arbitrarily defined daytime and night-time)

	CoQ ₁₀		Placebo		Advantage to CoQ ₁₀ †	P-value‡
	Baseline	12-weeks	Baseline	12-weeks		
Nocturnal fall§						
SBP, mmHg	12.1 ± 2.3	12.9 ± 2.3	12.9 ± 2.1	16.4 ± 2.5	2.6 (-3.9, 9.2)	0.42
DBP, mmHg	8.6 ± 1.1	9.3 ± 1.6	7.9 ± 1.1	11.3 ± 1.5*	2.7 (-2.3, 7.8)	0.28
% Nocturnal fall§						
SBP, %	8.1 ± 1.5	8.8 ± 1.6	8.6 ± 1.4	11.0 ± 1.6	1.7 (-2.4, 5.9)	0.40
DBP, %	10.7 ± 1.4	11.1 ± 1.8	9.8 ± 1.3*	13.6 ± 1.7*	3.4 (-2.3, 9.1)	0.23

Values are mean ± SEM.

†Difference between mean change after placebo and CoQ₁₀ (95% CI).

‡ANOVA with repeated measures for comparison of between group changes.

§Daytime and night-time defined arbitrarily.

*P<0.05 using paired t-test for within-group changes from baseline.

SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; HR, heart rate.

Appendix 8. Effect of CoQ₁₀ and placebo on nocturnal fall in blood pressures (participant defined daytime and night-time)

	CoQ ₁₀		Placebo		Advantage to CoQ ₁₀ †	P-value‡
	Baseline	12-weeks	Baseline	12-weeks		
Nocturnal fall§						
SBP, mmHg	13.4 ± 2.8	15.5 ± 2.5	15.3 ± 2.6	18.3 ± 2.7	0.9 (-5.0, 6.8)	0.76
DBP, mmHg	9.4 ± 1.3	11.2 ± 1.6	10.1 ± 1.4	12.2 ± 1.4	0.4 (-4.2, 4.9)	0.88
% Nocturnal fall§						
SBP, %	9.0 ± 1.9	10.4 ± 1.7	10.2 ± 1.7	12.3 ± 1.8	0.6 (-3.1, 4.3)	0.74
DBP, %	11.5 ± 1.6	13.4 ± 1.8	12.3 ± 1.7	14.7 ± 1.7	0.5 (-4.6, 5.6)	0.83

Values are mean ± SEM.

†Difference between mean change after placebo and CoQ₁₀ (95% CI).

‡ANOVA with repeated measures for comparison of between group changes.

§Daytime and night-time defined by participant's bed and rise times.

SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; HR, heart rate.

Appendix 9. Effect of CoQ₁₀ and placebo on arbitrarily defined daytime and night-time ambulatory blood pressure and heart rate variability

	CoQ ₁₀		Placebo		Advantage to CoQ ₁₀ †	P-value‡
	Baseline	12-weeks	Baseline	12-weeks		
Daytime SD§						
SBP	17.5 ± 0.7	17.5 ± 0.9	18.4 ± 0.6	17.8 ± 0.8	-0.7 (-3.3, 2.0)	0.63
DBP	13.4 ± 0.8	13.5 ± 0.8	13.0 ± 0.7	14.0 ± 0.9	0.8 (-1.6, 3.3)	0.49
HR	12.4 ± 0.9	10.9 ± 0.8*	11.3 ± 0.8	10.5 ± 0.8	0.8 (-1.7, 3.2)	0.53
Night-time SD§						
SBP	15.4 ± 0.7	15.7 ± 1.0	17.1 ± 0.9	15.4 ± 0.7	-1.9 (-4.9, 1.1)	0.20
DBP	10.0 ± 0.6	11.2 ± 0.9	11.2 ± 0.8	9.9 ± 0.7	-2.6 (-5.2, -0.0)	0.05
HR	6.4 ± 0.7	6.4 ± 0.6	6.5 ± 0.6	6.3 ± 0.4	-0.1 (-1.7, 1.5)	0.88

Values are mean ± SEM.

†Difference between mean change after placebo and CoQ₁₀ (95% CI).

‡ANOVA with repeated measures for comparison of between group changes.

§Daytime and night-time defined arbitrarily.

*P<0.05 using paired t-test for within-group changes from baseline.

SD, standard deviation of the mean; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate.

Appendix 10. Effect of CoQ₁₀ and placebo on participant defined daytime and night-time ambulatory blood pressure and heart rate variability

	CoQ ₁₀		Placebo		Advantage to CoQ ₁₀ [†]	P-value [‡]
	Baseline	12-weeks	Baseline	12-weeks		
Daytime SD [§]						
SBP	16.9 ± 0.6	17.0 ± 0.9	17.7 ± 0.7	17.5 ± 0.8	-0.3 (-2.8, 2.3)	0.82
DBP	13.1 ± 0.8	13.3 ± 0.9	13.2 ± 0.7	13.8 ± 0.9	0.5 (-2.0, 3.0)	0.69
HR	12.0 ± 0.9	10.4 ± 0.8 [†]	10.6 ± 0.8	10.0 ± 0.8	1.0 (-1.6, 3.6)	0.45
Night-time SD [§]						
SBP	14.8 ± 0.6	14.9 ± 0.9	16.2 ± 0.8	14.4 ± 0.7	-1.9 (-4.6, 0.8)	0.16
DBP	9.9 ± 0.5	10.5 ± 0.9	9.4 ± 0.6	9.5 ± 0.7	-0.5 (-2.9, 1.8)	0.64
HR	5.5 ± 0.4	5.5 ± 0.5	5.4 ± 0.5	5.6 ± 0.4	-0.2 (-1.0, 1.4)	0.74

Values are mean ± SEM.

[†]Difference between mean change after placebo and CoQ₁₀ (95% CI).

[‡]ANOVA with repeated measures for comparison of between group changes.

[§]Daytime and night-time defined by participant's bed and rise times.

SD, standard deviation of the mean; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate.

Appendix 11. *Post-hoc* subgroup analyses of blood pressure and heart rate parameters, indicating those subgroup-variable combinations where there was a significantly different response to CoQ₁₀

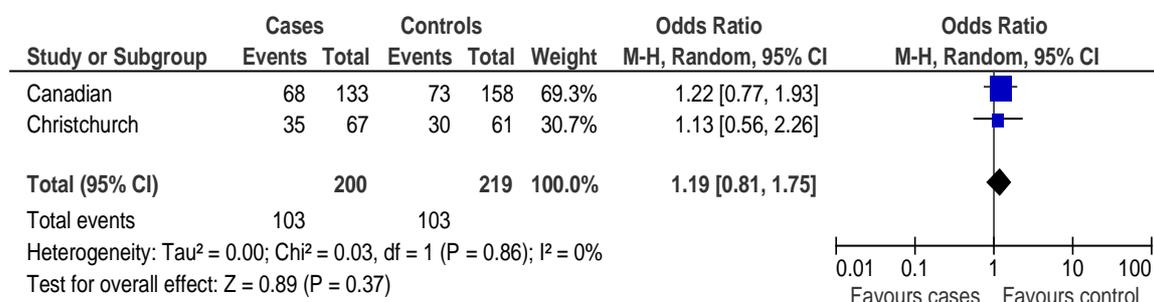
Subgroup	Mean BP and HR	Min / Max BP and HR	Min/Max Ratio	Morning Surge /Nocturnal Fall	BP Load	BP and HR Variability
CoQ ₁₀ achieved (≥ 2.9 $\mu\text{mol/L}$)	-	$\Delta\text{Max HR}$ ^{Night Arb, ESC, P} $\Delta\text{Min HR}$ ^{Night ESC}	ΔSBP ^{Day Arb} ΔHR ^{Night ESC}	-	-	ΔSDHR ^{Night ESC, P}
CoQ ₁₀ achieved (≥ 4.1 $\mu\text{mol/L}$)	-	$\Delta\text{Max DBP}$ ^{Night Arb} $\Delta\text{Max MAP}$ ^{Night Arb} $\Delta\text{Max HR}$ ^{Night ESC}	ΔDBP ^{Night Arb} ΔHR ^{Night ESC}	-	-	ΔSDHR ^{Night ESC}
BL ambulatory SBP >median	ΔSBP ^{Night P} ΔPP ^{Night P} ΔHR ^{Night ESC}	-	-	$\Delta\text{DBP PSUR}$	-	ΔSDSBP ^{Night P}
BL ambulatory DBP >median	ΔSBP ^{Night P} ΔHR ^{Night ESC, P}	$\Delta\text{Min DBP}$ ^{Night Arb, ESC} $\Delta\text{Min MAP}$ ^{Night Arb, ESC} $\Delta\text{Min HR}$ ^{Night ESC} $\Delta\text{Max SBP}$ ^{Night Arb, P} $\Delta\text{Max PP}$ ^{Night P}	-	$\Delta\text{SBP SSUR}$	$\Delta\text{DBP}>75$ ^{Night ESC} $\Delta\text{DBP}>70$ ^{Night ESC}	-
Nocturnal SBP fall <10%	-	$\Delta\text{Max SBP}$ ^{Day Arb}	ΔDBP ^{Night P}	-	-	-
Nocturnal DBP fall <10%	-	$\Delta\text{Min DBP}$ ^{Night Arb, P} $\Delta\text{Min HR}$ ^{Night P}	ΔDBP ^{Night P} ΔHR ^{Night P}	-	-	-
Treatment with statin	ΔHR ^{Day Arb, ESC}	$\Delta\text{Max SBP}$ ^{Night P}	ΔSBP ^{Night P}	-	-	-

