STATIN-INDUCED MUSCLE MITOCHONDRIAL TOXICITY

by

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ABSTRACT

Statins are the mainstay of cholesterol-lowering therapy and are taken by millions of people worldwide. These drugs are generally well-tolerated but can cause myopathy ranging from mild muscle pain to fatal rhabdomyolysis. The mechanism of statin-induced myopathy (SIM) is not fully understood and there is currently no convenient and reliable marker of SIM, but mitochondrial dysfunction has been implicated.

We sought to investigate the effect of statins on mitochondrial DNA (mtDNA) levels in order to gain information on the mechanism of SIM and to explore the possibility of utilizing changing mtDNA levels as a marker of SIM.

Several approaches were used. First, mtDNA levels were quantified in skeletal muscle biopsies collected from a previously published 8-week clinical trial of high-dose simvastatin or atorvastatin versus placebo. Forty-eight hypercholesterolemic subjects were randomly assigned to receive placebo (N=16), high dose atorvastatin 40mg/day (N=16), or high dose simvastatin 80mg/day (N=16) for 8 weeks. Muscle mtDNA content was assessed by real-time PCR at baseline and after 8-weeks on statin treatment and found to be significantly reduced in the group receiving simvastatin (P=0.005) but not the other two. In addition, a significant positive correlation was observed between mtDNA and muscle ubiquinone in all groups (R=0.63, P<0.01), with the strongest association found in the simvastatin-treated subjects (R=0.75, P=0.002). Next, in an attempt to determine whether statin-induced muscle pain may be associated with muscle mtDNA depletion, archived muscle biopsies collected from statin users with muscle complaints were sought through a review of a muscle biopsy database and possible study samples were identified; however, this was put on hold as too much information was
missing from the pathology reports. Third, a series of cell culture experiments were carried out
in which human skeletal muscle myotubes were exposed to various concentrations of simvastatin
or atorvastatin, in order to determine an appropriate dose range for subsequent mitochondrial
toxicity experiments. Lastly, mtDNA content and expression was quantified in skeletal muscle
biopsies collected from 10 patients with statin-induced rhabdomyolysis (SIR) and compared to 8
healthy controls to investigate whether muscle mtDNA is altered in rhabdomyolysis. No
differences in mtDNA content or expression were observed between the two study groups, but
this may have been be due to the SIR subjects’ marked heterogeneity.

Statin therapy can be associated with considerable alterations in mtDNA content, which may
play a role in the aetiology of SIM. MtDNA levels alterations with statin exposure should be
investigated further to explore the involvement of mitochondrial alterations in the mechanism of
SIM, and determine whether these may represent a useful clinical tool for assessing statin-
induced muscle toxicity.
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<tr>
<td>4S</td>
<td>Scandinavian Simvastatin Survival Study</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ApoA1</td>
<td>apolipoprotein A1</td>
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<tr>
<td>ApoB</td>
<td>apolipoprotein B</td>
</tr>
<tr>
<td>AFCAPS/TexCAPS</td>
<td>Air Force / Texas Coronary Atherosclerosis Prevention Study</td>
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<tr>
<td>AIF</td>
<td>apoptosis-inducing factor</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine transaminase</td>
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<tr>
<td>ASCOT-LLA</td>
<td>Anglo-Scandinavian Cardiac Outcomes Trial – Lipid Lowering Arm</td>
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<tr>
<td>ASPG</td>
<td>accessory subunit of the human polymerase gamma</td>
</tr>
<tr>
<td>AST</td>
<td>aspartate transaminase</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>AZT</td>
<td>zidovudine</td>
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<tr>
<td>BL</td>
<td>baseline</td>
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<tr>
<td>CARE</td>
<td>Cholesterol and Recurrent Events</td>
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<tr>
<td>CCOI</td>
<td>cytochrome c oxidase subunit I</td>
</tr>
<tr>
<td>CK</td>
<td>creatine kinase</td>
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<tr>
<td>Cmax</td>
<td>peak concentration</td>
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<tr>
<td>COQ2</td>
<td>coenzyme Q2</td>
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<tr>
<td>COX</td>
<td>cytochrome c oxidase</td>
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<tr>
<td>CV</td>
<td>coefficient of variation</td>
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<tr>
<td>CYP</td>
<td>cytochrome P450</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>-----------------------------------------------------------------------------</td>
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<td>ETC</td>
<td>electron transport chain</td>
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<td>EXCEL</td>
<td>Expanded Clinical Evaluation of Lovastatin</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>GAPDH</td>
<td>human glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>HDL-C</td>
<td>high-density lipoprotein cholesterol</td>
</tr>
<tr>
<td>HFH</td>
<td>heterozygous familial hypercholesterolemia</td>
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<tr>
<td>HMG CoA</td>
<td>3-hydroxy-3-methyl-glutaryl-Coenzyme A</td>
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<tr>
<td>HPS</td>
<td>Heart Protection Study</td>
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<tr>
<td>hSkMC</td>
<td>human skeletal muscle cells</td>
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<tr>
<td>IQR</td>
<td>interquartile range</td>
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<tr>
<td>LC</td>
<td>LightCycler®</td>
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<tr>
<td>LDL-C</td>
<td>low-density lipoprotein cholesterol</td>
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<tr>
<td>LIPID</td>
<td>Long-Term Intervention with Pravastatin in Ischaemic Disease</td>
</tr>
<tr>
<td>MELAS</td>
<td>mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes</td>
</tr>
<tr>
<td>MERRF</td>
<td>myoclonus epilepsy associated with ragged-red fibres</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>MIRACL</td>
<td>Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>mtDNA</td>
<td>mitochondrial DNA</td>
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<td>mtRNA</td>
<td>mitochondrial RNA</td>
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<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide</td>
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<tr>
<td>nDNA</td>
<td>nuclear DNA</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>NRTI</td>
<td>nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>OCT</td>
<td>optimal cutting temperature compound</td>
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<tr>
<td>OXPHOS</td>
<td>oxidative phosphorylation</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PRIMO</td>
<td>Prediction du Risque Musculaire en Observationnel</td>
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<tr>
<td>PROVE-IT</td>
<td>Pravastatin or Atorvastatin Evaluation and Infection Therapy</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RPDCT</td>
<td>randomized placebo-controlled double-blind controlled trial</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SkGM</td>
<td>skeletal muscle growth medium</td>
</tr>
<tr>
<td>SIM</td>
<td>statin-induced myopathy</td>
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<tr>
<td>SIR</td>
<td>statin-induced rhabdomyolysis</td>
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<tr>
<td>TNT</td>
<td>Treating to New Targets</td>
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<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>UBC</td>
<td>University of British Columbia</td>
</tr>
<tr>
<td>ULN</td>
<td>upper limit of normal</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
</tr>
<tr>
<td>VGH</td>
<td>Vancouver General Hospital</td>
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<tr>
<td>VLDL-C</td>
<td>very-low density lipoprotein cholesterol</td>
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<tr>
<td>WOSCOPS</td>
<td>West of Scotland Coronary Prevention Study</td>
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DEDICATION

To my family
CHAPTER 1: INTRODUCTION

1.1 THE BURDEN OF CARDIOVASCULAR DISEASE

Despite tremendous advancements in medicine, cardiovascular disease (CVD) continues to be a major public health problem around the world. Indeed, CVD is estimated to be the leading global cause of death and disability (Murray and Lopez, 1997); in 1998 it accounted for an estimated 31% of all deaths, in addition to 10% of the total disease-related burden in terms of disability-adjusted life year loss (The World Health Report, 1999). In the past few decades, the age-standardized rate of CVD mortality has declined in several developed nations, mostly in North America and Western Europe. This is due to a variety of reasons but can be in part attributed to the availability and utilization of numerous medications including aspirin, angiotensin-converting enzyme inhibitors, beta-adrenoreceptor antagonists, and the cholesterol-lowering drugs 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitors (statins) (Yusuf, 2002). Nevertheless, CVD remains a heavy burden in these regions. For example, in the United States (US) CVD is still the leading cause of death, today responsible for approximately 1 out of every 5 deaths (Gibbons et al, 2003).

In addition, the rate of CVD has risen considerably in many developing nations. The World Health Organization estimates that 80% of the current global burden of CVD is in the developing world in such areas as Latin America, the Middle East, and sub-Saharan Africa (Murray and Lopez, 1997; Callow, 2006). In developing nations, heart disease is projected to increase from 1990 to 2020 by 137% in men and 120% in women, compared to only 48% and 29%, respectively, in developed nations; a similar pattern is expected for cerebrovascular disease mortality (Ounpuu et al, 2001). Possible factors for the trend of increased CVD in developing countries include decreased mortality rates from infectious diseases with an accompanying older
population, and increased urbanization, industrialization, and automation with accompanying lifestyle changes associated with CVD risk factors, such as sedentary lifestyle (Ounpuu et al, 2001). Rising incidences in cigarette smoking, elevated body weight, and diabetes, in addition to a decrease in physical activity are also likely partially accountable for this trend.

1.2 LINKING CVD TO CHOLESTEROL

Since the mid-twentieth century, intensive research efforts including basic science laboratory analyses, clinical trials, and observational epidemiological surveys of entire populations have identified numerous individual causal risk factors for the development of CVD. These include cigarette smoking, high blood pressure, and dyslipidemia.

The Framingham study began in the 1950s and eventually demonstrated a firm correlation between elevated plasma cholesterol and CVD mortality (Kannel, 1995). Later studies attributed increased rates of CVD deaths to elevated blood low-density lipoprotein cholesterol (LDL-C), which typically comprises approximately 70% of total plasma cholesterol. From these studies came the 'lipid hypothesis': that an increased concentration of LDL-C in the blood is causally related to the development of CVD, and that reduced LDL-C would reduce the risk for CVD including myocardial infarction (MI).

A recent major study that strongly supports this notion is INTERHEART, a case-control study of acute MI carried out in 52 countries of 15,152 cases and 14,820 controls (Yusuf et al, 2004). The two most important factors were smoking and abnormal blood lipids; together these two risk factors accounted for approximately two-thirds of the risk for MI. In all geographic regions, the most important risk factor was apolipoprotein B (apoB) / apolipoprotein A1 (apoA1) ratio. ApoB is found in atherogenic particles, mainly LDL-C (Schaefer et al, 1994; Graziani et al, 1998; Westerveld et al, 1998), while ApoA1 is found in the anti-atherogenic high-density lipoprotein (HDL) particles (O’Brien et al, 1995; Garfagnini et al, 1995; Francis and Frohlich,
2001). It is now well-recognized that blood lipid levels, in particular elevated LDL-C, play a major role in the formation and progression of the atherosclerotic process.

By the mid-1980s enough evidence had accumulated for the US National Institutes of Health (NIH) to announce that lowering LDL-C, through dietary alterations or with drug therapy would reduce the risk of coronary events (NIH Consensus Development Conference, 1985). The discovery of statins would revolutionize the treatment of dyslipidemia.

1.3 THE DEVELOPMENT OF STATINS

Mammalian cholesterol synthesis is a complex process involving over 30 enzymes, and although it can occur in most cells, it takes place mainly in hepatocytes (Figure 1.1).
Figure 1.1: The cholesterol biosynthesis pathway.
Statins inhibit HMG CoA reductase, a microsomal enzyme which catalyzes this pathway's rate-limiting step: the conversion of HMG CoA to mevalonate. Intracellular mevalonate formation is thus inhibited, as is the downstream product cholesterol. However, mevalonate is a precursor for numerous other compounds, with varying functions indicated in *italics*. One of these compounds is ubiquinone, a key cofactor of the electron transport chain in the mitochondrion. This is a simplified schematic of the actual pathway which involves over 30 enzymes; for example there are 19 steps between squalene and cholesterol. References: Corsini *et al*, 1999; Tobert, 2003.
The rate-limiting step in cholesterol biosynthesis is the conversion of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG CoA) reductase to mevalonate by the enzyme HMG CoA reductase. The first HMG CoA reductase inhibitor (statin) was discovered by Dr. Akiro Endo in the mid-1970s (Endo et al, 1976; Endo et al, 1977), and in 1978 Merck Research Laboratories developed lovastatin, the first statin commercially marketed for clinical use (Alberts et al, 1980).

Numerous randomized, double-blind, placebo-controlled clinical trials showed lovastatin to be effective at reducing LDL-C in healthy volunteers (Tobert, 1982), patients with heterozygous familial hypercholesterolemia (HFH) (Havel et al, 1987), and in patients with coronary heart disease and without HFH (Lovastatin Study Group II, 1986). In 1987 lovastatin was approved by the US Food and Drug Administration (FDA) (Tobert, 2003).

Lovastatin (Mevacor®) revolutionized dyslipidemia treatment due to its ability to reduce LDL-C by an average of 40% at its maximal recommended dose of 80 mg daily, its excellent safety profile, and its ease of use (Lovastatin Study Group II, 1986; Havel et al, 1987; Lovastatin Study Group III, 1988; Lovastatin Study Group IV, 1990). Prior to the arrival of lovastatin for clinical use in 1987, clinicians had a limited selection of lipid-altering therapies at hand. Dietary advice, bile-acid sequestrants (cholestryamine, colestipol), niacin, fibrates (eg. gemfibrozil, fenofibrate, bezafibrate), and probucol were available, but were neither efficacious nor universally well-tolerated. Other statins began to be approved soon after: simvastatin (Zocor®) in 1988, pravastatin (Pravachol®) in 1991, fluvastatin (Lescol®) in 1994, atorvastatin (Lipitor®) in 1997, cerivastatin (Baycol®) in 1998, and rosuvastatin (Crestor®) in 2003, and the early statin mega-trials showed undeniable evidence of the efficacy and safety of this class of drugs (Figure 1.2).
Figure 1.2: Chemical structures of the statins.
The HMG CoA-like moiety of each statin acts on the target enzyme: HMG CoA reductase. Lovastatin and pravastatin are derived naturally from fungi, simvastatin is a semi-synthetic derivative of lovastatin, and these 3 statins are structurally similar. Simvastatin and lovastatin are administered as inactive pro-drugs that are converted to lactone form while pravastatin is administered in active form with an open acid structure. All other statins are completely synthetic and have the open acid form. Inter-statin structural differences account for the variable pharmacologic properties of these drugs. HMG CoA = hydroxymethylglutaryl coenzyme A. Reproduced from Shitara and Sugiyama, 2006.
1.4 THE STATIN TRIALS

The Scandinavian Simvastatin Survival Study (4S) of 4,444 patients with CVD and moderate LDL-C elevations was a landmark trial that dispelled any doubts about the ‘lipid hypothesis’ by unequivocally demonstrating in a randomized placebo-controlled double-blinded clinical trial (RPDCT) that using a statin to reduce blood cholesterol reduced all-cause mortality (Scandinavian Simvastatin Survival Study Group, 1994). In the West of Scotland Coronary Prevention Study (WOSCOPS), another large RPDCT, pravastatin lowered LDL-C levels by 26% and significantly reduced non-fatal MI and death from cardiovascular causes in 6,595 hypercholesterolemic men with no history of MI, with an average follow-up of 4.9 years (P <0.001) (Shepherd et al, 1995). Numerous other large-scale clinical trials also showed the efficacy and safety of statins (Plosker and Wagstaff, 1996; Sacks et al, 1996; Downs et al, 1998; LIPID Study Group, 1998) (Figure 1.3).