Appendix 11. *Post-hoc* subgroup analyses of blood pressure and heart rate parameters, indicating those subgroup-variable combinations where there was a significantly different response to CoQ₁₀ (continued)

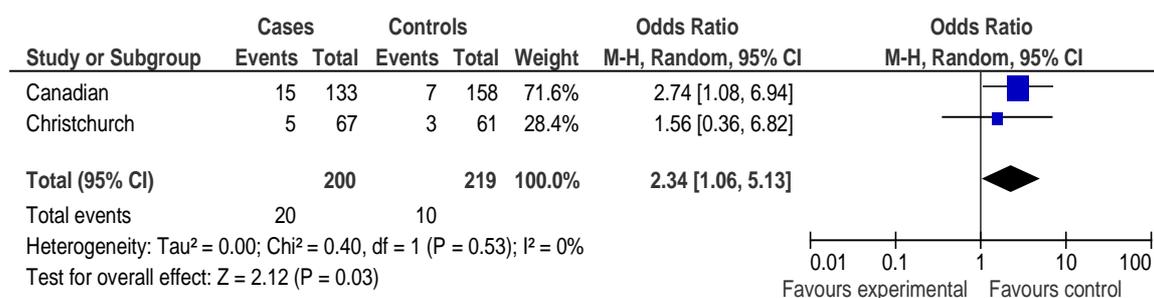
Subgroup	Mean BP and HR	Min / Max BP and HR	Min/Max Ratio	Morning Surge /Nocturnal Fall	BP Load	BP and HR Variability
Treatment with ACE inhibition	Δ SBP ^{Night Arb} Δ DBP ^{Night Arb} Δ MAP ^{Night Arb}	Δ Min SBP ^{Night P} Δ Min DBP ^{Night Arb, ESC} Δ Min MAP ^{Night Arb, ESC, P}	-	-	Δ DBP>90 ^{Day ESC, P}	-
Treatment with beta blocker	Δ DBP ^{Day Arb} Δ MAP ^{Day Arb}	Δ Min HR ^{24hr, Night P}	Δ DBP ^{Night Arb}	-	-	-
Treatment with metformin	Δ HR ^{Day ESC, P}	Δ Min DBP ^{24hr, Night Arb} Δ Min MAP ^{24hr} Δ Max SBP ^{Day Arb, ESC} Δ Max HR ^{Night Arb, P}	Δ HR ^{Night Arb}	-	-	Δ SDSBP ^{Day ESC} Δ SDHR ^{Night Arb, P}
Presence of type 2 diabetes	Δ HR ^{24hr, Day Arb, ESC} Δ HR ^{Night Arb, ESC} Δ HR ^{Day P}	Δ Max SBP ^{Day Arb} Δ Max HR ^{Night Arb, ESC, P}	-	-	-	Δ SDSBP ^{Day ESC} Δ SDHR ^{Night ESC, P}
Presence of CVD	-	Δ Max SBP ^{Night P} Δ Max DBP ^{24hr, Night Arb, P} Δ Max PP ^{Night P}	Δ SBP ^{Night P}	-	-	Δ SDDBP ^{Night Arb}

BL, baseline; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; HR, heart rate; PSUR, pre-awakening morning surge; SSUR, sleep through morning surge; SD, standard deviation; Arb, arbitrarily defined daytime or night-time; ESC, European Society of Cardiology Guidelines for daytime or night-time; P, participant defined daytime or night-time.

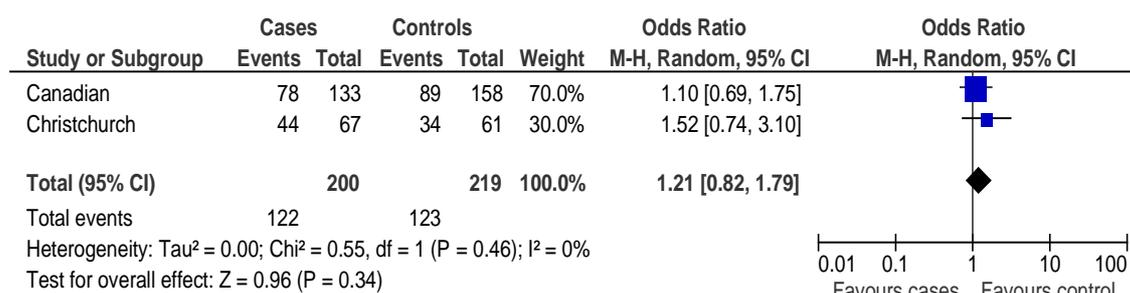
Appendix 12. Forest plots of comparison for dominant and recessive genetic models of association with statin intolerance (RevMan 5.0)



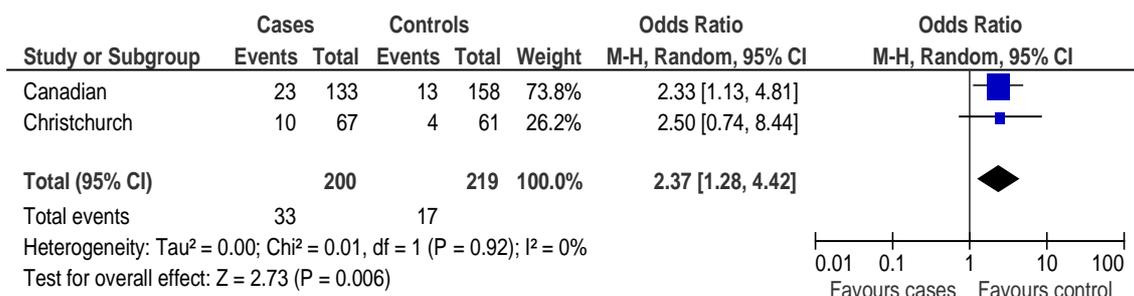
Forest plot of comparison: *COQ2* SNP1 A-dominant, outcome: cases vs controls



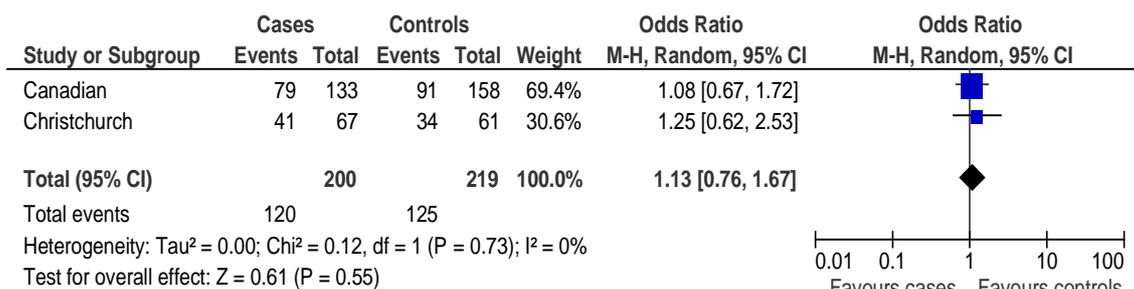
Forest plot of comparison: *COQ2* SNP1 A-recessive, outcome: cases vs controls



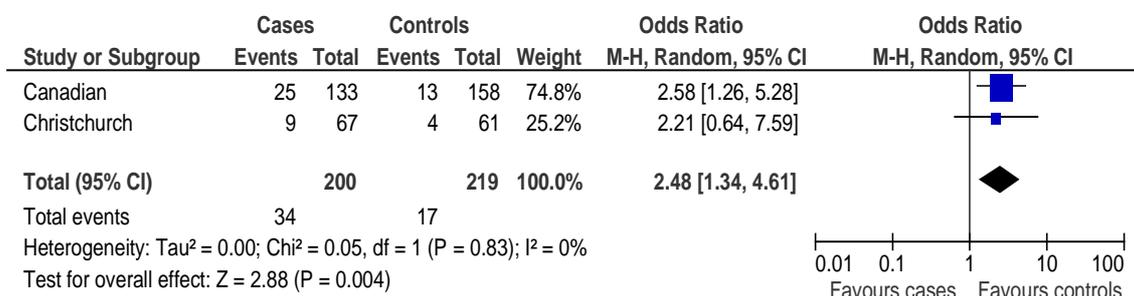
Forest plot of comparison: *COQ2* SNP2 G-dominant, outcome: cases vs controls



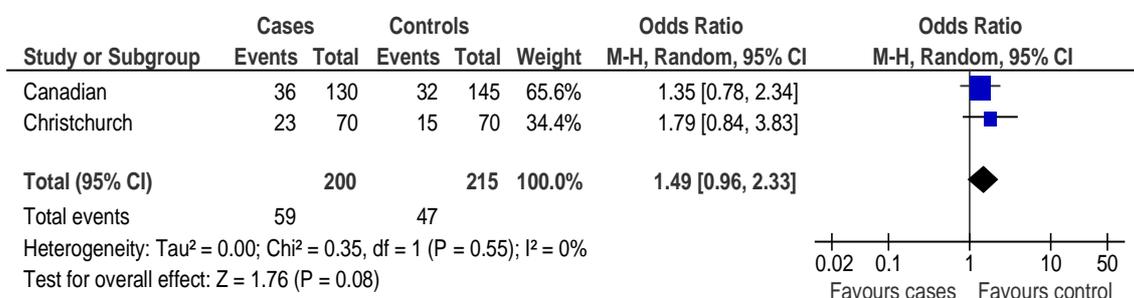
Forest plot of comparison: *COQ2* SNP2 G-recessive, outcome: cases vs controls



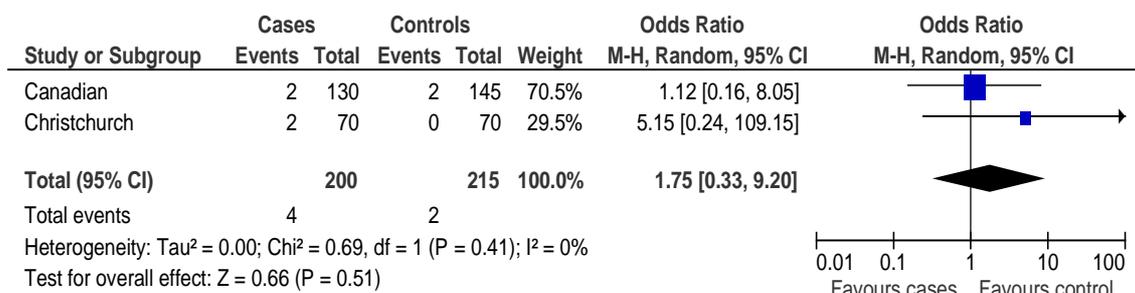
Forest plot of comparison: *COQ2* haplotype non-1 dominant, outcome: cases vs controls



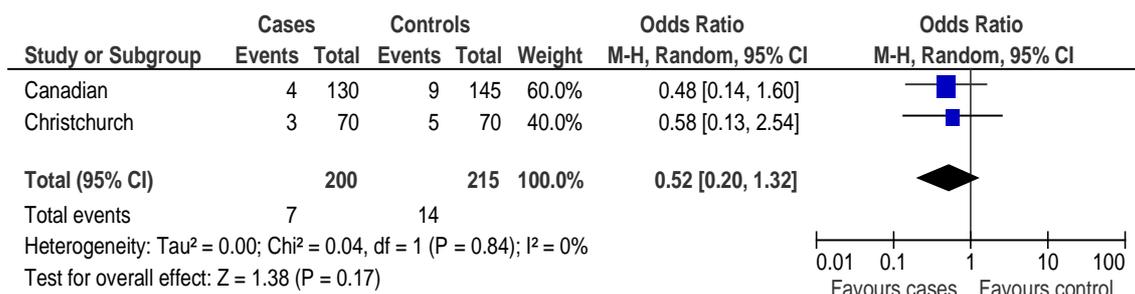
Forest plot of comparison: *COQ2* haplotype non-1 recessive, outcome: cases vs control



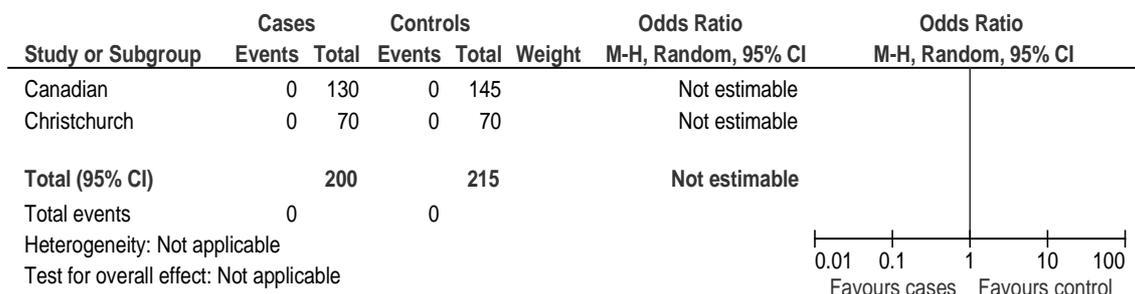
Forest plot of comparison: *AMPD1* Q12X T-dominant, outcome: cases vs controls



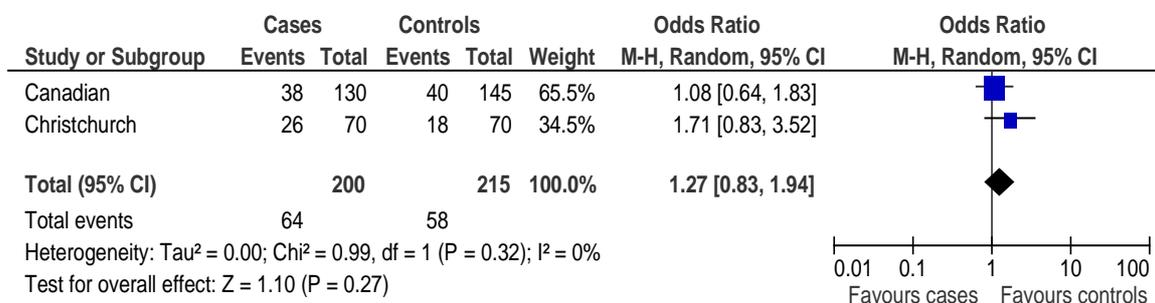
Forest plot of comparison: AMPDI Q12X T-recessive, outcome: cases vs controls



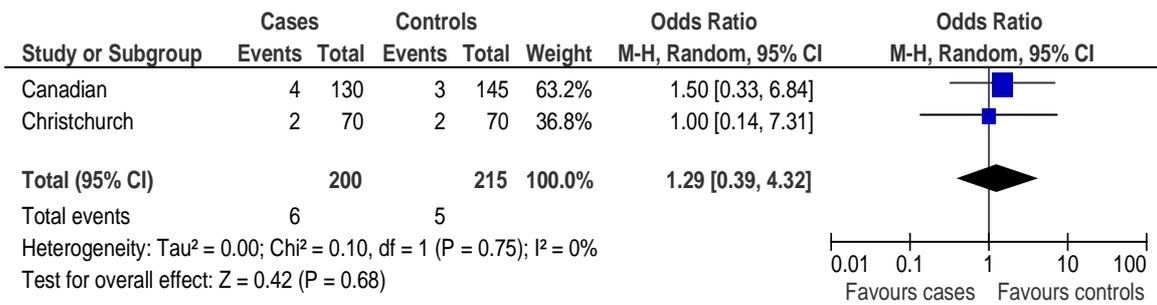
Forest plot of comparison: AMPDI K287I T-dominant, outcome: cases vs controls