A growing number of studies continue to demonstrate the benefits of statin therapy, including the Treating to New Targets (TNT) trial (Waters DD et al, 2004), the Anglo-Scandinavian Cardiac Outcomes Trial – Lipid Lowering Arm (ASCOT-LLA) study (Sever et al, 2003), and the Heart Protection Study (HPS) (Heart Protection Study Collaborative Group, 2002).
Figure 1.3: Landmark statin trials.
The major statin trials are presented in descending order of patient risk for cardiovascular disease (CVD). At the top of the pyramid is the Scandinavian Simvastatin Survival Study (4S), an early trial of simvastatin in high-risk patients, followed by the Heart Protection Study (HPS), the Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) study, and the Cholesterol and Recurrent Events (CARE) trial; all of these included higher-risk patients and were studies of secondary prevention. The lower 3 trials, the West of Scotland Coronary Prevention Study (WOSCOPS), the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT), and the Air Force / Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS), were primary prevention studies of lower-risk individuals. References are in the main text.
Thus, the therapeutic principle that lowering blood cholesterol reduces CVD risk has been well-established in numerous large trials. Adverse side effects in muscle and other tissues were noticed but occurred at very low frequencies (see below section, “Statin-induced myopathy”) (LaRosa et al, 1999). Accordingly, statins are well-recognized as an effective and safe method of anti-atherosclerotic therapy, and these drugs continue to be recommended as first-line treatment for dyslipidemia, as in the latest US National Cholesterol Education Program guidelines (National Cholesterol Education Program, 2002).

1.5 STATINS’ MECHANISM OF ACTION

The primary mechanism of statin therapy’s anti-CVD effect is by inhibiting cholesterol synthesis and reducing circulating levels of LDL-C. These drugs target the liver, which is where the majority of endogenous cholesterol synthesis occurs in mammals (Grundy, 1978). Statins reversibly, selectively, and competitively inhibit the enzyme that catalyses the rate-limiting step of cholesterol synthesis, HMG CoA reductase. This enzyme is thought to be localized in the endoplasmic reticulum (ER) and in peroxisomes (Hogenboom et al, 2002). The HMG-like moiety of these drugs occupies the HMG binding site of HMG CoA reductase, thus sterically inhibiting substrate binding. Statins’ affinity for the active site of HMG is several orders of magnitude higher than HMG CoA (Istvan and Deisenhofer, 2001). This inhibition reduces intracellular levels of mevalonate (Pappu and Illingworth, 1989; Naoumova et al, 1996) which leads to a decreased amount of sterols, which in turn stimulates upregulation of several molecules including HMG CoA reductase (Brown and Goldstein, 1980) and other enzymes involved in cholesterol biosynthesis (Balasubramaniam et al, 1977; Bergstrom et al, 1984). Importantly, the LDL receptor is also upregulated (Kovanen et al, 1981; Ma et al, 1986; Reihner et al, 1990). These changes result in increased uptake of LDL-C by LDL receptors on
hepatocytes and thus a decreased amount of LDL-C in the bloodstream. Statin-induced LDL-C lowering thus decreases CVD events such as MI by slowing the progression of the atherosclerotic plaque development and preventing atheroma formation in the coronary arterial wall (Illingworth and Tobert, 1994) (Figure 1.4).
Figure 1.4: Statins’ mechanism of action.
Simplified schematic illustrating the major way that statins reduce the risk for cardiovascular diseases (CVD) such as heart attack and stroke. Statins target the liver which is where the majority of endogenous cholesterol synthesis occurs, along the mevalonate pathway. These drugs are competitive inhibitors of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, an enzyme that catalyzes the rate-limiting step in the cholesterol synthesis pathway. This action leads to reduced levels of cholesterol within hepatocytes, which in turn stimulates hepatocytes to upregulate the expression of low-density lipoprotein cholesterol (LDL-C) receptors on their surface. LDL-C (the “bad cholesterol”) is subsequently removed from the blood by hepatocytes. Decreased concentration of blood LDL-C confers reduced risk for CVD.
1.6 STATIN POPULARITY

Statins are a multibillion dollar industry. In 2004 in the US, statins outsold any other type of prescription medication with an estimated $15.5 billion in sales and 12% growth in that year; Lipitor® (atorvastatin) alone earned $7.7 billion and grew by 14%, representing 50% of the class (IMS Health, 2005). Estimates of the current total number of patients on statin therapy are variable but in 2002 approximately 25 million people worldwide were being treated with statins (Mitka, 2003), and at present there are an estimated 14 million statin users in the US (Boltan et al, 2007). Canadian physicians filled 15 million statin prescriptions in 2003 (CBC News, 2004). Currently, Lipitor® and Zocor® (simvastatin) are respectively the #1 and #2 most prescribed drugs in the US (Consumer Reports, 2007).

Statins' massive popularity is attributed mostly to the widespread prevalence of dyslipidemia, their well-documented efficacy and good safety profile, and massive marketing campaigns targeting both clinicians and patients (Tobert, 2003). In addition, recent scientific evidence suggests that statins have non-lipid-altering beneficial health effects.

1.7 STATIN PLEIOTROPY

Numerous non-lipid-related properties of statins have been reported (Mason et al, 2005). Some studies have associated statin therapy with improved endothelial function in the coronary arteries, which is associated with relief from the symptoms associated with myocardial ischemia (Treasure et al, 1995; Anderson et al, 1995). Others have provided evidence suggesting statins interfere with the adhesion of monocytes to the arterial wall, an early step in the process of the formation of an atherosclerotic plaque (Weber et al, 1997). As early as 1993, statins were reported to inhibit the modification, such as oxidation, of LDL-C particles (Kleinveld et al, 1993). Statin therapy is also known to increase the amount of “good cholesterol,” high-density lipoprotein cholesterol (HDL-C), in blood by approximately 5 to 10% (Downs et al, 1998;...
Vreder et al, 2003). In addition, statin treatment is associated with significant reductions in blood concentrations of triglycerides (Stein et al, 1998; Wierzbicki et al, 2000). It is becoming increasingly well-recognized that statins can have a stabilizing effect on atherosclerotic plaques (Paradiso-Hardy et al, 2003). Finally, accumulating evidence suggests that statins possess potent anti-inflammatory properties that have beneficial effects on vascular endothelial function, immune cells, smooth muscle cells, and platelets (see Jain and Ridker, 2005).

Indeed, statin therapy has been associated with improvements in a variety of conditions, including cerebrovascular disease and stroke (Sever et al, 2003), dementia (including Alzheimer's disease) and osteoporosis (Waldman and Kritharides, 2003), and cancer (Heart Protection Study Collaborative Group, 2002; Weis et al, 2002). Other suggested statin-pleiotropy conditions include multiple sclerosis, diabetes, depression, peripheral artery disease, idiopathic dilated cardiomyopathy, and ventricular arrhythmias (Raja and Dreyfus, 2004). At present the precise mechanisms for these supposed pleiotropic effects are unknown.

1.8 STATIN TOLERABILITY

Statin therapy is well tolerated by most patients. The following clinical trials showed no increased rate of adverse events compared to placebo: Expanded Clinical Evaluation of Lovastatin (EXCEL) (Bradford et al, 1991), 4S (Scandinavian Simvastatin Survival Study Group, 1994), Cholesterol and Recurrent Events Trial (CARE) (Sacks et al, 1996), WOSCOPS (Shepherd et al, 1995), Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS) (Downs et al, 1998), Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering (MIRACL) study (Schwartz et al, 1998), and the HPS (Heart Protection Study Collaborative Group, 2002).

Unfortunately, the same high level of tolerability does not appear to be present in the “real-world” setting of clinical practice. This may be due to rigorous clinical trials exclusion
criteria that likely skew the data by selecting a specific healthy population. Individuals with factors that may increase the risk for any side effect are usually excluded from participating in trials of new drugs. This typically includes the elderly, polypharmacy patients, patients with medical conditions, especially hepatic or renal dysfunction and, in the case of statin trials, those with pre-existing muscle symptoms or high creatine kinase (CK) levels. Other patient groups may be under-represented in clinical trials, such as women, children, non-caucasians, patients with a personal or family history of muscle pain or elevated CK levels, and heavy alcohol users. All of these patients are at higher risk for developing muscle pain while on statin therapy, in addition to other drug intolerance (Thompson et al, 2006).

1.9 STATIN SIDE EFFECTS (NON-MUSCLE)
Statins are associated with a variety of adverse side effects in humans, including liver (Heuer et al, 2000; Pelli and Setti, 2004; Wolters and Van Buuren, 2005; Bays, 2006; Jacobson, 2006), and kidney (Bays, 2006; Kasiske et al, 2006) impairment, peripheral neuropathy (Brass et al, 2006), and non-serious reports of constipation, flatulence, dyspepsia, and generalized gastrointestinal discomfort (Evans and Rees, 2002). Muscle pain, however, is the statin side effect that has received by far the most attention from researchers.

1.10 STATIN-INDUCED MYOPATHY
Statin therapy has been associated with a variety of muscle-related complaints, and this adverse side effect, referred to as statin-induced myopathy (SIM), is the most serious, has the highest frequency, and has received by far the most attention (Thompson et al, 2006). These complaints range from mild weakness or pain to full-blown and potentially fatal rhabdomyolysis, characterized by severe muscle breakdown, myoglobinuria, myoglobinemia, acute renal failure
and CK level greater than 10 times the ULN (Pasternak et al, 2002; Bellosta et al, 2004; Rosenson, 2004).

In terms of typical clinical management of SIM, statin therapy is ceased until muscle symptoms resolve. Barring suspicions of severe myopathy, patients are re-challenged with either the same or a different statin, as described in clinical guidelines (Pasternak et al, 2002). The subsequent return of symptoms confirms the association between statin treatment and muscle pain, and alternate treatment options are explored including fibrates, niacin, resins, and cholesterol absorption inhibitors. A common alternative is a combination of fluvastatin, the statin with the lowest associated rate of myopathy, and ezetimibe, an absorption inhibitor. Patients with prolonged or complicated muscle problems associated with statin treatment may be referred to a neurologist or rheumatologist and have a muscle biopsy performed to confirm the diagnosis and exclude other possible underlying conditions (Evans and Rees, 2002; Omar and Wilson, 2002). In some cases, muscle symptoms and CK elevations persist long after statin withdrawal, but this is quite rare (Thompson et al, 2003; Hansen et al, 2005). The time of onset of SIM upon initiation of statin therapy is very variable (Hansen et al, 2005), but most commonly occurs at about 1 month (Bruckert et al, 2005).

SIM is definitely of concern in tertiary lipid clinics with their selected patient populations. However, it is also of increasing concern to family physicians who now routinely prescribe statins, not only for secondary but also for high-risk primary prevention.

1.11 CERIVASTATIN

The existence and clinical relevance of SIM was underscored when cerivastatin was withdrawn on 08/08/2001. Bayer introduced cerivastatin (Baycol®) in 1998 and voluntarily withdrew it in 2001 due an unacceptably high rate of rhabdomyolysis. Fifty-two cases of rhabdomyolysis-related death in cerivastatin users were documented worldwide (Farmer, 2001). Most of these
patients had been taking high doses (0.8 mg/day; cerivastatin is much more potent than the other statins) (Furberg and Pitt, 2001). Twelve were co-treated with gemfibrozil (Staffa _et al_, 2002; Roca _et al_, 2002), a fibrate known to increase peak blood concentrations of cerivastatin (Backman _et al_, 2002). Cerivastatin carried a much higher risk, 16 to 20 times higher, of fatal rhabdomyolysis than the other statins (Ballantyne _et al_, 2003; Raine, 2001), for unknown reasons (Tobert, 2003).

**1.12 SIM NOMENCLATURE**

Inconsistent nomenclature is a major feature of SIM literature. Numerous terms, including myopathy, myalgia, myositis, muscle toxicity/weakness/cramps/pains, rhabdomyolysis and others have been used. In 2006 the US National Lipid Association’s Muscle Safety Expert Panel issued the following SIM terminology:

- **Myopathy**: a general term for all the potential muscle problems listed below.
- **Symptomatic myopathy**: complaints referable to skeletal muscle including myalgia (muscle pain), weakness (by complaint or objective testing), and cramps.
- **Asymptomatic myopathy**: CK elevations without symptoms or objective evidence of weakness.
- **Clinically important rhabdomyolysis**: any evidence of muscle cell destruction or enzyme leakage, regardless of the CK level when measured, considered to be causally related to a change in renal function.

**1.13 THE INCIDENCE OF SIM**

Data from the statin trials suggest that statins do not cause myalgia. In the HPS, for example, the rate of reported muscle pain was the same in the simvastatin-treated patient group as in the placebo group (Heart Protection Study Group, 2002). In addition, pooled data from trials using
pravastatin (Pfeffer et al, 2002), atorvastatin (Newman et al, 2003; Mohler et al, 2003),
lovastatin (Bradford et al, 1991; Downs et al, 1998), and fluvastatin (Serruys et al, 2002;
Holdaas et al, 2003) show no increase of myalgias in the statin-treated groups.

The incidence of severe statin-induced rhabdomyolysis as described above is very rare,
estimated to be approximately 3.4 per 100,000 person-years (Law and Rudnicka, 2006). Fatal
cases of statin-induced rhabdomyolysis have an estimated incidence of 0.15 deaths per 1 million
statin prescriptions (Staffa et al, 2002).

However, reports from clinical practice and observational studies suggest that mild
symptoms and complaints of muscle pain are more common, occurring in approximately 5 to 7%
of statin users (Lambrecht and Malini, 1993; Jokubaitis, 1994; Bertolini et al, 1997; Dart et al,
1997; Bruckert et al, 1999; Farmer and Torre-Amione, 2000; Ucar et al, 2000).

In today’s clinical practice, because of the sheer number of people treated, muscle pain is
a common problem among patients taking statins. For example, between 10 and 20% of patients
referred to the Healthy Heart Program / Lipid Clinic at St. Paul’s Hospital in Vancouver, a
specialty clinic for primary and secondary prevention of vascular disease, are referred because of
SIM. In absolute numbers this represents approximately 80 to 100 new referrals each year.

A recent large-scale French cross-sectional observational study titled Prediction du
Risque Musculaire en Observationnel (PRIMO) investigated the frequency of muscle pain
associated with high-doses of a variety of statins in the clinical setting (Bruckert et al, 2005).
The rate of mild and moderate muscle symptoms in 7,924 patients was 10.5% overall, 18.2% in
simvastatin users (N = 1027) and 14.9% in atorvastatin users (N = 1844). The average time of
onset following statin treatment initiation was 1 month. Thirty-nine percent of those reporting
muscle pain used an analgesic medication to relieve muscle discomfort, and 38% claimed their
symptoms prevented them from doing everyday activities of even moderate exertion. In 4% of
cases the muscle pain was to the extent that patients were unable to work or even leave their bed.
The PRIMO study findings are consistent with a preliminary study by the same group of investigators (Franc et al, 2003), and these data may provide a more relevant and accurate picture of the “real-world” incidence of muscle complaints associated with statin treatment, at least with high-dose treatment, as opposed to data from controlled clinical trials.

Thus, it appears that in the clinical setting, the incidence of SIM is more common than in the clinical trial setting. This is especially true for mild to moderate muscle symptoms that do exert a noteworthy impact on patients’ quality of life. Muscle pain with CK levels greater than 10x the ULN occurs in as many as 0.5% of statin users (Shek and Ferrill, 2001). A recent study of the incidence of statin-induced rhabdomyolysis study found 24 affected individuals in more than 250,000 patients treated with statins (Graham et al, 2004). In the US there are 36 million people with indications for statins, and only approximately 11 million on therapy (Fedder et al, 2002; Foley et al, 2004; O’Meara et al, 2004); therefore about 500,000 of these patients may experience muscle pain (Gruer et al, 1999).

The frequency of the mild forms of SIM is probably underestimated as these mild muscle symptoms are often not detected by clinicians (Sinzinger et al, 2002). The PRIMO investigators suggested that these mild symptoms are not necessarily reported by patients unless they are specifically asked by their physicians, especially in the clinical trials (Bruckert et al, 2005). It has been suggested that as many as 25% of statin users who exercise vigorously may experience muscle pains, and that these complaints are largely dismissed by both patient and physician (Tomlinson and Mangione, 2005; see also Sinzinger and O’Grady, 2004).