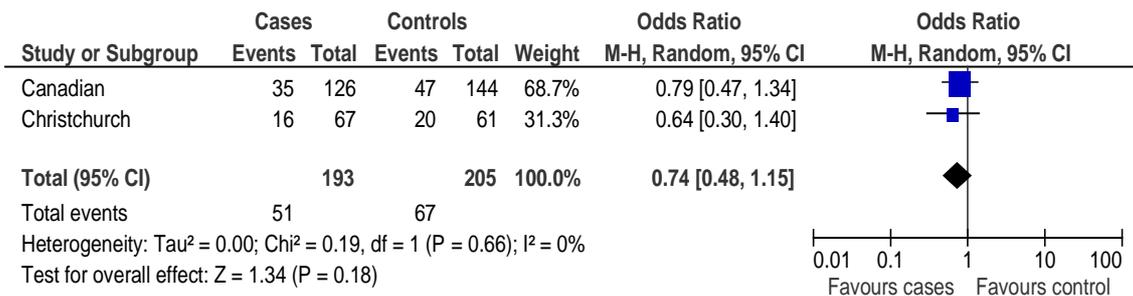
Forest plot of comparison: AMPDI K287I T-recessive, outcome: cases vs controls



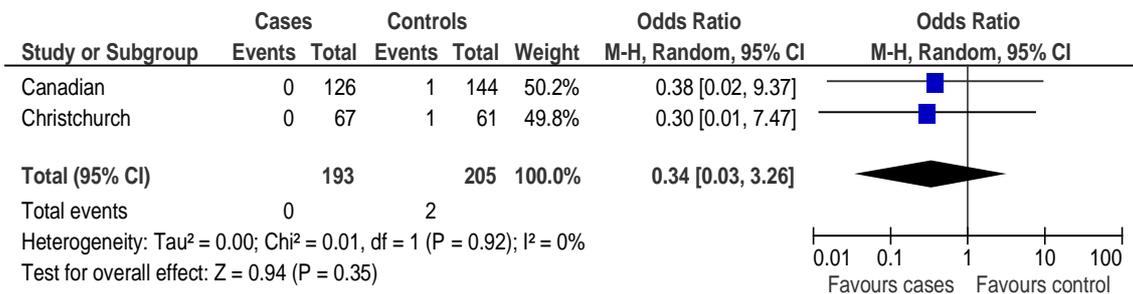
Forest plot of comparison: AMPDI haplotype non-1 dominant, outcome: cases vs controls



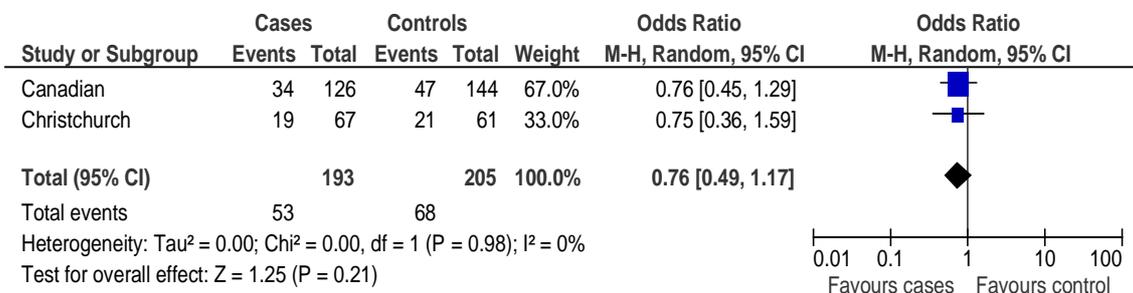
Forest plot of comparison: *AMPDI* haplotype non-1 recessive, outcome: cases vs control



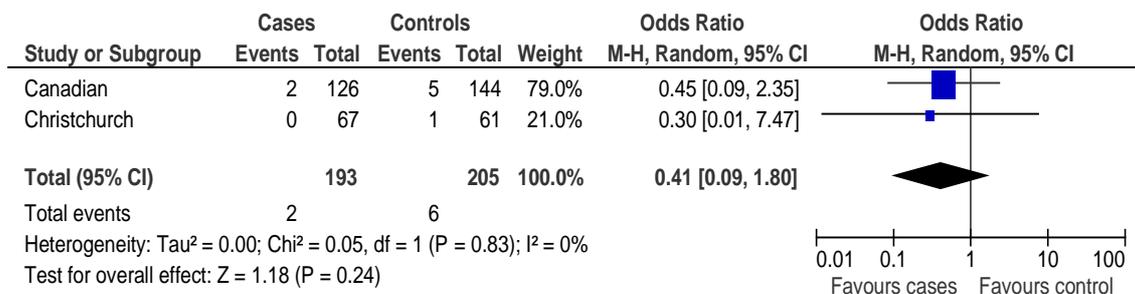
Forest plot of comparison: *SLCO1B15 C-dominant, outcome: cases vs controls**



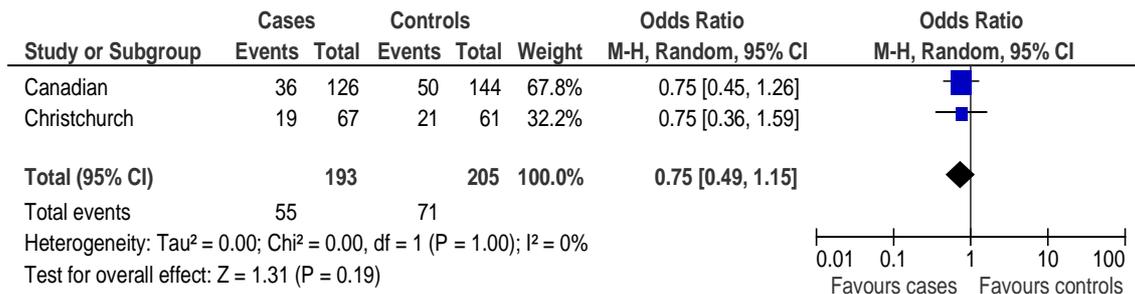
Forest plot of comparison: *SLCO1B15 C-recessive, outcome: cases vs control**



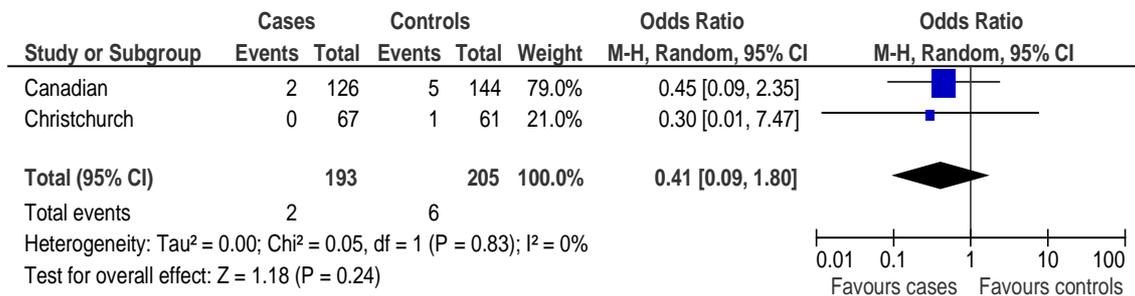
Forest plot of comparison: *SLCO1B1* C-dominant, outcome: cases vs controls



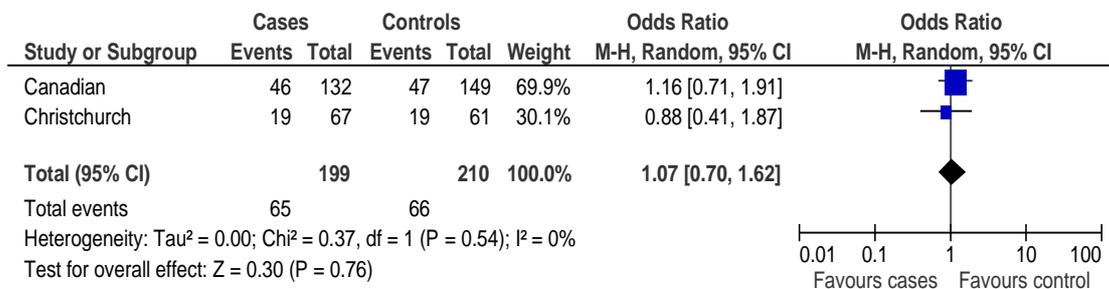
Forest plot of comparison: *SLCO1B1* C-recessive, outcome: cases vs controls



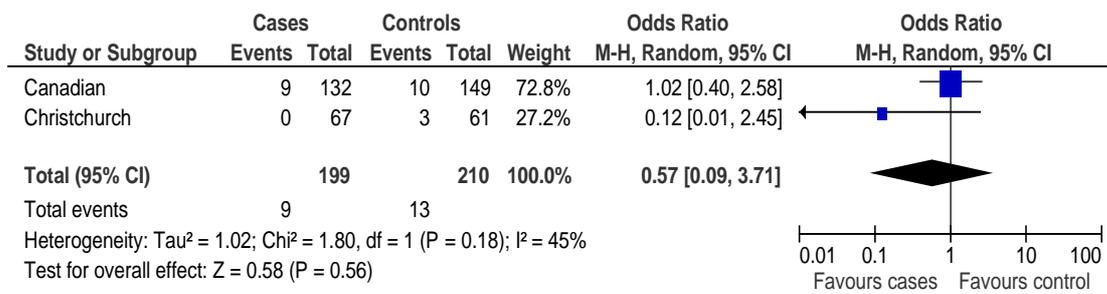
Forest plot of comparison: *SLCO1B1* haplotype non-1 dominant, outcome: cases vs controls



Forest plot of comparison: *SLCO1B1* haplotype non-1 recessive, outcome: cases vs control

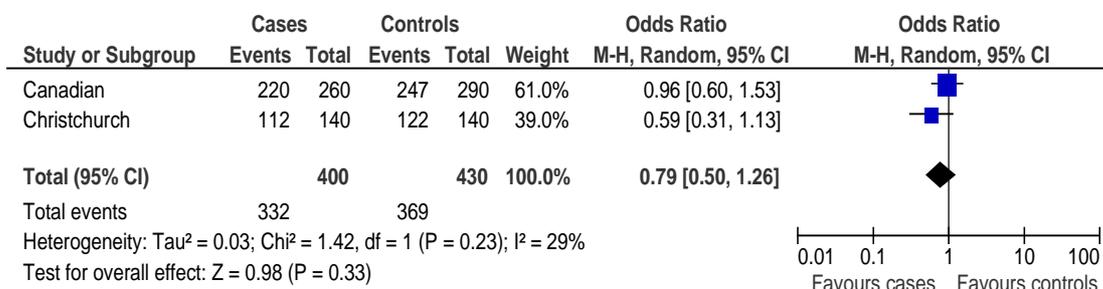


Forest plot of comparison: *CYP2D6*4* 4-dominant, outcome: cases vs controls

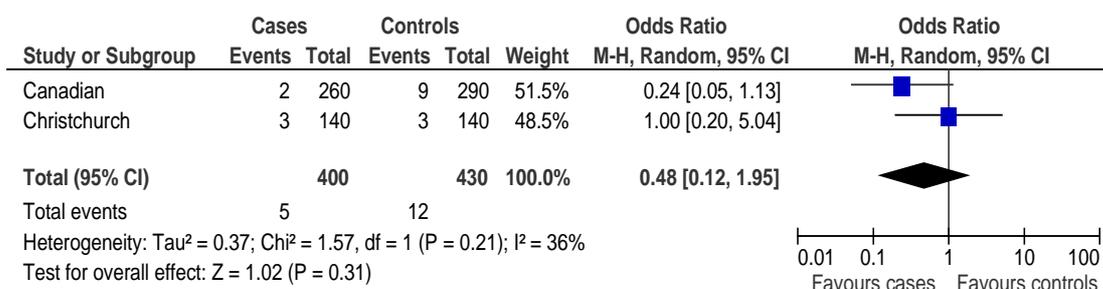


Forest plot of comparison: *CYP2D64 4-recessive, outcome: cases vs control**

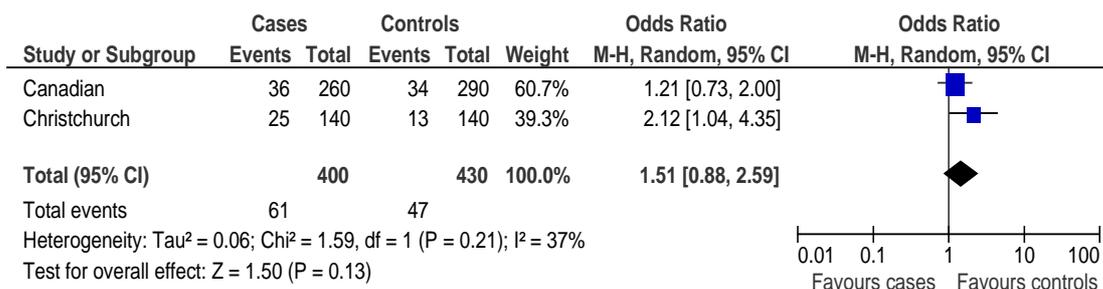
Appendix 13. Forest plots of comparison for single haplotype associations with statin intolerance (SHEsis-predicted haplotypes) (RevMan 5.0)



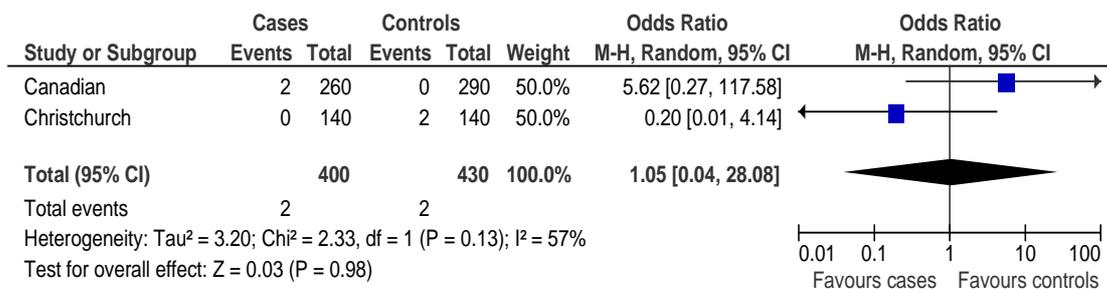
Forest plot of comparison: *AMPDI* haplotype 1, outcome: cases vs controls



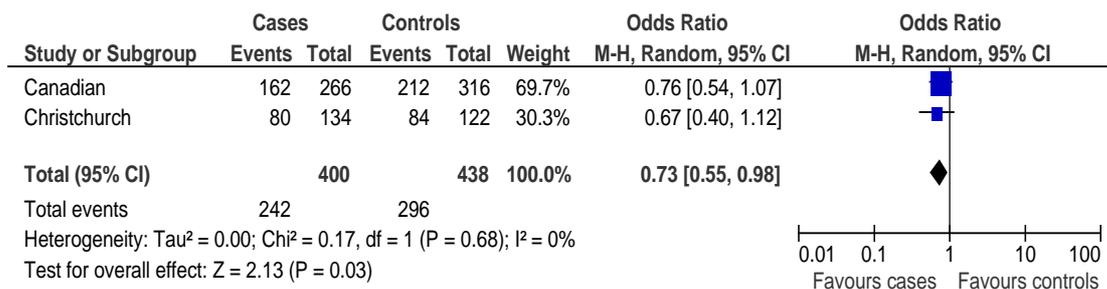
Forest plot of comparison: *AMPDI* haplotype 2, outcome: cases vs controls



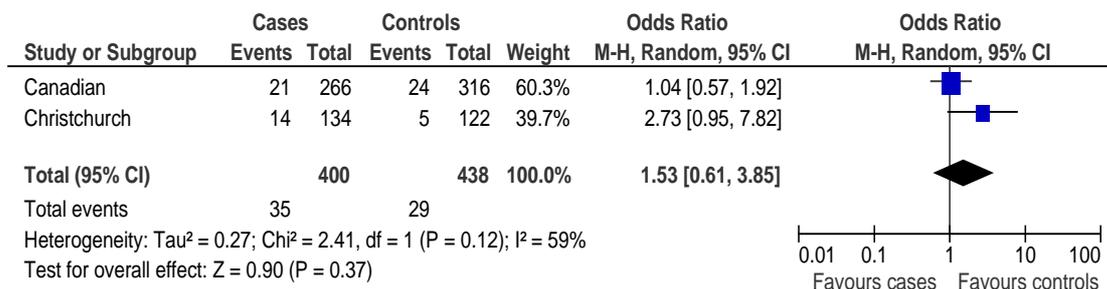
Forest plot of comparison: *AMPDI* haplotype 3, outcome: cases vs controls