1.14 WHY SIM IS A PROBLEM

For the individual patient, SIM decreases quality of life. It can affect a patient’s ability to perform simple tasks such as open a jar, rise from a chair, climb stairs, or exercise (Dirks and Jones, 2006). It can also prevent affected people from being able to work and can even confine
them to bed (Bruckert et al, 2005). The PRIMO investigators found that SIM typically manifested in the thighs and calves as cramps, stiffness or heaviness (Bruckert et al, 2005; Franc et al, 2003); it may also present as a more generalized pain (Christopher-Stine, 2006).

For the prescribing clinician, SIM complicates patient management, and is the rate-limiting factor in the proper use of these highly beneficial drugs. Fear of serious muscle toxicity associated with statin treatment continues to be a considerable clinical barrier. SIM is not trivial, even mild forms of it, as patients who experience muscle pains due to statin therapy are more likely to cease treatment (Bruckert et al, 1999). In addition, a recent study suggests that more than half of elderly patients who start a statin may obtain no or minimal benefit due to being forced to discontinue treatment because of poor tolerance (Jackevicius et al, 2002).

1.15 WHY SIM WILL BECOME A BIGGER PROBLEM

For numerous reasons, SIM is expected to become a more significant clinical problem in the near future. Recent studies have shown that using statins and other therapeutic measures to lower LDL to very low levels corresponds with even lower risk for CVD (Heart Protection Study Collaborative Group, 2002; Cannon et al, 2004). The US National Cholesterol Education Program now recommends the use of higher statin doses to lower LDL-C to 1.81 mmol/L or lower in high-risk patients (Grundy et al, 2004). It is well known that SIM is dose-dependent (Ballantyne et al, 2003; de Lemos et al, 2004). Less than half of the patients eligible for statin treatment are actually currently treated (Foley et al, 2003; O’Meara et al, 2004). Furthermore, some statins may soon become available without a prescription, albeit at low doses, in Canada (Rashid, 2007), the US, and other countries (Tobert, 2003). Statin use may also increase due to recent reports of the beneficial pleiotropic effects of statins; thus, there may be an expansion of the indications for statin use. In addition, there is a rising prevalence of dyslipidemia and CVD.
and their diagnosis in an aging population, and physicians are becoming more comfortable prescribing these drugs.

Risk factors for SIM are also anticipated to increase in the near future. Such factors include living longer, the effect of the aging process on skeletal muscle tissue, and comorbid conditions and concomitant medications (Rosenson, 2004). The increasing prevalence of diabetes (National Cholesterol Education Program, 2002) and the metabolic syndrome (Ford et al., 2002) represent a growing clinical need to treat the full spectrum of complex dyslipidemias associated with these conditions, including elevated LDL-C and triglycerides, and decreased HDL-C levels. This will call for more combination therapy, and polypharmacy is a risk factor for SIM (Rosenson, 2004).

1.16 THE KNOWN RISK FACTORS FOR SIM

Although the mechanism(s) of SIM are not fully understood, there are many known factors and medications that increase the risk for myopathy in statin-treated patients (Table 1.1; Table 1.2).
Table 1.1: Factors known to increase the risk for SIM.
Old age likely increases SIM risk due to declining renal function, female gender as statin dosage guidelines are typically based on males who usually have more body mass, biliary obstructive liver disease because most statins are eliminated through bile metabolic routes, and polypharmacy due to interactions with drug clearance mechanisms. References: Tobert, 1988; Christians et al, 1998; Pasternak et al, 2002; Sica and Gehr, 2002; Rosenson, 2004; Antons et al, 2006; Vladutiu et al, 2006; Christopher-Stine, 2006.

<table>
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<th>Higher dose</th>
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<td>Old age</td>
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<td>Female gender</td>
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<td>Renal insufficiency</td>
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<td>Hepatic dysfunction</td>
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<td>Polypharmacy</td>
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<td>Strenuous exercise</td>
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<td>Frailty / small body frame</td>
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<td>Multisystem disease</td>
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<td>Perioperative period</td>
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<td>Biliary obstructive liver diseases</td>
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<td>Diabetes</td>
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<td>Alcoholism</td>
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<td>Infections</td>
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<td>Underlying metabolic disease</td>
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<td>Carnitine palmityl transferase II deficiency</td>
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<td>McArdle disease</td>
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<td>Myoadenylate deaminase deficiency</td>
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Table 1.2: Medications known to increase the risk for SIM.
References: Mastaglia, 1982; Le Quintrec and Le Quintrec, 1991; Pasternak et al, 2002; Christopher-Stine, 2006.

| Fibrates, especially gemfibrozil |  |
| Nicotinic acid (rarely) |  |
| Cyclosporine |  |
| Azole antifungals |  |
| Itraconazole and ketoconazole |  |
| Macrolide antibiotics |  |
| Erythromycin and clarithromycin |  |
| HIV protease inhibitors |  |
| Nefazodone |  |
| Verapamil |  |
| Amiodarone |  |
| Grapefruit juice (large amounts) |  |
| Antibiotics |  |
| Beta-blockers |  |
| Corticosteroids |  |
It is well-known that the risk for SIM increases with higher dose statin treatment (de Lemos et al, 2004; Bruckert et al, 2005) and concurrent therapy with cytochrome P450-inhibiting drugs or substrates (Mukhtar and Reckless, 2005; Gotto, 2006). Increased statin bioavailability increases potential for myotoxicity; thus, anything that increases the concentration of statin in blood, such as concomitant medications that are metabolized through the same enzymes as statins, may increase the potential for myopathy (Kantola et al, 1998). Hypertension increases SIM risk 5-fold, and diabetes 2.5 fold; the reason for this is unknown (National Cholesterol Education Program, 2002). Medical conditions such as biliary obstructive liver diseases (Tobert, 1988) or renal insufficiency (Christians et al, 1998; Pasternak et al, 2002; Sica and Gehr, 2002) that reduce statin metabolism and excretion could contribute to increased statin blood concentration. In a recent review Mukhtar and Reckless (2005) listed over 90 drugs that can interact with statins through the CYP pathway and may increase risk for myopathy.

1.17 THE MECHANISM OF SIM

The mechanism of SIM is not fully understood but many theories exist (Baker, 2005; Chapman and Carrie, 2005; Mukhtar and Reckless, 2005; Owczarek et al, 2005; Bays, 2006; Laaksonen, 2006).

Initially it was thought that SIM may be a result of decreased membrane cholesterol content. Cholesterol has numerous functions, one of which is being an important structural component and fluidity regulator of biological membranes. By blocking cholesterol synthesis, statins may disturb the dynamic equilibrium of membrane and plasma lipids and reduce intracellular cholesterol concentrations in skeletal muscle cells and possibly other cell types. This may result in reduced membrane lipid content, and subsequent modified membrane fluidity and decreased cell proliferation, and contribute to the aetiology of SIM (Levy et al, 1992; Lijnen et al, 1994; Morita et al, 1997). Altered electrical properties may occur as a result; indeed,
Electrical myotonia has been observed in the skeletal muscle of statin-treated rabbits (Sonoda et al., 1994). Altered membrane composition as a result of an intracellular lipid decrease may lead to impaired membrane sodium-potassium channel function and irreversible cell damage (Lijnen et al., 1994), or impaired calcium intake (Muhktar and Reckless, 2005) and subsequent impaired myofibril contractility (Hodel, 2002). Simvastatin-induced increases of intracellular calcium concentrations in human skeletal muscle fibres, in addition to increases of mitochondrial NADH content and mitochondrial membrane depolarization, have been observed and suggested to play a role in SIM (Sirvent et al., 2005a). Large calcium release into the cytoplasm from the sarcoplasmic reticulum have also been observed in mouse and rat muscle exposed to cerivastatin (Inoue et al., 2003). Disturbed calcium homeostasis may lead to depolarization of the mitochondrial membranes and subsequent mitochondrial dysfunction and possibly SIM (Endo, 2005; Sirvent et al., 2005a).

Decreased inner mitochondrial membrane lipid content may also interfere with inter-mitochondrial fusion, as this process is active and is catalyzed by proteins located at the inner membrane (Lyamzaev et al., 2004). Additionally, mitochondrial fusion inhibition has been suggested to be an early step of apoptosis, and it may be a necessary step in the mitochondrial release of cytochrome c into the cytosol (Frank et al., 2001; Karbowski et al., 2004).

This theory is supported by the fact that muscle complaints can arise in patients treated with non-statin lipid-altering therapies, such as clofibrate (Langer and Levy, 1968; Smals and Beex, 1977) and niacin (Gharavi, 1994). Also, up to 60% reduction of cholesterol content has been observed in simvastatin-treated patients’ myocytes (Morita et al., 1997), and reduced cholesterol has been observed in erythrocyte and platelet membranes of pravastatin-treated patients (Lijnen et al., 1994).

Also supporting the reduced intracellular cholesterol theory is evidence from animal studies which show that mevalonate supplementation can improve statin-muscle effects.
(Muhktar and Reckless, 2005). For example, mevalonate supplementation in rats exposed to statins reduced muscle toxicity (Westwood et al, 2005); this argues that it is not statins per se that cause myopathy, but rather their metabolic effects. Other studies have similar results with varying experimental models (Bifulco, 1993; Flint, 1997; Sacher et al, 2005).

However, blocking cholesterol synthesis with squalene synthase inhibitors does not produce myotoxicity in cultured rat myocytes (Flint et al, 1997), or in rat or human myocytes (Johnson et al, 2004). Furthermore, cholesterol or squalene supplementation does not reduce statin myotoxicity in various cell models (Soma, 1992; Kim et al, 1990; O'Donnell et al, 1993), and a rabbit model found that statin myotoxicity is not related to cholesterol-lowering (Nakahara et al, 1998). Taken together, these findings suggest alternative mechanisms.

A more recent avenue of SIM mechanism research is that of enhanced apoptosis. Apoptosis, or programmed cell death, allows for the death of one cell without damage to surrounding cells (Kerr et al, 1972). Statins have been observed to enhance apoptosis in numerous studies and cell types (Corsini et al, 1996; Negre-Aminou et al, 1997; Stark et al, 1998; Axel et al, 2000) including differentiated primary human skeletal muscle cells (Sacher et al, 2005) and human and rat skeletal myotubes (Johnson et al, 2004), both in a dose-dependent manner. This effect has also been observed in vascular smooth muscle cells (Guijarro et al, 1998; Blanco-Colio et al, 2002; Li et al, 2002; Erl, 2005), cardiac myocytes (Demyanets et al, 2006), pericytes (Boucher et al, 2006), rheumatoid synovial cells and several types of cancer cells (Zhong et al, 2003; Graaf et al, 2004; Cafforio et al, 2005; Fromigue et al, 2006).

It is rather apparent that statins can induce apoptosis in cell culture models, but little in vivo data exist. One in vivo study found that asymptomatic exercising humans treated with statins had enhanced ubiquitin proteosome pathway gene expression (Urso et al, 2005). Another reported increased apoptosis in the degenerative muscle fibers of a patient with statin-induced rhabdomyolysis (Salarieh et al, 2004).
A rat model study, however, suggests that statins do not induce apoptosis *in vivo* (Seachrist *et al*, 2005). Rats were treated with cerivastatin to induce muscle damage, but levels of cleaved caspase-3, an apoptotic protein and common marker of apoptosis, did not differ from controls. This particular study is limited by the fact that protein levels were measured after only 24 hours of statin treatment and also because other indicators of apoptosis such as nuclear DNA fragmentation were not determined.

The mechanism of statin-associated apoptosis in the context of SIM is not fully understood (Baker, 2005; Owczarek *et al*, 2005) but may be related to statin-induced mevalonate depletion, accompanying isoprenoid depletion, and subsequent lack of production of GTP-binding proteins such as Ras, Rac, and Rho, which are involved in carrying out programmed cell death (Takemoto and Liao, 2001). The translocation of Bax to the mitochondria in cells exposed to statins has been observed, which may lead to cytochrome *c* release and activation of mitochondrial-mediated apoptosis (Sacher *et al*, 2005).

Another major theory of the mechanism of SIM is that statins can cause mitochondrial dysfunction (or impaired ATP production) by depleting ubiquinone (Langsjoen and Langsjoen, 2003; Hargreaves *et al*, 2005; Nawarskas, 2005; Levy and Kohlhaas, 2006; Littarru and Langsjoen, 2007; Marcoff and Thompson, 2007). Statins reduce the synthesis of mevalonate via their inhibitory action on HMG CoA reductase, but mevalonate is a precursor for not only cholesterol but also many other products, including ubiquinone. Ubiquinone, or coenzyme Q10, is a steroid isoprenoid found in the hydrophobic areas of cell membranes. In humans approximately 50% of it is produced endogenously, the rest is obtained from the diet. Its major function is as an electron transport vector in the mammalian mitochondrion’s oxidative phosphorylation (OXPHOS) process (Crane, 2007). Recent work suggests that it also has an important intracellular antioxidant role, in that it functions to protect the cell against free radicals that cause oxidative stress (Bentinger *et al*, 2007). Ubiquinone may also be involved in aging,
stress responses, apoptosis (Navas et al, 2007), membrane permeability pores, and uncoupling proteins (Dallner and Stocker, 2005).

Since it is a key cofactor of mitochondrial function (Lenaz et al, 2007), a statin-induced ubiquinone depletion could lead to mitochondrial dysfunction, reduced mitochondrial ATP synthesis, and subsequent myocyte instability and disturbed muscle function. This may contribute to the aetiology of SIM, however, studies have been somewhat inconsistent (Levy and Kohlhass, 2006). For example, early studies demonstrated that statin therapy can decrease levels of ubiquinone in blood (Folkers et al, 1990; Ghirlanda et al, 1993; Watts et al, 1993) and that ubiquinone supplementation can reverse this effect (Bargossi et al, 1994). However, subsequent studies that took into account the fact that ubiquinone is transported on LDL-C particles found unaffected blood ubiquinone levels in statin patients (Laaksonen et al, 1996; Bleske et al, 2001). In addition, skeletal muscle ubiquinone levels have been found to be both decreased (Baker and Tarnopolsky, 2001; Paiva et al, 2005) and unaffected (Laaksonen et al, 1995a; Laaksonen et al, 1995b; Laaksonen et al, 1996; Koumis et al, 2004) by statin treatment.

Ubiquinone supplementation for SIM amelioration continues to be explored as a possible therapeutic intervention (Koumis et al, 2004). There are currently at least two US patents for ubiquinone supplementation in the clinical management of SIM. Ubiquinone was used to treat SIM in two clinical trials studying the effectiveness of high-dose lovastatin in treating solid tumors; in both studies the incidence was unaffected, but symptom intensity was decreased (Thibault et al, 1996; Kim et al, 2001). A recent double-blind trial of 32 patients on a statin with myopathy and who were given either vitamin E or ubiquinone supplementation found that after 1 month, pain severity decreased by 40% in the ubiquinone group (N = 18); in contrast, no pain severity changes occurred in the vitamin E group (N = 14) (Caso et al, 2007). But overall, however, there is currently a lack of conclusive data from large prospective studies on the potential benefit of ubiquinone supplementation in statin users with muscle complaints, and
therefore this notion should not yet be put into widespread practice (Antons et al, 2006; Christopher-Stine, 2006).

There is considerable evidence that mitochondrial dysfunction, possibly due to a ubiquinone reduction, participates in SIM (England et al, 1995; Negre-Aminou et al, 1997; Nakahara et al, 1998; Sugiyama, 1998; Baker and Tarnopolsky 2001; Evans and Rees, 2002; Thompson et al, 2003; Phillips et al, 2002; Arenas et al, 2003; Bergman et al, 2003; Gambelli et al, 2004; Lamperti et al, 2005; Seachrist et al, 2005; Sirvent et al, 2005a; Sirvent et al, 2005b; Westwood et al, 2005; Velho et al, 2006). Mitochondrial proliferation in the context of SIM has been reported (Diaczok and Shali, 2003; Caner et al, 2007); this is presumably an attempt by the cell to compensate for defective OXPHOS (Shoubridge and Molnar, 2000). Gambelli et al (2004) observed mitochondrial alterations including cytochrome c oxidase (COX)-negative fibres and ultrastructural abnormalities in muscle biopsies obtained from patients experiencing muscle pain while on statin therapy. Sirvent et al found that human skeletal muscle samples treated with simvastatin underwent a series of cellular events originating from mitochondria and resulting in altered calcium homeostasis, and that the simvastatin-induced mitochondrial impairment resulted from the inhibition of complex I of the electron transport chain (Sirvent et al, 2005a; Sirvent et al, 2005b). Statin users have been shown to have a higher blood ratio of lactate to pyruvate (De Pinieux et al, 1996), indicative of increased anaerobic respiration due to mitochondrial dysfunction. Light microscopy of needle biopsies from simvastatin or pravastatin users with myopathy showed signs of mitochondrial myopathy, possibly the result of reduced ubiquinone as suggested by investigators (Schalke et al, 1992; Giordano et al, 1997). Accumulation of lipids or impaired fatty acid oxidation and ragged-red fibres, hallmarks of mitochondrial dysfunction, have also been observed in muscle biopsies collected from statin users with muscle complaints (Phillips et al, 2002). In addition, muscle cells cultured from patients with SIM show impaired fatty acid oxidation in response to statin exposure, compared to
control muscle cells (Phillips et al, 2004). Reduced ubiquinone has been observed in some other mitochondrial myopathies (DiMauro et al, 1998). Intracellular ubiquinone depletion has been associated with congenital myopathies including mitochondrial myopathy, encephalopathy, lactacidosis, stroke (MELAS), myoclonus epilepsy associated with ragged-red fibres (MERRF), and Kearns-Sayre syndrome (Koumis et al, 2004).

Statin-induced mitochondrial dysfunction, due or not due to a ubiquinone depletion, and statin-induced myofibre apoptosis may very well be related, as the mitochondrion plays a central regulatory role in the induction of programmed cell death by releasing pro-apoptotic compounds such as cytochrome c, apoptosis-inducing factor (AIF), and endonuclease G into the cytosol (Garrido et al, 2006).

Taken together, these observations suggest that statins may induce mitochondrial toxicity that could be, at least partially, responsible for their adverse effects on skeletal muscle.

1.18 CREATINE KINASE, A POOR MARKER OF SIM

In addition to the mechanism of SIM not being fully understood, currently there is no marker of SIM that is both convenient (e.g. accessible in blood, not only in the affected tissue skeletal muscle) and reliable. Serum CK has been used widely as a marker of SIM (Pasternak et al, 2002; McKenney et al, 2006), but appears to be inconsistent (please see below).

CK is an enzyme expressed by various tissues that catalyzes the conversion of creatine to phosphocreatine, consuming adenosine triphosphate (ATP) and producing adenosine diphosphate (ADP) in the process. It is used clinically in blood tests as an indicator of muscle damage, especially in cases of MI, acute rhabdomyolysis, and acute renal failure, as muscle breakdown leads to the release of CK (Evans and Rees, 2002).

Although serum CK levels have been and continue to be used as an indicator of muscle damage caused by statin therapy, mounting evidence strongly suggests that it is not a reliable
marker of SIM. For example, Phillips et al (2002) clearly documented normal serum CK levels in individuals treated with statins and with muscle biopsy-confirmed myopathy. The muscle biopsies collected from these patients showed several of the main signs of mitochondrial pathology including increased intracellular lipid accumulation and ragged red fibres. Ragged red fibres are muscle fibres that contain an abnormally high amount of mutated genomes and many proliferating structurally altered mitochondria (Sarnat and Marin-Garcia, 2005). They are revealed by modified Gomori trichrome stain, and are usually found in the subsarcolemmal region where altered mitochondria typically localize and accumulate (Sarnat and Marin-Garcia, 2005). Ragged red fibres usually indicate the presence of altered functional proteins (DiMauro and Schon, 2001). COX-negative fibres were also observed in these patients. COX (also known as Complex IV) is a large transmembrane protein enzyme located inside the mitochondrion, and is the last protein of the electron transport chain (Figure 1.5); when muscle fibres do not stain for COX activity it is a strong indication of mitochondrial dysfunction (Filosto et al, 2007).
Figure 1.5: The mitochondrial respiratory chain.
Simplified schematic of the electron transport chain (ETC) in mitochondria. Electrons are transported (thick solid arrows) down the ETC through the enzyme complexes. Complex I accepts electrons from the Krebs cycle electron carrier NADH and passes them ubiquinone (CoEnzyme Q10; labeled “Q10”). Complex II also donates electrons to ubiquinone. Ubiquinone transfers electrons to Complex III which passes them to cytochrome c (labeled “cyto c”) which moves them to Complex IV. This process pumps protons (thin dashed arrows, H+) from the mitochondrial matrix into the inter-membrane space, thus establishing an electrochemical proton gradient across the inner mitochondrial membrane (IMM). Complex V, or ATP synthase, utilizes this H+ gradient to generate ATP from adenosine diphosphate (ADP) and an inorganic phosphate (not shown). Reactive oxygen species (ROS) are generated by the ETC and pose a danger to the cell, especially to the structural integrity of the mitochondrial genome (mtDNA) which is located nearby in the matrix. References: Hargreaves et al, 2005; Shults, 2005.
Several other such reports have since surfaced. Four related Norwegian patients (three siblings and their mother) were recently described (Troseid et al, 2005). All complained of muscle pain after initiating statin therapy, and these symptoms resolved upon statin discontinuation. Two of the four had myopathy confirmed by electromyogram and muscle biopsy laboratory assays including light and electron microscopy (EM), and a third had slight myopathic findings in these tests. During the myopathic episodes, all four patients had normal serum CK levels.

Similarly, Bennett et al (2003) reported 5 hypercholesterolemic patients who developed muscle complaints on simvastatin treatment, 20 to 40 mg daily. Physical examinations found considerable weakness in all 5 patients, and muscle symptoms resolved within 3 to 4 weeks after discontinuation of statin treatment. Again, CK levels were within the normal reference range.

Additionally, in a recent study of patients with muscle complaints attributable to statin therapy, 5/18 (28%) had no CK elevation and 6/18 (33%) had only a minor elevation (Soininen et al, 2006).

These reports and others (Sinzinger et al, 2002; Hyman, 2003; Torgovnick and Arsura, 2003) provide strong evidence that serum CK is not a reliable marker of SIM for many patients. The physicians making these reports emphasized that some statin users can experience significant myalgias, cramps, and weaknesses without having marked CK elevations, and called for the continued study of surveillance methods of statin therapy and its safety in terms of muscle toxicity, in light of their findings. Another important indication that CK may lack sensitivity and specificity as a marker of SIM is that serum CK concentrations are often increased in the absence of a statin (Bradford et al, 1991).

Recently the US National Lipid Association’s Muscle Safety Expert Panel called for an alternative validated measurement instrument for statin-related muscle complaints (Thompson et al, 2006).
1.19 GENETIC BASIS OF SIM

In the field of SIM, some important gene association studies have been published recently that should be briefly discussed as they provide convincing evidence for a genetic predisposition to SIM.

In a cross-sectional study of 136 SIM patients, Vladutiu et al (2006) found an increased rate of underlying metabolic muscle diseases compared to 3 appropriate control groups (N > 100 in each group). In the affected group, 10% were heterozygous or homozygous for mutations that cause metabolic myopathies compared to only 3% in the control group (P = 0.04), and compared to the general population, the number of carriers of mutations for McArdle disease and carnitine palmitoyltransferase II deficiency was increased 20- and 13-fold, respectively. In addition, in support of the theory that mitochondrial dysfunction may play a role in SIM, 52% of muscle biopsies from the affected group analyzed (N = 106) showed signs of mitochondrial or fatty acid metabolism dysfunction, including ubiquinone deficiency (the most common defect found in the biopsies) and mitochondrial respiratory chain defects. Serum CK was normal in 60% of the patients with defective mitochondrial respiratory chain function, providing further evidence that serum CK is not a reliable marker of SIM. Given these findings, a synergetic interaction between pharmacologic and genetic factors may trigger the clinical manifestation of presymptomatic metabolic myopathies; in other words, statins may “unmask” sub-clinical myopathies.

A similar recent investigation (Oh et al, 2007) studied COQ2 in 133 patients with SIM and 158 matched controls on a statin with no muscle complaints. The COQ2 gene encodes the second enzyme in the ubiquinone synthesis pathway, and alterations of it are associated with acute genetic myopathy. In this study such alterations were found to be associated with statin muscle intolerance, suggesting that genetic variations in the COQ2 gene may be one of likely many genetic factors that predispose to SIM. This is consistent with the notion that SIM is
associated with an individual’s genetic makeup. It may be that SIM is associated with COQ2 mutations and/or perhaps also with mutations in other genes such as those involved in the ubiquinone pathway.

These two studies support the notion that statin intolerance can be at least partially attributed to underlying genetic metabolic predisposition. These studies are reinforced by others (Arenas et al, 1996; Barth et al, 2003; Delgado-Lopez et al, 2004; Livingstone et al, 2004; Vermes and Vermes, 2004), including the abovementioned PRIMO study which found that a family history of muscle problems, with or without cholesterol-lowering therapy, was a significant predictor of SIM. Finally, a number of recent expression-profile studies in varying experimental models have been used to investigate the metabolic pathways affected by statins. Numerous genes and systems related to signal transduction, protein synthesis, and degradation have been shown to be altered upon statin exposure, but results have been inconsistent (Morikawa et al, 2002; Morikawa et al, 2004; Morikawa et al, 2005; Urso et al, 2005; Laaksonen, 2006).

1.20 WHY IT IS IMPORTANT TO STUDY SIM

It is very important that statins be truly safe and well-tolerated, for numerous reasons. The burden of CVD is large, and hypercholesterolemia and atherosclerosis are chronic conditions which typically require life-long cholesterol-lowering treatment, of which statins are the mainstay. Some patients will never experience a CVD endpoint such as MI but due to being at an increased risk for such an endpoint, they will take a statin drug for their life-time. The molecular basis of SIM, the most important adverse effect of these highly beneficial drugs, is not fully understood. An enhanced understanding of the mechanism of SIM may improve our ability to overcome this clinical problem and treat hypercholesterolemic patients at risk for CVD more effectively. It may lead to the development of better clinical strategies to avoid myopathy or
possibly even to the design of new statins with reduced myotoxic potential. It may also lead to improved identification of high-risk individuals who are particularly susceptible to SIM, by exploring possible markers of SIM other than CK, and a higher standard of care for the millions of dyslipidemic patients worldwide on statin therapy. Given that some studies have linked SIM with mitochondrial dysfunction, and that serum CK has been shown to be an inconsistent marker of SIM, new research exploring alternative markers and the effects of statins on mitochondria is warranted.

1.21 HIV MITOCHONDRIAL DNA RESEARCH

In another setting, nucleoside reverse transcriptase inhibitors (NRTI) drugs, such as zidovudine (AZT), have been observed to have an unwanted side effect of mitochondrial toxicity. This was initially believed to be a result of their inhibition of the human mitochondrial polymerase gamma which is responsible for replicating mitochondrial DNA (mtDNA), but more recently alternative mechanisms have been discussed (Côté, 2005; Côté, 2007). In this context, decreased levels of mtDNA have been observed in peripheral blood mononuclear cells (Côté et al, 2002), subcutaneous fat (Cherry et al, 2006), liver (Walker et al, 2004) and importantly in skeletal muscle (Fleischman et al, 2007). These studies have given valuable information on the mechanism of NRTI toxicity, and also have led to additional research into the prospect of utilizing changing or reduced levels of mtDNA in surrogate tissue such as blood cells, as a clinical biomarker of mitochondrial toxicity.

Interestingly, the symptoms of NRTI-therapy associated mitochondrial toxicity are strikingly similar to the symptoms of statin-therapy associated muscle toxicity. AZT has been associated with skeletal myopathy (Gherardi et al, 1994) which manifests clinically as proximal and symmetric muscle weakness that develops subacutely (Authier et al, 2005). In addition, laboratory tests of muscle biopsies from patients with AZT-induced myopathy show similar
signs of mitochondrial abnormalities as in SIM. Various mitochondrial abnormalities include ragged red fibres (Dalakas et al, 1990; Chariot and Gherardi, 1991; Mhiri et al, 1991; Pezeshkpour et al, 1991; Chariot et al, 1993a; Grau et al, 1993; Rosenfeldt et al, 2005), respiratory chain dysfunction (Mhiri et al, 1991), ultrastructural abnormalities (Brinkman et al., 1998; Côté et al, 2006), and COX deficiency (complex IV of the mitochondrial respiratory chain) (Chariot and Gherardi, 1991; Chariot et al, 1993a). High blood lactate/pyruvate ratio is also observed in AZT-myopathy (Chariot et al, 1994), as is decreased skeletal muscle mtDNA (Arnaudo et al, 1991).

1.22 MITOCHONDRIAL DNA AND SIM

The mitochondrion is the organelle responsible for most of the cell’s energy production by generation of adenosine triphosphate (ATP). It is also involved in pyruvate oxidation, the Krebs cycle, and the metabolism of amino acids, fatty acids, and steroids (Elston et al, 1998). Cells can contain 1000s of mitochondria, and each mitochondrion contains about 2 to 10 copies of the mitochondrial genome (Johns, 1995). The number of mtDNA copies per cell varies from tissue to tissue (Bogenhagen and Clayton, 1974). The maternally-inherited human mtDNA, located in the mitochondrial matrix, is a circular double-stranded molecule with 16,569 base pairs encoding 37 genes. Of these, 13 are polypeptide subunits which constitute part of the five enzyme complexes of the OXPHOS system, located on the inner mitochondrial membrane, as well as a portion of the mitochondrial protein synthesis apparatus (Anderson et al, 1981). mtDNA also encodes 22 tRNA (transfer RNA) and 2 rRNA (ribosomal RNA) (Schapira, 2002). Most gene products in mitochondria, however, are encoded by nuclear DNA (nDNA) and are imported from the cytoplasm (Chinnery et al, 1999). In addition, mtDNA is vulnerable to oxidative damage due to a lack of protective histones, poor repair mechanisms in comparison to nDNA, and because of its proximity to the inner mitochondrial membrane with the electron transport
chain which results in increased exposure to ROS (reactive oxygen species) (Fromenty and Pessayre, 1995; Bogenhagen, 1999). Since 13 mitochondrial proteins, all involved in mitochondrial respiration, and several tRNA and rRNA are encoded by the mtDNA genome, changes in mtDNA have the potential to affect mitochondrial proteins and ultimately mitochondrial function; ATP production by mitochondrial OXPHOS is heavily dependent on mtDNA being intact (Hudson and Chinnery, 2006).

mtDNA depletion is a rare condition but it has been observed elsewhere. Mutations in nuclear genes that regulate mtDNA replication or deoxynucleotide pools can reduce mitochondrial biogenesis and mtDNA copy number (Bai et al, 2004). These include mutations that result in deoxyguanosine kinase deficiency (Mandel et al, 2001) and thymidine kinase deficiency (Saada et al, 2001), both of which typically present in childhood with myopathy and hypotonia (low muscle tone). mtDNA depletion is also observed in individuals with a mutation in the gene MPV17 which codes for a protein localized in the inner mitochondrial membrane; myopathy is one of the main clinical features (Spinazzola et al, 2006). Reduced mtDNA levels may also result from a defective protein preventing proper mtDNA replication. AZT-induced mtDNA depletion has been well-documented (Arnaudo et al, 1991; Lewis and Dalakas, 1995; Chariot and Gherardi, 1991). If other proteins are defective, such as mitochondrial-specific single-strand-binding protein (mtTFA) or RNA polymerase, this may also inhibit replication of the mitochondrial genome (Marin-Garcia and Goldenthal, 2000). Reduced mtTFA level, for example, has been observed in infants with mitochondrial myopathies related to mtDNA depletion (Poulton et al, 1994).