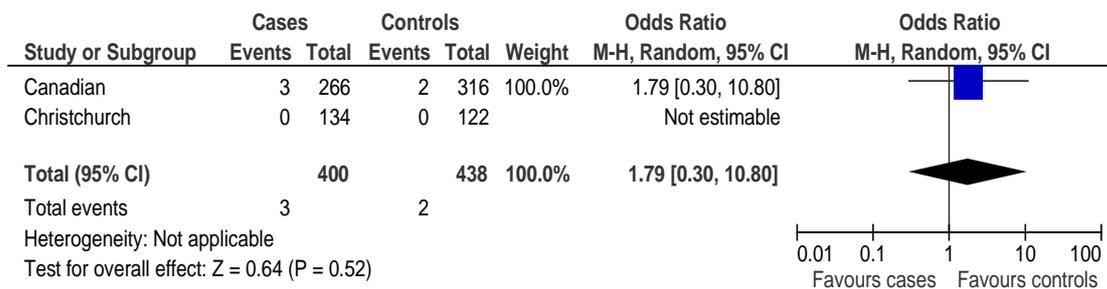
Forest plot of comparison: AMPD1 haplotype 4, outcome: cases vs controls



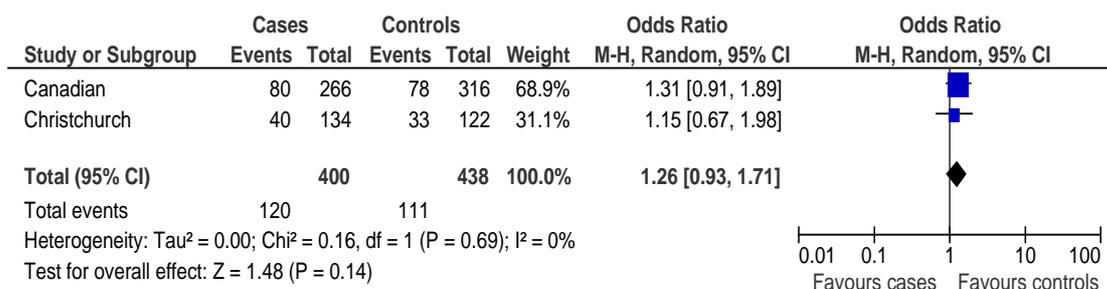
Forest plot of comparison: COQ2 haplotype 1, outcome: cases vs controls



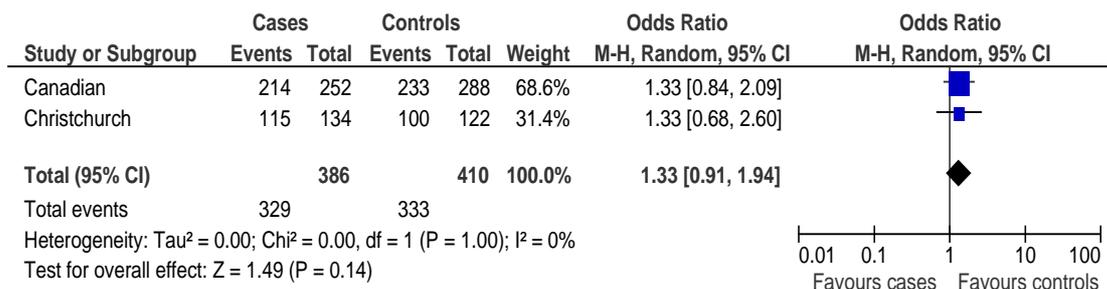
Forest plot of comparison: COQ2 haplotype 2, outcome: cases vs controls



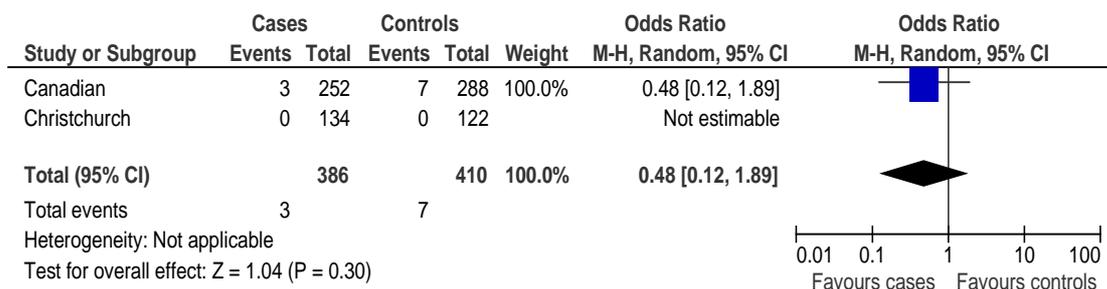
Forest plot of comparison: COQ2 haplotype 3, outcome: cases vs controls



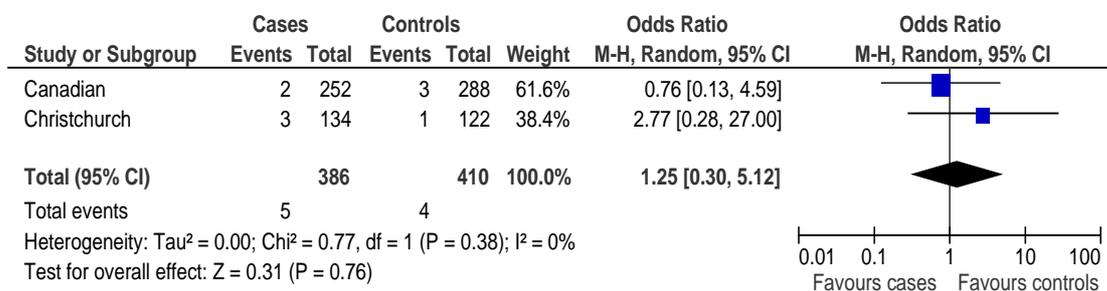
Forest plot of comparison: *COQ2* haplotype 4, outcome: cases vs controls



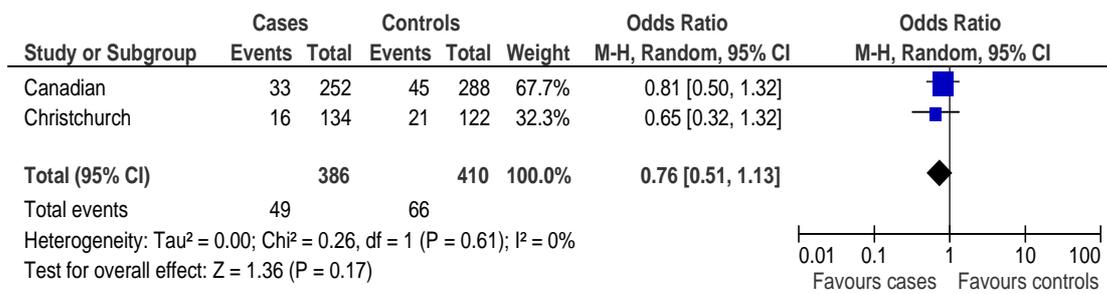
Forest plot of comparison: *SLCO1B1* haplotype 1, outcome: cases vs controls