There is no published clinical study of the effect of statin treatment on levels of mtDNA in skeletal muscle or in any other tissue type. If mitochondrial toxicity is indeed a pathogenic mechanism underlying SIM, then quantifying mitochondrial damage may be useful to evaluate and/or monitor statin users with muscle complaints.
For these reasons we set out to determine the effect of statins on levels of mtDNA, to
give information on the mechanism of SIM and also to explore the possibility of utilizing
mtDNA as a clinical marker of statin muscle damage.
CHAPTER 2: DECREASED SKELETAL MUSCLE MITOCHONDRIAL DNA IN PATIENTS TREATED WITH HIGH-DOSE SIMVASTATIN

2.1 STUDY OVERVIEW

At the outset of this masters thesis, our main research question was to determine whether statin therapy can affect mtDNA content in skeletal muscle, the affected tissue of SIM. In this chapter, which was recently published (Schick et al, 2007), we were able to answer this question due to an international collaboration with a group of investigators in Finland. mtDNA was retrospectively quantified in 86 skeletal muscle biopsy specimens collected as part of a previously published (Paiva et al, 2005) 8 week RPDCT of high-dose simvastatin or high-dose atorvastatin versus placebo.

2.2 METHOD

2.2.1 Study population

The original RPDCT enrolled 48 hypercholesterolemic subjects (33 men, 15 women) aged 31 to 69 years. Study participants were recruited from the University Hospital of Tampere and Primary Health Care Centres of nearby municipalities of Tampere, Finland. Mean serum concentrations were 3.66 mmol/L for LDL-C and < 4.5 mmol/L for triglycerides. No subjects had received prior statin therapy and they were instructed to maintain their normal diet during the study. Exclusion criteria were: familial hypercholesterolemia, serum total cholesterol > 7.0 mmol/L at initial screening, use of concomitant lipid-lowering medication, antioxidant vitamins or medications known to alter the metabolism of atorvastatin or simvastatin, and hepatic or renal dysfunction. Women of childbearing potential were also excluded. The Ethics Committee of the
University Hospital of Tampere approved this study’s protocol and written informed consent was obtained from all participants.

### 2.2.2 Trial design

In this 8 week placebo-controlled, double-blinded clinical trial, subjects were randomized into 3 treatment groups: simvastatin 80 mg/day, atorvastatin 40 mg/day and placebo (Figure 2.1).

![Figure 2.1: Trial design.](image)

Forty-eight mildly hypercholesterolemic patients who had never been on statin therapy were randomized into three treatment groups to receive either simvastatin 80 mg/day, atorvastatin 40 mg/day or placebo for 8 weeks. This was a double-blinded trial, and a block-of-18 randomization scheme was employed. mg = milligrams; adapted from Paiva et al, 2005.
A block-of-18 randomization scheme was employed, meaning that with every 18 recruitments, 6 were assigned to each of the 3 treatment groups. Atorvastatin and simvastatin were chosen as they are the most commonly used statins (Figure 2.2). The dosages were selected based on their equivalent lipid-lowering potency (Roberts, 1997; Jones et al, 1998; McClure et al, 2007) and were aimed to have an equipotent LDL-C-lowering effect.

Figure 2.2: Market share of the different statins. Pie chart showing statin prescription frequencies: atorvastatin, 42.8%; simvastatin, 20.2%; simvastatin/ezetimibe, 11.3%; lovastatin, 9.4%; rosuvastatin, 8.6%; pravastatin, 5.9%; fluvastatin, 1.8%. Atorvastatin and simvastatin occupy approximately 75% of the total statin market. These data represent statin prescriptions in the US between June and December, 2006 (Consumer Reports, 2007).
Needle biopsy muscle specimens were obtained at baseline and at the end of the 8-week treatment period from the quadriceps femoris muscle, with the patient under local anesthesia (Tru-Cut, Baxter; McGaw Park, IL, USA) (Figure 2.3).
Figure 2.3: Flowchart of study design.
Percutaneous skeletal muscle biopsy specimens were collected from study participants at baseline and after the 8 week treatment period, from the quadriceps using a biopsy needle and with the patient under local anesthesia. Total DNA was extracted from these specimens, and real-time PCR with fluorescent probes was utilized to quantify the number of copies of a mitochondrial gene (human cytochrome c oxidase subunit I, CCOI) and a nuclear gene (accessory subunit of the human polymerase gamma, ASPG). The results are expressed as the ratio of mitochondrial DNA (mtDNA) copy number / nuclear DNA (nDNA) copy number, and considered to be an accurate indication of the amount of mtDNA, as the number of copies of nDNA is presumed to be constant at two copies per nucleus while the mtDNA copy number will fluctuate under different intracellular physiological environments.
Muscle biopsies were frozen immediately in liquid nitrogen and stored at -80°C until analyzed. At baseline and at 1, 2, 4 and 8 weeks, blood samples were collected and stored at -80°C.

### 2.2.3 Total DNA extraction

The muscle biopsy samples had been homogenized by the Finnish group, 3 X 20 seconds using an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, Germany) as described (Paiva et al, 2005). The homogenates were then centrifuged, and the majority of the supernatant was removed. We were sent aliquots of the homogenized muscle pellets for mtDNA analyses. Total DNA was extracted from these muscle homogenate pellets with the QIAamp DNA Mini Kit (QIAGEN; Mississauga, Canada). We were also sent aliquots of the supernatant but we found it contained insufficient mtDNA copies and thus continued our analyses with the homogenized muscle pellets only.

### 2.2.4 mtDNA quantification

An in-house real-time polymerase chain reaction (PCR) assay was used to quantify mtDNA content in relation to nDNA, in the skeletal muscle biopsies (Côté et al, 2002). The number of copies of the nuclear gene human polymerase gamma accessory subunit (ASPG) and the mitochondrial gene human cytochrome-c oxidase subunit I (CCOI) were quantified in separate 4 uL aliquots of the muscle biopsy total DNA extracts, by real-time PCR using fluorescent-labelled hybridization probes (Proligo, Singapore), on a Roche LightCycler (LC) 1.2 (Pensberg, Germany). The LC FastStart DNA Master Hybridization Probes kit (Roche Applied Science) was used to assay each gene in duplicate. Primer and probe sequences are as follows: for the mitochondrial gene (CCOI), the forward primer used was 5'-TTGCCTACCTGTTGGC-3', the reverse primer was 5'-AAGTTTATTACAAATGCTGGG-3', the hybridization fluorescein probe was 5'L-AACGACCACATCTCAGGGTTATGTCAC-P3', and the
hybridization LC Red 640 probe was 5'-AACGACCACATCTACAACGTTATCGTCAC-P3'; for the nuclear gene (ASPG), the forward primer used was 5'-GAGCTGTTGACGGAAAGGAG-3', the reverse primer was 5'-CAGAAGAGAATCCCGGCTAAG-3', the hybridization fluorescein probe was 5'GAGGCGCTGTTAGAGATCTGTCAGAGA-P3', and the hybridization LC Red 640 probe was 5'-GGCATTTCCTAAGTGGAAGCAAGCA-P3'. The PCR reactions contained the following: 5 mM magnesium chloride, 1.0 μM of each primer, 0.2 μM 3' fluorescein probe, 0.4 μM 5' LC Red 640 probe, and 4 μL of the DNA extract in the QIAGEN elution buffer (Buffer AE, QIAGEN DNA Mini Kit). Amplification conditions were as follows: one denaturation-enzyme-activation step (10 minutes at 95°C), 45 cycles (0 seconds at 95°C; 10 seconds at 60°C; 5 seconds at 72°C). The temperature-transition rate was 20°C per second. At the end of each annealing step, a single fluorescence acquisition was done.

Seven point standard curves were built from serial 10-fold dilutions (15 to 15,000,000 copies) of plasmid DNA (pCR®2.1-TOPOvector®; Invitrogen, Burlington, ON, Canada) containing the cloned nuclear (ASPG) and mitochondrial (CCOI) genes amplified. The standard curves were included in each run and used for both ASPG and CCOI gene quantitation. The second-derivative maximum of each amplification reaction was related to its standard curve to obtain the result, or copy number. All values fell between 150 and 15,000 copies, which was an appropriate concentration range that yielded a constant mtDNA/nDNA ratio for a given sample.

Samples were assayed blindly in duplicate, with the standard curve, a negative and two internal controls included in each run. Muscle and plasmid DNA showed similar PCR efficiencies and the inter-run coefficient of variation was < 15%. For each DNA extract, results are expressed as mtDNA/nDNA, shorthand for the ratio of the average mtDNA copy number of the duplicate 4 μL aliquots to the average nDNA copy number ratio of the duplicate 4 μL aliquots.
2.2.5 Labwork from our research collaborators in Finland

Muscle biopsy specimens had been oxidized and muscle ubiquinone assayed as described (Paiva et al., 2005). In addition, the following variables in blood samples were assayed by the Finns: ubiquinone, CK, lipids and lipoproteins. In muscle, the Finns measured: lathosterol, campesterol, sitosterol, and ubiquinone. In addition, in selected patients, mitochondrial respiratory chain enzyme activities were assessed, including complex II (succinate coenzyme Q reductase), complex II + III (succinate cytochrome c reductase), complex III (ubiquinol-cytochrome c reductase), complex IV (COX), and citrate synthase. Complex I analyses were not done due to insufficient sample material.

Serum CK values as measured by the Finnish group remained normal in all study participants throughout the study (Paiva H et al., 2005). No overall (R = 0.177, P = 0.262) or intra-group (placebo, R = 0.184, P = 0.529; atorvastatin, R = 0.162, P = 0.563; simvastatin, R = 0.089, P = 0.773) correlation was observed between changes in mtDNA and serum CK.

Muscle mitochondrial respiratory chain enzyme complex activities were assayed in selected participants (N = 18 of the 43 total participants, 6 from each group). The six simvastatin-treated individuals with the largest decreases in muscle ubiquinone at follow-up (from - 24% to - 74%) were selected and assayed. Twelve age- and gender-matched participants from the placebo (N = 6) and atorvastatin (N = 6) treatment groups were also assayed, as controls. The 6 simvastatin subjects had reduced activities of complexes II, II + III, III, IV, and citrate synthase after 8 weeks of high-dose treatment. However, the ratio between the activities of the complexes and citrase synthase activity did not change.

2.2.6 Statistical analyses

The Kruskal-Wallis test was used to compare the baseline muscle mtDNA/nDNA ratios and changes in the ratios among the three treatment groups. Changes from baseline to follow-up
within groups were tested using the Wilcoxon Signed Ranks test. Pearson’s coefficient (R) was used to determine the correlation between muscle ubiquinone and mtDNA/nDNA ratio. Statistical results are displayed as medians and inter-quartile ranges (IQR). A two-sided P-value < 0.05 was considered statistically significant. All statistical analyses were conducted using SPSS® Version 13.0 (SPSS®, Chicago, IL, USA) and Microsoft Excel® (Microsoft Corporation®, Seattle, WA, USA).

2.3 RESULTS

In this longitudinal retrospective study, the mtDNA/nDNA ratio was determined in human quadricep muscle biopsies collected before and after 8 weeks of treatment with placebo (N = 14), high-dose atorvastatin 40 mg/day (N = 15) or high-dose simvastatin 80 mg/day (N = 14). Forty-three of the 48 study participants were included in the final analyses, as 4 subjects dropped out (3 for personal reasons, one due to unspecific symptoms) and one sample was misplaced. A total of 86 muscle biopsies were analyzed. No subjects reported muscle pain or weakness during the study, and none had elevated serum CK at follow-up. Plasma lipid and sterol values, and muscle respiratory chain enzyme activities were previously published (Paiva et al, 2005).

2.3.1 mtDNA

The in-house assay used to quantify changes in the mtDNA content used real-time PCR with fluorescent probes to determine the number of copies of the mitochondrial genome in relation to the number of copies of the nuclear genome. The 7-point standard curves showed linearity in the range studied ($R^2 = 0.99$). The inter-individual mtDNA/nDNA coefficient of variation (CV) was 40%, based on the baseline values (N = 43).
At baseline, mtDNA/nDNA ratios were not different between the three treatment groups (median [IQR]; placebo, 1705 [1502 – 2654]; atorvastatin, 2107 [1546 – 2721]; simvastatin, 2117 [897 – 3289]; P = 0.42) (Table 2.1).

Table 2.1: Study population characteristics and muscle mtDNA/nDNA ratios.
Age data is presented as (mean ± standard deviation). All continuous variables are reported as median [inter-quartile range]. All tests of significance are non-parametric Kruskal-Wallis.
mtDNA = mitochondrial DNA; nDNA = nuclear DNA

<table>
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<th>Simvastatin 80 mg/day</th>
<th>Atorvastatin 40 mg/day</th>
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<td>14</td>
<td>15</td>
<td>14</td>
<td>--</td>
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<tr>
<td>Age in years</td>
<td>60 ± 15</td>
<td>57 ± 10</td>
<td>57 ± 16</td>
<td>0.72</td>
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<td>% female</td>
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<td>20</td>
<td>43</td>
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</tr>
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<td>Baseline mtDNA/nDNA</td>
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<td>2107.0 ± 1175.0</td>
<td>1704.5 ± 1152.5</td>
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<td>Follow-up mtDNA/nDNA</td>
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<td>1404.0 ± 1063.5</td>
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<tr>
<td>Change in mtDNA/nDNA</td>
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<tr>
<td>Intra-group Baseline vs. Follow-up P value</td>
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<td>0.16</td>
<td>0.20</td>
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</tbody>
</table>

The main finding in this study was that a significant median decrease (- 47%) in muscle mtDNA levels was observed in the simvastatin group between baseline and the 8-week follow-up (-987 [-1944 – -249], P = 0.005) (Figure 2.4).
Figure 2.4: Changes in mtDNA/nDNA ratio after 8 weeks of high-dose statin treatment

Comparative box-plots illustrating the change in muscle mitochondrial DNA/nuclear DNA (mtDNA/nDNA) ratio in the placebo (N = 14), atorvastatin (N = 15), and simvastatin (N = 14) groups between baseline and the 8-week follow-up. The vertical lines represent the maximal and minimal mtDNA/nDNA ratios observed within each group, the top and bottom of the boxes indicate the inter-quartile ranges (25th and 75th percentiles), and the bold horizontal lines within the boxes represent the medians; the “o” above the atorvastatin boxplot represents an outlier in that group. Small non-significant decreases were observed in the placebo (P = 0.16) and atorvastatin (P = 0.20) groups, but a significant decrease (median change -47%, P = 0.005) occurred in the simvastatin-treated participants.
Indeed, 7/14 patients in the simvastatin group showed > 50% decrease in mtDNA at the 8-week follow-up, compared to only 2/15 such decreases in the atorvastatin group and 0/14 in the placebo group. No significant changes in mtDNA at follow-up were observed in the placebo or atorvastatin groups.

2.3.2 Ubiquinone

As was the case with muscle mtDNA, baseline muscle ubiquinone levels (units: umol/kg) had been found in the original study (Paiva et al, 2005) to be not significantly different between groups (median [IQR]; placebo, 0.036 ± 0.02; atorvastatin, 0.040 ± 0.03; simvastatin, 0.035 ± 0.02; P = 0.55; Table 2.2). Ubiquinone was significantly decreased at follow-up in the simvastatin group (-26%; P = 0.028) but there were no significant changes in the atorvastatin or placebo groups (Table 2.2, Figure 2.5). No change in plasma ubiquinone was observed when adjustments were made for the change in serum total cholesterol concentration.

Table 2.2: Muscle ubiquinone data from the original Finnish study.
Data are presented as median ± inter-quartile range, and the ubiquinone units are umol/kg. * indicates P = 0.001 for difference between baseline and follow-up.

<table>
<thead>
<tr>
<th></th>
<th>Simvastatin 80 mg/day</th>
<th>Atorvastatin 40 mg/day</th>
<th>Placebo</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>14</td>
<td>15</td>
<td>14</td>
<td>--</td>
</tr>
<tr>
<td>Baseline ubiquinone</td>
<td>0.035 ± 0.02</td>
<td>0.040 ± 0.03</td>
<td>0.036 ± 0.02</td>
<td>0.55</td>
</tr>
<tr>
<td>Follow-up ubiquinone</td>
<td>0.021 ± 0.01*</td>
<td>0.038 ± 0.02</td>
<td>0.036 ± 0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Change in ubiquinone</td>
<td>-0.0090 ± 0.017</td>
<td>-0.0035 ± 0.017</td>
<td>-0.0035 ± 0.019</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Figure 2.5: Changes in muscle ubiquinone after 8 weeks of high-dose statin treatment.
Comparative box-plots illustrating the change in muscle ubiquinone in the placebo (N = 14), atorvastatin (N = 15), and simvastatin (N = 14) groups between baseline and the 8-week follow-up. The vertical lines represent the maximal and minimal ubiquinone levels observed within each group, the top and bottom of the boxes indicate the inter-quartile ranges (25th and 75th percentiles), and the bold horizontal lines within the boxes represent the medians. Small non-significant decreases (P > 0.05) were observed in the placebo and atorvastatin groups, but a significant decrease (median change -26%, P = 0.028) occurred in the simvastatin-treated participants (Paiva et al, 2005).
2.3.3 mtDNA and ubiquinone correlation

Thus, the relationship between muscle ubiquinone and muscle mtDNA levels was investigated and a significant overall correlation between the change in ubiquinone and the change in mtDNA/nDNA ratios was observed (R = 0.63, P < 0.01) (Table 2.3; Figure 2.6). This relationship was strongest within the simvastatin group (R = 0.76, P = 0.002).

Table 2.3: Correlations between the changes in mtDNA/nDNA and ubiquinone within each group.
A significant correlation between the change in muscle ubiquinone levels and the change in mtDNA/nDNA ratios was observed in all groups combined (R = 0.63, P < 0.01), and this relationship was strongest within the simvastatin group (R = 0.76, P = 0.002).

<table>
<thead>
<tr>
<th></th>
<th>Pearson coefficient (R)</th>
<th>P value</th>
<th>R squared</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall (N = 43)</td>
<td>0.63</td>
<td>&lt; 0.01</td>
<td>0.40</td>
</tr>
<tr>
<td>Placebo (N = 14)</td>
<td>0.60</td>
<td>0.024</td>
<td>0.36</td>
</tr>
<tr>
<td>Atorvastatin 40mg/day (N = 15)</td>
<td>0.37</td>
<td>0.173</td>
<td>0.14</td>
</tr>
<tr>
<td>Simvastatin 80mg/day (N = 14)</td>
<td>0.76</td>
<td>0.002</td>
<td>0.57</td>
</tr>
</tbody>
</table>
Figure 2.6: Correlation between change in mtDNA/nDNA ratio and change in ubiquinone.
Scatter-plot demonstrating the overall positive correlation ($R = 0.63$, $P < 0.01$) between changes in muscle mtDNA/nDNA ratio and changes in muscle ubiquinone from baseline to 8-week followup in the 3 treatment groups. The simvastatin-treated subjects ($N = 14$) fall predominantly in the lower-left quadrant, illustrating the significant correlation ($R = 0.76$, $P = 0.002$) observed between mtDNA/nDNA ratio and ubiquinone changes in that group.

**All groups:**

$R = 0.63$

$P < 0.01$

**Simvastatin group only:**

$R = 0.76$

$P = 0.002$
In addition, the relationship between baseline muscle mtDNA content and muscle ubiquinone was determined. A significant correlation was observed overall (R = 0.55; P = 0.0001) but this relationship was specific to the simvastatin group (R = 0.68; P = 0.008) (Table 2.4).

In addition, a strong relationship was observed between the amount of baseline mtDNA content and mtDNA loss, in all groups combined (R = 0.51, P = 0.01), but especially in the simvastatin group alone (R = 0.90, P < 0.0001). Such an association did not reach significance in the atorvastatin group (R = 0.47, P = 0.086), and was not observed in the placebo group (R = 0.22, P = 0.87) (Table 2.4). Similar data were observed in terms of the relationship between the baseline mtDNA level and the % change in mtDNA (Table 2.4).

Table 2.4: Additional correlations between muscle mtDNA/nDNA and muscle ubiquinone. R = Pearson’s coefficient; BL = baseline; mtDNA = mitochondrial DNA/ nuclear DNA ratio; vs. = versus.

<table>
<thead>
<tr>
<th></th>
<th>Overall (N = 43)</th>
<th>Simvastatin (N = 14)</th>
<th>Atorvastatin (N = 15)</th>
<th>Placebo (N = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>P value</td>
<td>R</td>
<td>P value</td>
</tr>
<tr>
<td>BL mtDNA vs. BL ubiquinone</td>
<td>0.55</td>
<td>0.0001</td>
<td>0.68</td>
<td>0.008</td>
</tr>
<tr>
<td>BL mtDNA vs. mtDNA loss</td>
<td>0.51</td>
<td>0.01</td>
<td>0.90</td>
<td>&lt;</td>
</tr>
<tr>
<td>BL mtDNA vs. % change in mtDNA</td>
<td>0.38</td>
<td>0.012</td>
<td>0.61</td>
<td>0.021</td>
</tr>
</tbody>
</table>
CHAPTER 3: ARCHIVED MUSCLE BIOPSIES CHART REVIEW

3.1 STUDY OVERVIEW

In the previous chapter, mtDNA levels were shown to be altered by high-dose simvastatin treatment, thus answering our initial research question. However, in spite of considerable mtDNA reductions (median decrease of 47% in fourteen 80 mg simvastatin users after only 8 weeks of treatment), the individuals studied in the previous chapter did not report any muscle complaints and did not show abnormal CK values. Therefore, our next research question was: if mtDNA levels can be reduced in some statin users without muscle pain, then do patients on statin therapy with muscle pain have even more severely reduced levels of skeletal muscle mtDNA? In an attempt to address this question, we set out to quantify mtDNA/nDNA ratios in archived skeletal muscle biopsies that had been collected from statin users during an acute episode of myopathy, and compare these with appropriate controls. Unfortunately, we were unable to obtain adequate samples, as described below.

3.2 METHOD

3.2.1 Study design

This was to be a retro- and prospective cross-sectional study. The intent was to compare mtDNA/nDNA ratios in quadricep muscle biopsies collected from 4 groups of patients (N = 20 per group) to explore the relationship between statin therapy and muscle pain in terms of mtDNA/nDNA ratio. The quadricep muscle was chosen for purposes of comparing these data to data generated from Chapter 2. Biopsy specimens from Groups 1 and 2 were to be obtained retrospectively, from the 8000+ archive of muscle biopsies stored for research purposes at Vancouver General Hospital (VGH). These samples are preserved at -80°C in OCT (optimal
cutting temperature compound), a substance used to preserve tissue architecture during freezing and subsequent sectioning of frozen tissue. Groups 3 and 4 were to be enrolled prospectively from the Healthy Heart Program / Lipid Clinic at St. Paul’s Hospital and the general population, respectively (Figure 3.1). The subjects were to be age-matched as evidence suggests that skeletal muscle mtDNA levels can be affected by age (Masuyama et al, 2005).

Group 1: Statin users with muscle pain due to statin therapy as determined by the pathologist who analyzed the biopsy. Obtained retrospectively from the VGH archive.

Group 2: Non-statin users with muscle pain but the specimen was determined to be non-pathologic by the examining pathologist. Obtained retrospectively from the VGH archive and age-gender-matched to Group 1.

Group 3: Statin-users without muscle pain recruited prospectively from the Healthy Heart Program / Lipid Clinic at St. Paul’s Hospital patient database and invited to donate a quadricep needle biopsy specimen. Age-gender- and statin-matched to Group 1.

Group 4: Non-statin users without muscle pain (healthy controls) recruited prospectively from the general population and invited to donate a quadricep needle biopsy specimen. Age-gender-matched to Group 1.
RETROSPECTIVELY
Identify quadriiceps muscle biopsies from the 8000+ muscle biopsy archive at Vancouver General Hospital

GROUP 1
Statin users with myopathy.
N=20

GROUP 2
Non-statin users with myopathy, no pathology (age-gender-matched to GROUP 1)
N=20

GROUP 3
Statin users without myopathy (age-gender-statin-matched to GROUP 1)
N=20

GROUP 4
Healthy controls not on statin and without myopathy (age-gender-matched to GROUP 1)
N=20

PROSPECTIVELY
Collect needle muscle biopsies from subjects recruited from the 6000+ Healthy Heart Program patient database.

Collect needle muscle biopsies from controls recruited from general population

Primary endpoint: compare mtDNA in different groups.
Also do microscopy & histopathology on all samples.

Figure 3.1: Study design.
mtDNA/nDNA = mitochondrial DNA copy number / nuclear DNA copy number
Ethical Approval from The University of British Columbia (UBC) Clinical Research Ethics Board was obtained to access the muscle biopsy database at VGH in order to identify appropriate retrospective biopsy specimens for Groups 1 and 2, collect relevant demographic and clinical information from the associated pathology reports, and transport the specimens to the Côté laboratory at UBC Hospital for mtDNA quantification (Appendix A).

3.2.2 Subject identification

The first step of this research project was to identify Group 1 individuals using an electronic search of the VGH database with the word “statin” and variations such as “atorvastatin” as search terms. Appropriate quadriceps specimens with sufficient clinical and demographic information available were to be aliquoted on-site at VGH.

3.2.3 Clinical and demographic data collection

The following information variables were collected: name, age, date of birth, sex, biopsy collection date, examining pathologist, muscle from which the biopsy was collected (eg. quadriceps), the last statin they were on, if they ever had elevated CK due to statin treatment, if they were still experiencing muscle symptoms at time of biopsy collection, if they had a family or personal history of muscle disease, other medical conditions present, and the examining pathologist’s diagnosis.

3.2.4 Laboratory analyses

Approximately 25 mg of tissue were to be transferred into a new study tube and transported on dry-ice from VGH to the Côté laboratory for laboratory analysis. Total DNA was to be extracted from these biopsies and mtDNA/nDNA ratios determined as described above in Chapter 2.
The mean quadriceps mtDNA level of Group 1 patients was to be compared with that of the 3 other study groups, and also possibly to mtDNA data from the Chapter 2 study. The type of statistical analyses used was to depend on the distribution of the mtDNA/nDNA ratio data (eg. normal or non-normal distribution). The clinical and demographic information collected from the pathology reports was to be used for control matching, statistical adjustments and correlations.

3.3 RESULTS

The initial screen of the electronic database using the aforementioned search terms identified a total of 21 candidate biopsies, all of which had been collected after the year 2000. From this, one pathology report could not be retrieved, and I reviewed the remaining 20. Of those 20 reports, there were two from ineligible patients: in one there was no indication that the patient had ever been on statin therapy, in the other the patient had been off statin therapy for four years prior to the time of muscle biopsy. Of the 18 remaining candidates, the average age was 63.4 years, and 6/18 (33%) were female. There were four different pathologists who had analyzed these muscle specimens and generated pathology reports for them. Of the 18 candidates, only 5 had had their biopsy in the quadriceps; there were six biceps, three deltoid, one calf, and three unspecified.

Clinical characteristics of the 5 quadriceps biopsies are presented in Table 3.1.
Table 3.1: Clinical characteristics of the 5 quadriceps biopsies.

<table>
<thead>
<tr>
<th></th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at time of biopsy</td>
<td>57</td>
<td>54</td>
<td>52</td>
<td>79</td>
<td>67</td>
</tr>
<tr>
<td>Gender</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Last statin on</td>
<td>Simvastatin</td>
<td>Cerivastatin</td>
<td>Atorvastatin</td>
<td>Atorvastatin</td>
<td>Unspecified</td>
</tr>
<tr>
<td>Ever had statin-induced CK elevation?</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Muscle pain was statin-induced?</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Unspecified</td>
</tr>
<tr>
<td>Had muscle symptoms at time of biopsy?</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Family history of muscle disease?</td>
<td>No</td>
<td>Unspecified</td>
<td>Unspecified</td>
<td>Unspecified</td>
<td>Unspecified</td>
</tr>
<tr>
<td>Other medical conditions</td>
<td>Hypertension</td>
<td>Bipolar affective disorder</td>
<td>Type II Diabetes</td>
<td>Hypertension, Type II Diabetes</td>
<td>Unspecified</td>
</tr>
<tr>
<td>Summary of pathologist's diagnosis</td>
<td>Occasional muscle fibre atrophy and necrosis</td>
<td>Myopathic changes with type II muscle fibre atrophy</td>
<td>Inflammatory myopathy</td>
<td>Inflammatory myopathy, ragged red fibres indicative of mitochondrial dysfunction, mild type II muscle fibre atrophy</td>
<td>Denervation atrophy</td>
</tr>
</tbody>
</table>
Due to the small number of quadriceps biopsies identified (N = 5) and the lack of much necessary information in the pathology reports (such as type of statin, duration and dosage of statin therapy, concomitant medications and medical conditions, the presence or absence of a personal or family history of muscle disease, CK data, and even the muscle from which the biopsy was obtained), this study was postponed. The prospective component of this study was not conducted, and ethical approval was not sought for it, due to insufficient funding.
CHAPTER 4: CELL CULTURE EXPERIMENTS

4.1 STUDY OVERVIEW

In the previous chapter we attempted to measure mtDNA levels in muscle biopsies collected from statin-treated individuals during a period of muscle pain, but were unsuccessful. Given the difficult experience we had in acquiring appropriate clinical samples (human skeletal muscle tissue), we then decided to turn to an in vitro experimental approach. In this chapter, human primary skeletal muscle cells were exposed to statins in an attempt to reproduce the effect seen in the in vivo clinical data described in Chapter 2, namely, decreased mtDNA in simvastatin users. The main research question in this chapter was: in this in vitro cell model system, does statin exposure decrease levels of mtDNA in human skeletal muscle cells? If so, is it a dose-dependent or statin-dependent effect? Three different cell culture experiments were carried out, subsequently, as described below.

4.2 METHOD

4.2.1 Drugs

Two types of statins were used in the cell culture experiments: simvastatin and atorvastatin. These drugs were selected given the overall goal of these cell culture experiments which was to attempt to mimic the Chapter 2 in vivo findings of decreased mtDNA in high-dose simvastatin users and unaffected mtDNA in high-dose atorvastatin users.

Simvastatin was purchased from a commercial vendor (Calbiochem, EMD Chemicals Inc; Darmstadt, Germany) in its active acid, form: simvastatin sodium salt. This carboxylate form of simvastatin, molecular weight 458.6 grams per mole (g/mol), is in powder form and is
soluble in dimethyl sulfoxide (DMSO) or ethanol. Atorvastatin (base-compound) was a gift from Pfizer Canada. It also came as a powder, molecular weight 558.64 g/mol.

Both of these compounds were dissolved in DMSO (Sigma) rather than ethanol as alcohols such as ethanol may have an unwanted toxic effect on mtDNA and/or mitochondria (Mansouri et al., 1997; Mansouri et al., 1999). The final concentration of DMSO in the cell culture media was 0.1% (eg. 1 uL per 1 mL), a concentration low enough to likely be non-toxic to the cells (Nachshon-Kedmi et al., 2004; Zhang Y et al., 2005).