Forest plot of comparison: *SLCO1B1* haplotype 2, outcome: cases vs controls

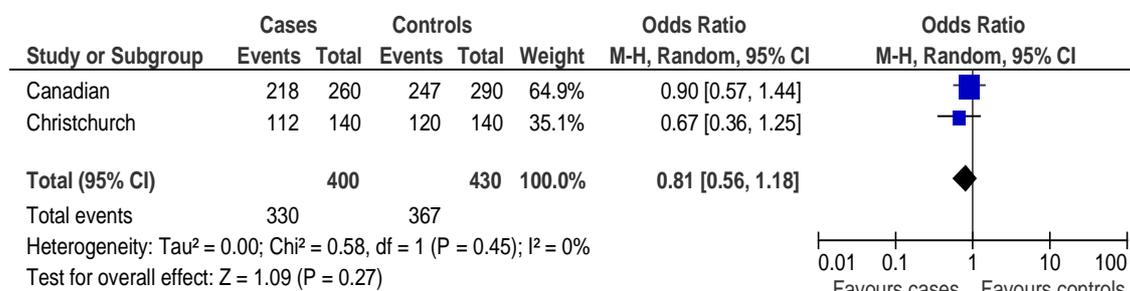


Forest plot of comparison: *SLCO1B1* haplotype 3, outcome: cases vs controls

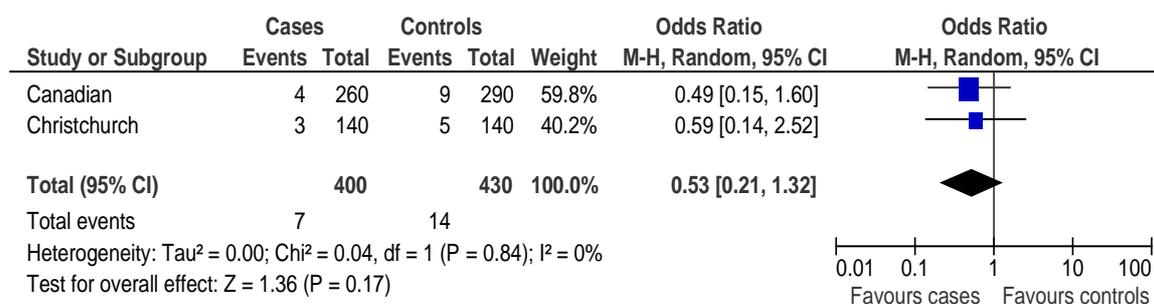


Forest plot of comparison: *SLCO1B1* haplotype 4, outcome: cases vs controls

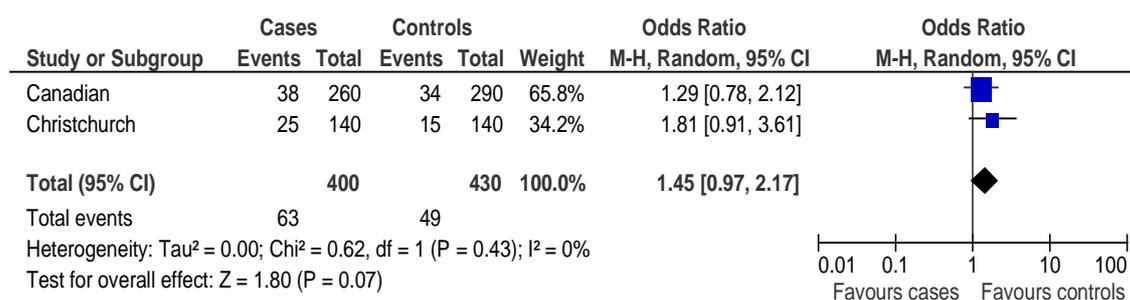
Appendix 14. Forest plots of comparison for single haplotype associations with statin intolerance (PHASE-predicted haplotypes) (RevMan 5.0)



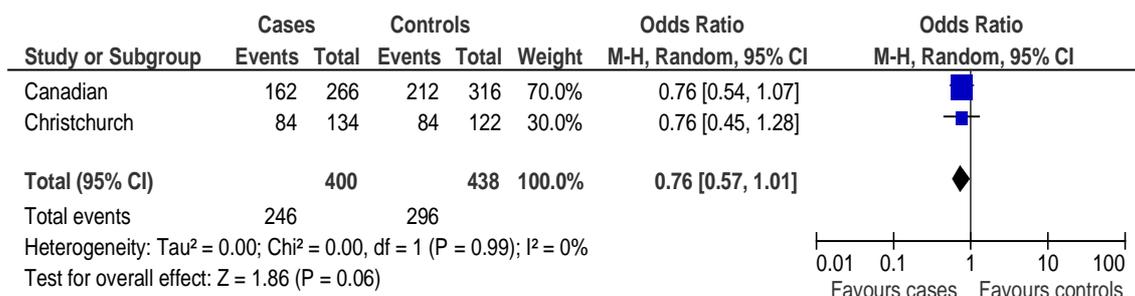
Forest plot of comparison: *AMPDI* haplotype 1, outcome: cases vs controls



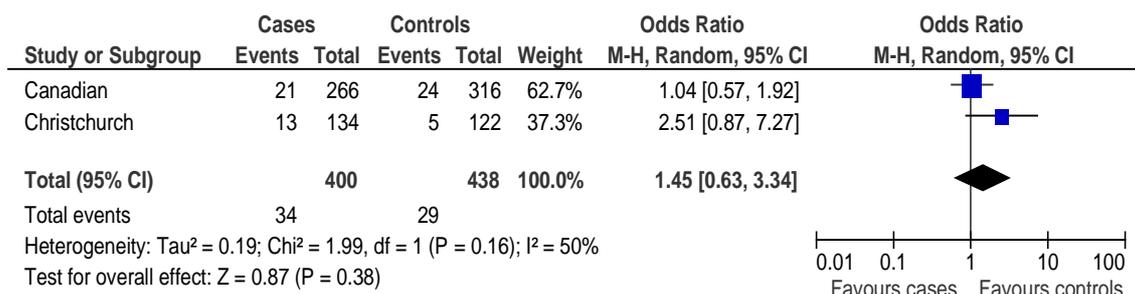
Forest plot of comparison: *AMPDI* haplotype 2, outcome: cases vs controls



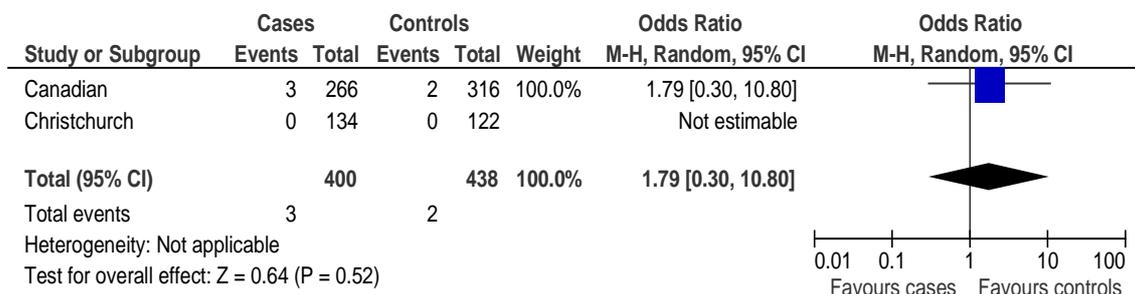
Forest plot of comparison: *AMPDI* haplotype 3, outcome: cases vs controls



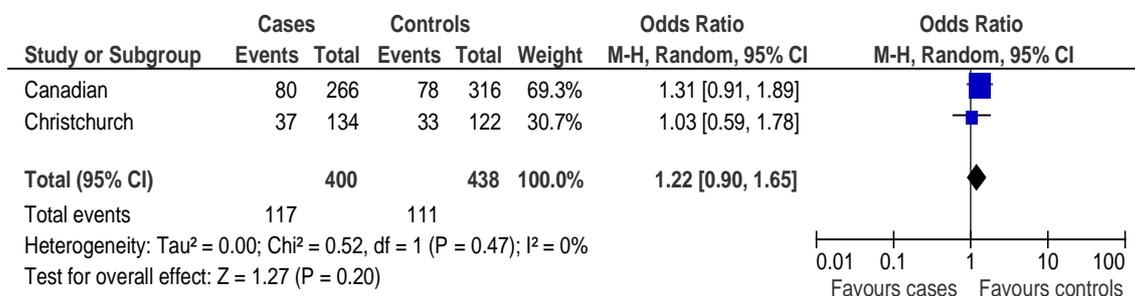
Forest plot of comparison: *COQ2* haplotype 1, outcome: cases vs controls



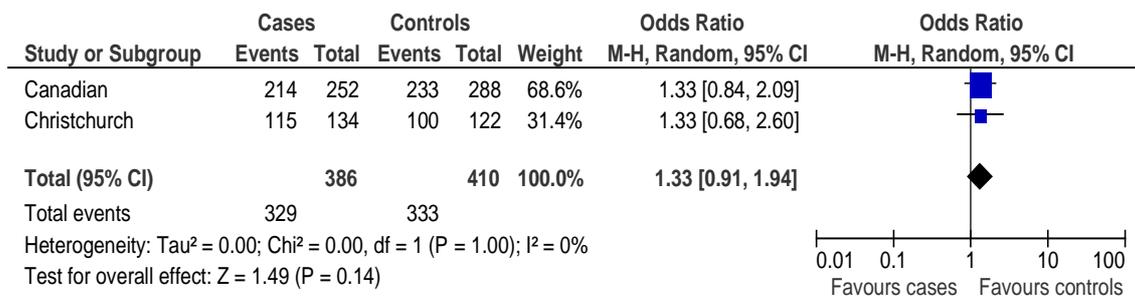
Forest plot of comparison: *COQ2* haplotype 2, outcome: cases vs controls