4.2.2 Cells

It currently is not possible to stabilize cultured human skeletal muscle cells. However, several commercial vendors have recently made available primary culture systems for the study of human skeletal muscle-like cells. The human skeletal muscle cells (hSkMC) used in these experiments were purchased from a US company (Part Code CC-2580; Clonetics® Human Skeletal Muscle Myoblasts; Cambrex Bio Science Walkersville, Inc, Baltimore, MD, USA). They were shipped frozen in cryovials as second-passage primary-derived normal human muscle cells (myoblasts), obtained from a 24 year-old Caucasian male.

4.2.3 Medium

As per the hSkMC manufacturer’s recommendations, the SkGM®-2 Skeletal Muscle Medium System Two was used (BulletKit®; Part Code: CC-3245; Cambrex). SkGM®-2 SingleQuots® (Cambrex) – growth factors, cytokines, and supplements – were added to 500 mL of the SkGM medium: rhEGF (recombinant human epiderman growth factor; a growth factor that plays an important role in cell growth, proliferation, and differentiation) 0.5 mL (Part Code: CC-4420W), dexamethasone 0.5 mL (CC-4421W), L-glutamine 10 mL (CC-4422W), and fetal bovine serum 50 mL (CC-4423W). The provided antibiotic (GA-1000 0.5 mL; Part Code CC-4419W) was not
added as recommended by the vendor, as antibiotics may have an undesirable toxic effect on mtDNA and/or mitochondria (Tune, 1989; Pritsos et al, 1997). DMEM:F-12 (Part Code 12-719F; Cambrex) supplemented with 2% horse serum (Part Code 14-403; Cambrex) was used to differentiate the myoblasts into multinucleated myotubes before subsequent drug treatment.

4.2.4 Differentiation procedure

The cells were cultured at 37°C in a 5% CO₂ atmosphere. Prior to initiation of drug treatment, the cells were handled as follows:

1) The frozen myoblasts cells were thawed and seeded into a T-175 flask (BD Biosciences, Falcon, San Jose, CA, USA) at 3,500 cells/cm² as per the manufacturer's recommendation, then brought to 60% confluence which took between 3 and 5 days. The SkGM medium was replaced every 48 hours.

2) The myoblasts were then passaged into 6-well plates (Falcon), again at a seeding density of 3,500 cells/cm², and brought up to 60% confluence which took between 3 and 5 days.

3) At this point the regular medium was removed and replaced with the differentiation, or fusion, medium. The myoblasts were treated with the fusion medium for 5 days in order to differentiate them into myotubes.

4) After 5 days in the fusion medium, the drug-containing regular SkGM medium was added.
Thaw frozen cells, plate in flask 3-5 days 60% confluence, passage into 6-well plates 3-5 days 60% confluence, add fusion medium 5 days Remove fusion medium, initiate treatment

mtDNA/nDNA ratio

Total DNA extraction

Harvest cells at end of treatment

Cells viable for up to 14 days

Figure 4.1: Flowchart of the myoblast differentiation and treatment procedure.

mtDNA/nDNA ratio = mitochondrial DNA copy number / nuclear DNA copy number ratio
4.2.5 Harvesting procedure

The cells were harvested as per the manufacturer’s recommendation (Cambrex). Briefly:

1) The medium was aspirated from the well.
2) The cells were rinsed in the well with phosphate-buffered saline (PBS) (Gibco-Invitrogen) (1 ml PBS per well; 6-well plates were used).
3) To re-suspend these adherent cells, 0.25% trypsin (Gibco-Invitrogen) was applied to the cells in the well for 6 minutes (2 ml trypsin per well).
4) The vessel was rapped gently to release the cells.
5) The mixture of trypsin and released cells was transferred to a fresh 15 ml centrifuge tube (Falcon) containing an equal volume of SkGM medium to neutralize the trypsin.
6) A PBS rinse of the well was performed and the rinse added to the cells.
7) The wells were inspected under an inverted microscope (Nikon) to ensure a successful harvest (> 95% of cells released).
8) The cells were centrifuged at 220 g for 5 minutes to produce a pellet.
9) All but approximately 1 ml of the supernatant was aspirated from the 15 ml tube (Falcon).
10) The cells were resuspended in the supernatant, and this mixture was transferred to a 1.7 ml microcentrifuge tube (Falcon).
11) The cells were spun for 30 seconds at high speed.
12) All supernatant was carefully removed with a P1000 pipet (Gilson, Middleton, WI, USA).
13) A cell lysis buffer, QIAGEN Buffer RLT Plus, was added (350 uL) as per QIAGEN’s recommendation.
14) The harvested cells were stored at -80°C for subsequent nucleic acids extraction.
4.3 First cell culture experiment: Overview

The purpose of the first cell culture experiment was to treat the cells with a very high dose of statin and determine if any effect at all could be observed on levels of mtDNA in this in vitro system.

4.3.1 Design

The myoblasts were differentiated into myotubes as described above and brought to 100% confluence in 6-well plates. Differentiation was assessed by a direct inverted light microscope examination. Three treatments were used: 30 uM simvastatin acid, 30 uM atorvastatin, and DMSO (control). The treatment period was to last for 12 days, treatments were done in triplicate, and the drug-containing medium was replaced every 48 hours. The cells used in this experiment were at passage #2; according to the vendor, these cells are viable up to passage #10.

4.3.2 Results

After 4 days the simvastatin and atorvastatin treated cells had dropped from 100% to 5% confluence. The DMSO-only treated control cells remained at 100% confluence. From this it was concluded that the doses used were much too high and were cytotoxic.

4.4 Second cell culture experiment: Overview

The purpose of the second cell culture experiment was to treat the cells with simvastatin at various doses to determine an appropriate dose to use for subsequent experiments.

4.4.1 Design

The myotubes were brought to 100% confluence in 6-well plates as above. Six drug treatments of simvastatin were used: 5 uM, 1 uM, 0.1 uM, 0.025 uM, 0.005 uM, and DMSO. These doses
were selected based on the human physiological peak serum concentration (C<sub>max</sub>) associated with a 40 to 80 mg dose of simvastatin acid, which is approximately 5 ng/ml or 0.01uM (Ucar et al., 2004; Marino et al., 2000; Shitara and Sugiyama, 2006). The myotubes were harvested at four timepoints: baseline, 2, 6, and 12 days. The following treatments were done in triplicate: baseline untreated; DMSO 2 and 12 days; and 0.1 uM 2 and 12 days. All other treatments were done in single, due to contamination which occurred near the end of the differentiation step. The cells used in this experiment were at passage #4.

4.4.1.1 mtDNA quantification

At the end of drug-treatment, total DNA was extracted from the harvested cells using a QIAamp DNA Mini Kit (February 2003 edition, QIAGEN, Mississauga, ON, Canada). MtDNA quantification was done in duplicate as described in Chapter 2 with a LightCycler 480 and LightCycler 480 Probes Master kit (Roche Applied Science; Penzberg, Germany). As before, the mtDNA assays were performed in a blinded fashion, and results are expressed as the mtDNA/nDNA ratio.

4.4.2 RESULTS

4.4.2.1 Confluence

At baseline all wells contained multinucleated fully differentiated myotubes at 100% confluence (Table 4.1). After 2 days of drug treatment, the 1 uM cells had dropped to 90% confluence, and the 5 uM-treated cells had dropped to 80% confluence. At Day 6 and at Day 12, the 1 uM and 5 uM cells were at 60% and 5% confluence, respectively. All other treatments stayed at 100% confluence throughout the experiment. From this it was concluded that the selected doses were
not desirable, and that the doses used were still too high as the higher doses were causing too much cell death, and there were too few of the lower doses.

**Table 4.1: Confluence results from cell culture experiment 2.**

<table>
<thead>
<tr>
<th>% confluence</th>
<th>0 DAYS</th>
<th>2 DAYS</th>
<th>6 DAYS</th>
<th>12 DAYS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>5 uM</td>
<td>100</td>
<td>90</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>1 uM</td>
<td>100</td>
<td>80</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>0.1 uM</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.025 uM</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.005 uM</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

4.4.2.2 mtDNA

The baseline mtDNA/nDNA ratio was 159 (N = 3; SD = 32) (Table 4.2; Figure 4.2; Figure 4.3). The mtDNA in the DMSO control cells increased slightly over the treatment period. Similar data were observed in the cells treated with all simvastatin doses except the highest dose. The 5 uM-treated cells’ mtDNA/nDNA ratio experienced a marked increase: 314 at 2 days, 1011 at 6 days, and 2303 at 12 days.

**Table 4.2: mtDNA/nDNA ratio results from cell culture experiment 2.**

<table>
<thead>
<tr>
<th>mtDNA/nDNA ratio</th>
<th>0 DAYS</th>
<th>2 DAYS</th>
<th>6 DAYS</th>
<th>12 DAYS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>159</td>
<td>221</td>
<td>172</td>
<td>247</td>
</tr>
<tr>
<td>5uM</td>
<td>159</td>
<td>314</td>
<td>1011</td>
<td>2303</td>
</tr>
<tr>
<td>1uM</td>
<td>159</td>
<td>236</td>
<td>299</td>
<td>324</td>
</tr>
<tr>
<td>0.1uM</td>
<td>159</td>
<td>229</td>
<td>174</td>
<td>235</td>
</tr>
<tr>
<td>0.025uM</td>
<td>159</td>
<td>293</td>
<td>174</td>
<td>211</td>
</tr>
<tr>
<td>0.005uM</td>
<td>159</td>
<td>155</td>
<td>158</td>
<td>290</td>
</tr>
</tbody>
</table>
Figure 4.2: mtDNA/nDNA results from cell culture experiment 2
The following treatments were done in triplicate: baseline, DMSO 2 days, DMSO 12 days, 0.1 uM 2 days, 0.1 uM 12 days. All others were done in single due to contamination.
Figure 4.3: mtDNA/nDNA results from cell culture experiment 2 (different y axis)
The following treatments were done in triplicate: baseline, DMSO 2 days, DMSO 12 days, 0.1 uM 2 days, 0.1 uM 12 days. All others were done in single due to contamination. The DAY 6 and DAY 12 5 uM treatments continue upwards as indicated in the previous table.
4.5 Third cell culture experiment: Overview

The 3rd cell culture experiment was a repeat of the 2nd one, but with different simvastatin doses and harvest timepoints.

4.5.1 METHOD

4.5.1.1 Design

In this experiment, simvastatin was again the only drug used. Based on data from the second cell culture experiment, different doses were selected. There were 7 treatments: 1 uM, 0.5 uM, 0.1 uM, 0.05 uM, 0.01 uM, DMSO, and untreated (SkGM medium only). There were two timepoints: baseline (Day 0) and Day 12. Pictures of the cells at these timepoints were taken using a digital camera attached to an inverted microscope (Nikon). The cells used in this experiment were at passage #4. All treatments were performed in quadruplicate. Three wells were harvested for mtDNA quantitation, performed as described above in the second cell culture experiment, and the fourth was used for cell counting and viability analyses.

4.5.1.2 Cell counting and viability assessment

Cell counting was performed using a hemacytometer (Bright-Line; Hausser Scientific, Horsham, PA, USA) and cell viability was assessed using tryphan blue stain (Sigma) as described in the Cambrex hSkMC protocol (Cambrex).
4.5.2 RESULTS

4.5.2.1 Confluence, viability, and cell count

At baseline the confluence was 90% (Table 4.3). At Day 12, all treatments were at 100% confluence except the highest dose, 1 uM, which had dropped to 80%.

The cell viability at baseline and at Day 12 in all treatments was approximately 90% (Table 4.3). The reason for this is may be the nature of the viability assay. The cells that were assessed for viability were only those cells that remained adhered to the bottom of the well; all of the cells that had died, presumably, and were released into the medium were aspirated and not included in the analysis. Thus, viability was determined only in the adherent cells, and it is therefore understandable that all drug treatments and even the baseline cells exhibited almost identical viability.

At Day 12, the cell count was approximately equal in all seven treatments at about 975,000, excluding the highest simvastatin dose (1 uM) which counted only 351,000 (Table 4.3). This is likely due to the lower confluence observed in the 1 uM-treated cells. At baseline, the cell count was considerably higher at 2,604,000. This apparent disconnect between confluence and cell number may be due to the myoblasts not being fully fused together into myotubes at baseline. Photos of the cells taken at Day 12 are displayed in Figure 4.4.
Table 4.3: Day 12 cell count, confluence, viability, and mtDNA/nDNA ratio results from cell culture experiment 3.

mtDNA/nDNA ratio data are the averages of triplicate wells. SD = standard deviation; CV = coefficient of variation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Confluence (%)</th>
<th>Cell viability (%)</th>
<th>Cell count</th>
<th>mtDNA/nDNA ratio</th>
<th>Ratio</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>90</td>
<td>89</td>
<td>2,604,000</td>
<td>258</td>
<td>22</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>100</td>
<td>93</td>
<td>840,000</td>
<td>221</td>
<td>47</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>100</td>
<td>89</td>
<td>975,000</td>
<td>224</td>
<td>53</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>1.0uM</td>
<td>80</td>
<td>87</td>
<td>351,000</td>
<td>388</td>
<td>35</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>0.5uM</td>
<td>100</td>
<td>91</td>
<td>1,125,000</td>
<td>252</td>
<td>20</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>0.1uM</td>
<td>100</td>
<td>88</td>
<td>941,875</td>
<td>270</td>
<td>24</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>0.05uM</td>
<td>100</td>
<td>91</td>
<td>994,500</td>
<td>284</td>
<td>59</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>0.01uM</td>
<td>100</td>
<td>87</td>
<td>969,375</td>
<td>247</td>
<td>60</td>
<td>24</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.4: Images of cells at Day 12 in experiment 3

All images were taken at Day 12 (except baseline which was at Day 0) just prior to harvesting. Images were taken before the medium was removed (thus the medium is approximately 48 hours old in the images). There appear to be more floating white spherical cells (dead cells) in 1 uM image, probably reflecting increased statin cytotoxicity. The baseline image is a different colour due to a software modification utilized to clarify the image. UNTREATED = medium only.
4.5.2.2 mtDNA

At baseline the mtDNA/nDNA ratio was 258 (N = 3; triplicate ratio SD = 22; CV = 8%) (Table 4.3; Figure 4.5). At Day 12 the values were similar for all drug treatments excluding the highest dose (1 uM) at approximately 250; therefore, for almost all treatments the ratio did not change after 12 days of exposure to simvastatin acid. The mtDNA/nDNA ratio in the 1 uM-treated cells was increased at 388 (N = 3; SD = 35; CV = 9), but 20% of the cells had died and thus it may be unreliable to draw conclusions about this ratio in dying cells.
Figure 4.5: mtDNA/nDNA ratio results at Day 12 in experiment 3.
All treatments were performed in triplicate, and the values shown are the triplicate averages.
CHAPTER 5: STATIN-INDUCED RHABDOMYOLYSIS STUDY

5.1 STUDY OVERVIEW

The previous chapter described attempts to determine the effect of statins on mtDNA levels in vitro, in a human primary skeletal muscle cell system. While these studies did contribute to our knowledge of appropriate dose ranges to use for subsequent such experiments, they provided few data to help with our understanding of the mechanism of SIM or statin-induced mtDNA level alterations, and we were unable to reproduce in vitro our clinical in vivo data from Chapter 2. At this point we were fortunate that our collaborators shared with us a new batch of clinical samples, this time from statin users with severe muscle damage and a diagnosis of statin-induced rhabdomyolysis (SIR). In this chapter, mtDNA content was quantified in skeletal muscle biopsies that had been collected by another research group from patients with SIR (N = 10), and compared to an age-matched control group (N = 8). The purpose was to explore muscle mtDNA levels in cases with severe clinical myotoxicity, and the main research question was: do muscle biopsies collected from statin-users during an episode of rhabdomyolysis have severely reduced mtDNA content? In addition, gene expression levels of mtDNA-encoded genes, or mitochondrial RNA (mtRNA), were analyzed.