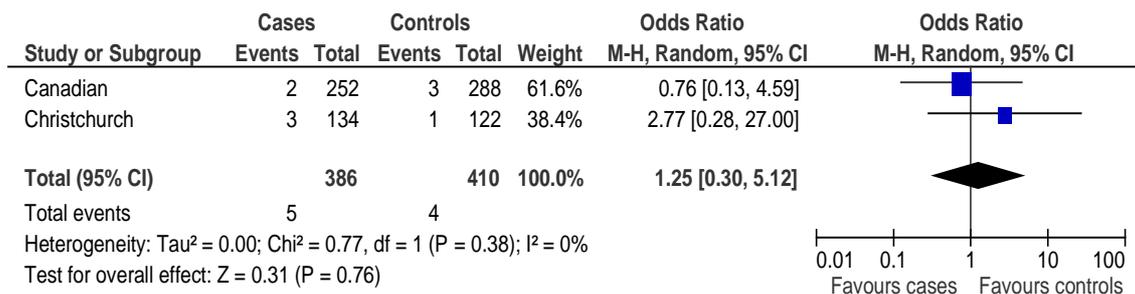
Forest plot of comparison: *COQ2* haplotype 3, outcome: cases vs controls



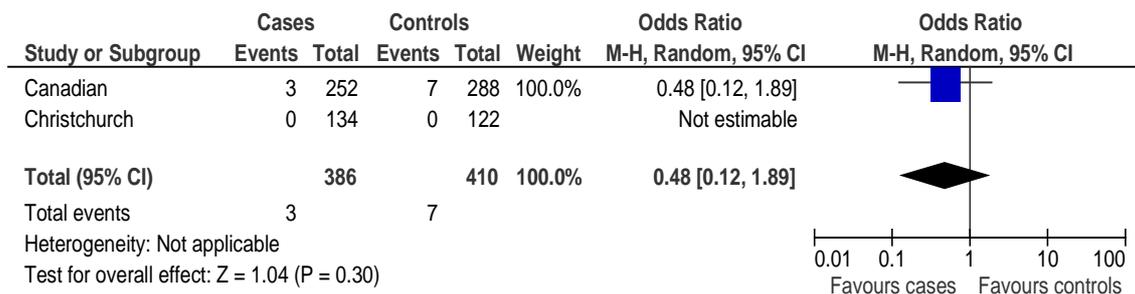
Forest plot of comparison: *COQ2* haplotype 4, outcome: cases vs controls



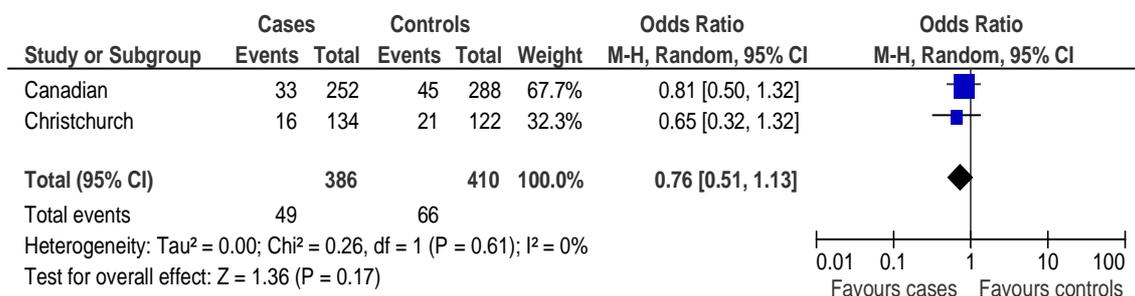
Forest plot of comparison: *SLCO1B1* haplotype 1, outcome: cases vs controls



Forest plot of comparison: *SLCO1B1* haplotype 2, outcome: cases vs controls

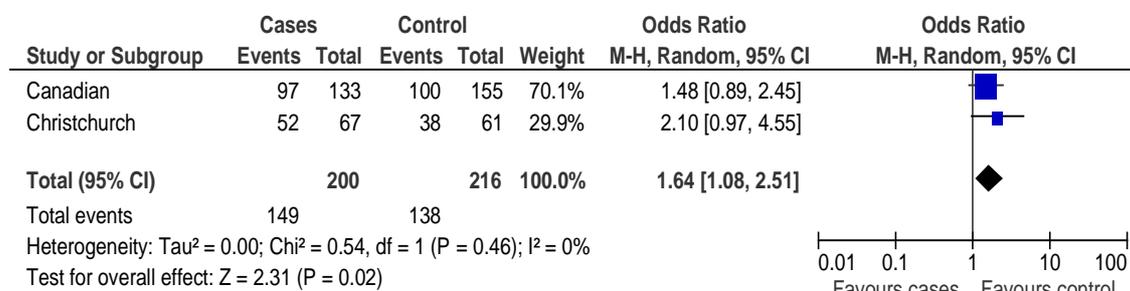


Forest plot of comparison: *SLCO1B1* haplotype 3, outcome: cases vs controls

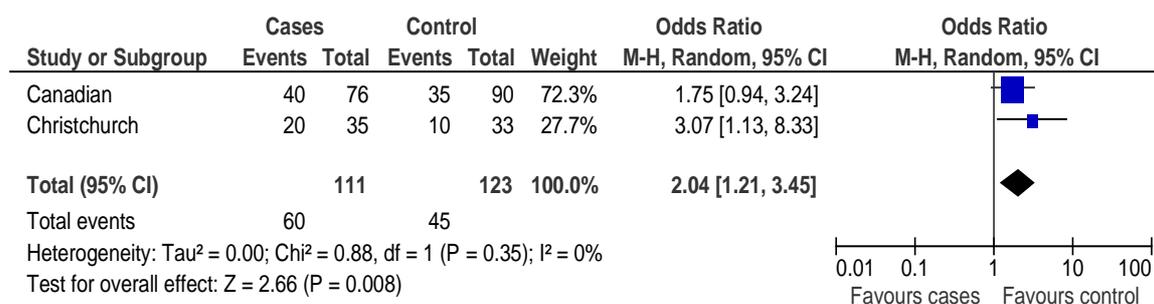


Forest plot of comparison: *SLCO1B1* haplotype 4, outcome: cases vs controls

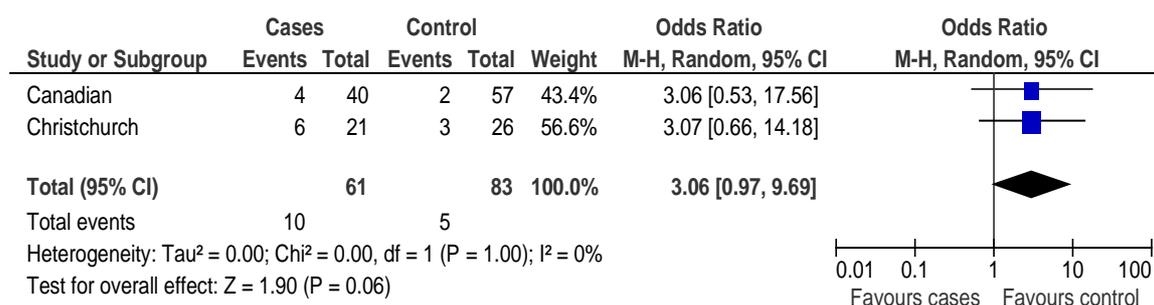
Appendix 15. Forest plots of comparison for interactions with variant alleles for *COQ2* SNP-2 and *AMPD1* Q12X (RevMan 5.0)



Forest plot of comparison: 0 vs 1 or more minor alleles, outcome: cases vs controls



Forest plot of comparison: 0 vs 2 or more minor alleles, outcome: cases vs controls



Forest plot of comparison: 0 vs 3 or more minor alleles, outcome: cases vs controls