5.2 METHOD

5.2.1 Patient population

All samples were collected by Dr. Paul S. Phillips at the Clinical Research Centre, Scripps Mercy Hospital in San Diego as part of research at that institution and shipped to Vancouver on dry ice. In the affected group (N = 10), percutaneous muscle biopsies were collected from patients with a clinical diagnosis of SIR. Inclusion criteria were: muscle pain or weakness due to
statin therapy, serum CK > 10X ULN, and normal thyroid stimulating hormone level. Exclusion criteria were: other diagnoses such as trauma, illicit drug use, or sepsis that could contribute to rhabdomyolysis. Individuals taking ubiquinone or carnitine supplements were excluded as well. In the SIR group there were 8 males and 2 females. All 10 SIR subjects were experiencing muscle pain at the time of biopsy.

Controls (N = 8) were statin-naïve individuals undergoing total knee replacement and willing to donate a specimen of quadriceps vastus medialis muscle. In the control group there were 2 males and 6 females.

The two study groups were similar in age (P = 0.76), with a median age of approximately 64 years in each group. This study was approved by the local ethics committee and all participants signed an informed consent form.

5.2.2 mtDNA quantification

Upon collection, muscle biopsies had been divided into 3 portions: one portion was processed for ultrastructure and histology testing, another was placed in HAMS media and prepared for cell culture analyses, and the third was snap-frozen in liquid nitrogen for subsequent biochemical analyses including mtDNA content and expression quantification. On a bed of dry ice, approximately 5 mg of snap-frozen dry unprocessed skeletal muscle, which had been stored at -80°C, was aliquoted into a separate cold 1.7 mL tube for DNA extraction, using a disposable scalpel and petri dish.

The QIAamp DNA Mini Kit (QIAGEN, February 2003 edition) was used for total DNA extraction from the 5 mg aliquots. With these unprocessed muscle samples, a prolonged lysis step was necessary during the extraction procedure; as per the manufacturer’s recommendation, the muscle aliquots were lysed overnight at 56°C in a solution of Proteinase K (20 uL) and QIAGEN Buffer ATL (180 uL).
mtDNA was quantified in a blinded fashion as described in Chapter 2 but with the LC 480 instrument and LC 480 Probes Master kit (Roche Applied Science, Penzberg, Germany). As before, results are expressed as the mtDNA/nDNA ratio.

5.2.3 mtRNA quantification

Total muscle RNA was extracted from 10 mg aliquots of biopsy material. Ten mg was selected as an appropriate amount of starting material based on the manufacturer’s recommendation (QIAGEN). The tissue was homogenized using a Polytron® System PT2100 (Lucerne, Switzerland) and total RNA was isolated using the QIAGEN RNeasy® Fibrous Tissue Mini Kit (Mississauga, Canada). This kit was more appropriate than the regular QIAGEN RNA isolation kit because skeletal muscle, like heart and aortic-tissue, is a fibre-rich tissue that contains contractile proteins, connective tissue, and collagen, all of which may interfere with proper cell lysis. The RNeasy® Fibrous Tissue kit contains a Proteinase K treatment step which removes these proteins. Six microlitres of the RNA extract was used to synthesize cDNA using the QIAGEN QuantiTect® Reverse Transcription Kit (Mississauga, Canada). As part of this kit, the ‘RT Primer mix,’ a random primer, was used. To quantify mtRNA, real-time PCR with fluorescent probes was performed as above but with the following modifications. A 6 point standard curve was constructed from serial 10-fold dilutions (84 to 8,400,000 copies / 2 uL in 10 uL final volume) of plasmid DNA containing the housekeeping (human glyeraldehyde-3-phosphate dehydrogenase, GAPDH) and mitochondrial (CCOI) genes studied. CCOI primer and probe sequences used were the same as above. GAPDH was chosen as a housekeeping gene because it has been shown to be appropriate for skeletal muscle studies (Barber et al, 2005) and unaffected by age (Touchberry et al, 2006). GAPDH primer and probe sequences were identified based on another study (Wagner et al, 2002) and were as follows: the forward primer used was 5’-TTGGTATCGTGGAAGGACTCA-3’, the reverse primer was 5’-
TGTCATCATATTTGTCAGGTTT-3', the hybridization fluorescein probe was 5'-TGTCCCACTGCAACGTGTCAG-3'-FL, and the hybridization LC Red 640 probe was 5'LCRed640-GGTGGACCTGACCTGCCGTCTAGA-3' (TIB Molbiol, Berlin, Germany). Real-time PCR conditions were the same as described in Chapter 2. The results are expressed as mitochondrial mRNA copy number / housekeeping gene mRNA copy number ratio (CCO1/GAPDH mRNA ratio). For both mtDNA and gene expression quantification, samples were assayed blindly in duplicate. The inter-run CV was < 15% and muscle and plasmid DNA exhibited similar PCR efficiencies.

5.2.4 Other laboratories’ assays

Numerous other tests were conducted by the various collaborators in this study (described in Phillips et al, in preparation). Pathology of all samples was determined by a single blinded pathologist utilizing a light microscope and a numerical ‘index of abnormality’ score was given for each sample for purposes of statistical comparison. EM was performed on the SIR samples only. The presence of morphological characteristics as degenerative/regenerative fibres, COX negative fibres, ragged red fibres, and lipid accumulation were determined. Fatty acid oxidation was measured in cell cultures of the biopsy specimens. Biochemical analyses such as mitochondrial respiratory chain enzyme activities, ubiquinone, sterols, prenylated proteins, geranylgeranylpyrophosphate levels, and lipidomics were included. Finally, atrogin-1 expression levels were measured. Atrogin-1 is a recently-discovered gene that is highly expressed during muscle atrophy, and its expression appears to increase with statin exposure (Gomes et al, 2001; Lecker et al, in preparation).

The majority of the other laboratory tests conducted on these biopsy specimens were unable to discern a statistically significant difference between the two groups of samples. These tests include: histopathology (P = 0.95); fatty acid oxidation response to statin treatment of
cultured myocytes from the biopsy specimens (P = 0.28); mitochondrial respiratory chain enzyme activities including citrate synthase (P = 0.23), succinate dehydrogenase (P = 0.50), and COX (P = 0.59); ubiquinone (P = 0.40); and finally measurements of intramuscular cholesterol (P = 0.89), lathosterol (P = 0.72), and desmosterol (P = 0.70).

The other methods, however, were able to distinguish between normal and SIR muscle samples: levels of sitosterol (P = 0.03), sitosterol/cholesterol (P = 0.015), and campesterol (P = 0.09; a trend but not quite statistically significant) were all increased in the affected group. In addition, atrogin-1 expression was non-significantly increased (P = 0.08), and the prenylated protein Ras was significantly decreased (P = 0.008) in the SIR group.

There was a non-significant trend toward reduced quantity of mitochondrial enzymes when the 5 SIR-simvastatin subjects were analyzed separately and compared to the 8 controls: citrate synthase (P = 0.07), succinic dehydrogenase (P = 0.07) and COX (P = 0.06).

5.2.5 Statistical analyses

The Shapiro-Wilk test was used to determine whether the data were normally distributed to ensure that parametric tests could then be used. The f- and one-tailed t-tests were used to determine statistical significance for comparisons between the affected and control individuals in terms of mtDNA content and expression. Correlations were determined using the Pearson coefficient and linear regression equations. A P value of < 0.05 was considered statistically significant. Microsoft Excel® (Microsoft Corporation®, Seattle, WA, USA) and R® (Vienna, Austria) were used for these statistical analyses.
5.3 RESULTS

5.3.1 Clinical characteristics

The clinical characteristics of the SIR study participants are presented in Table 5.1, including the marked variation in days from last statin dose to biopsy.

Table 5.1: Clinical characteristics of the 10 SIR subjects.
The normal reference range for serum creatine kinase is 55 – 170 units/litre in males and 30 – 135 units/litre in females (Lehmann and Henry, 2006). SD = standard deviation

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Peak serum creatine kinase (units/litre)</th>
<th>Statin (mg/day)</th>
<th>Other possibly contributing drugs</th>
<th>Time elapsed between last statin dose and biopsy (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>51</td>
<td>8,881</td>
<td>lovastatin 20</td>
<td>niacin 500</td>
<td>69</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>49</td>
<td>10,597</td>
<td>pravastatin 20</td>
<td>ezetimibe 10</td>
<td>27</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>82</td>
<td>51,080</td>
<td>simvastatin 40</td>
<td>ketoconazole 100</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>71</td>
<td>45,750</td>
<td>simvastatin 20</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>Male</td>
<td>67</td>
<td>104,000</td>
<td>simvastatin 40</td>
<td>niacin 500, ketoconazole 100</td>
<td>38</td>
</tr>
<tr>
<td>6</td>
<td>Female</td>
<td>62</td>
<td>39,000</td>
<td>simvastatin 40</td>
<td>itraconazole</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>Male</td>
<td>87</td>
<td>8,264</td>
<td>simvastatin 20</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>65</td>
<td>12,000</td>
<td>atorvastatin 20</td>
<td></td>
<td>45</td>
</tr>
<tr>
<td>9</td>
<td>Male</td>
<td>54</td>
<td>13,000</td>
<td>atorvastatin 20</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>Male</td>
<td>56</td>
<td>11,106</td>
<td>atorvastatin 80</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td></td>
<td>30,368 ± 30,755</td>
<td></td>
<td></td>
<td>20 ± 24</td>
</tr>
</tbody>
</table>

5.3.2 mtDNA content and expression

No significant difference was observed in mtDNA content (P = 0.36) (Figure 5.1) or expression, as expressed by the mitochondrial to GAPDH mRNA ratio (P = 0.75) (Figure 5.2), between the two study groups.
Figure 5.1: mtDNA levels in the two study groups
Comparative box-plots illustrating the muscle mitochondrial DNA/nuclear DNA (mtDNA/nDNA) ratio in the 8 healthy controls and the 10 statin-induced rhabdomyolysis patients. The vertical lines represent the maximal and minimal mtDNA/nDNA ratios observed within each group, the top and bottom of the boxes indicate the inter-quartile ranges (25th and 75th percentiles), and the bold horizontal lines within the boxes represent the medians; the "o" above the SIR boxplot represents an outlier in that group. There was no significant difference between the two groups \( (P = 0.36) \), though more variation was observed in the SIR group.
Figure 5.2: mtDNA expression levels in the two study groups
Comparative box-plots illustrating the muscle mitochondrial DNA expression levels, as expressed by the mitochondrial to GAPDH mRNA ratio in each of the two study groups (7 healthy controls and the 9 statin-induced rhabdomyolysis patients). One subject from each group was excluded from this analysis due to a suspicion of poor tissue preservation in two of the study samples. The vertical lines represent the maximal and minimal values observed within each group, the top and bottom of the boxes indicate the inter-quartile ranges (25th and 75th percentiles), and the bold horizontal lines within the boxes represent the medians. No significant difference was observed between the two groups ($P = 0.75$).
However, a sub-analysis of the 5 simvastatin-treated SIR subjects revealed a trend toward reduced mtDNA content in that group compared to the 8 controls (P = 0.09) (Figure 5.3). No such trend was observed in mtDNA expression in this simvastatin sub-analysis (P = 0.40) (Figure 5.4).
Figure 5.3: mtDNA levels in the SIR-simva and control groups

Comparative box-plots illustrating the muscle mitochondrial DNA / nuclear DNA (mtDNA/nDNA) ratio averages between the 8 healthy controls and the 5 statin-induced rhabdomyolysis patients treated with simvastatin (labelled “SIR-simva”). The vertical lines represent the maximal and minimal mtDNA/nDNA ratios observed within each group, the top and bottom of the boxes indicate the inter-quartile ranges (25th and 75th percentiles), and the bold horizontal lines within the boxes represent the medians; the “o” above the SIR-simva boxplot represents an outlier in that group. A non-significant trend toward reduced mtDNA was observed in the SIR-simvastatin patients (P = 0.09).
Figure 5.4: mtDNA expression levels in the SIR-simva and control groups
Comparative box-plots illustrating the muscle mitochondrial DNA expression levels, as expressed by the mitochondrial to GAPDH mRNA ratio, in 4 statin-induced rhabdomyolysis subjects and 7 healthy controls. One sample from each group was excluded from this analysis due to suspicions of poor sample preservation. The vertical lines represent the maximal and minimal values observed within each group, the top and bottom of the boxes indicate the inter-quartile ranges (25th and 75th percentiles), and the bold horizontal lines within the boxes represent the median. No significant difference was observed between the two groups ($P = 0.40$).
There was a moderate positive correlation between mtDNA content and expression in the SIR group (R = 0.62, P = 0.07, N = 9), and a strong positive correlation between these variables in the SIR-simva group (R = 0.88, P = 0.12, N = 4). In addition, a moderate negative correlation was observed between mtDNA content and serum CK in the SIR group (R = -0.52, P = 0.12, N = 10), and a weak negative correlation was found between mtDNA expression level and serum CK in the SIR group (R = -0.27, P = 0.48, N = 9).
CHAPTER 6: DISCUSSION

6.1 DISCUSSION

The major finding of this research was the Chapter 2 description of significantly decreased mtDNA levels in skeletal muscle biopsies collected from mildly hypercholesterolemic patients treated with high-dose simvastatin for 8 weeks, but not in those treated with an equivalent lipid-lowering dose of atorvastatin for the same length of time.

There are several studies that are consistent with this finding. First, simvastatin appears to be associated with a slightly higher rate of SIM compared to atorvastatin. As described above, the French PRIMO study observed a higher rate of muscle symptoms in high-dose simvastatin users (18.2%; N = 1,027) compared to high-dose atorvastatin users (14.9%; N = 1,844), though it is unclear whether this difference was statistically significant (Bruckert et al, 2005). A recent retrospective observational study of an administrative claims database in the US provides additional supportive evidence (Cziraky et al, 2006): 473,343 statin patients (490,988 person-years) were studied, and simvastatin users had a slightly higher, though not statistically significant, rate of myopathies compared to atorvastatin users: 3.39 versus 2.45 per 10,000 person-years respectively. In the same study, simvastatin use was also associated with slightly higher but non-significant rates of renal {54.6 (48.6 – 61.2) versus 30.97 (28.8 – 33.2)}, and hepatic events {12.87 (10 – 16.3) versus 9.83 (8.7 – 11.1)}, compared to atorvastatin. A recent meta-analysis of 86,000 participants in 199 statin studies found that more trials reported incidences of rhabdomyolysis, myositis, and CK elevations > 10 times the ULN in simvastatin users than atorvastatin users (McClure et al, 2007).

The recent SIM gene association study by Vladutiu et al (2006) described above also provides supportive evidence. Of all the statins studied, including atorvastatin, it was the simvastatin users that had the highest rate of fatty acid oxidation defects and mitochondrial
disorders including ubiquinone deficiency and respiratory chain defects (Vladutui et al, 2006). The ratio of atorvastatin to simvastatin users in this study was the same as that reported for these drugs between in 2001 and 2003 in the US (IMS Health Annual TRX, 2003).

A very recently published Australian study describes the effect of simvastatin on the petite-positive yeast *Candida glabrata* (Westermeyer and Macreadie, 2007). The investigators found that simvastatin exposure was associated with a complete loss of the mitochondrial genome, as determined by a staining (4’,6-diamidino-2-phenylindol) and fluorescence microscopy technique. This is the only published study to date other than Schick et al (2007) linking statins with mtDNA level. Unfortunately for this discussion, atorvastatin was not included in these experiments on yeast.

Data from the original clinical trial in Finland from which the Chapter 2 muscle biopsies were derived are also supportive. As described above, the simvastatin group alone had significantly decreased muscle ubiquinone. In addition, the selected simvastatin patients (N = 6) had reduced mitochondrial respiratory chain complex activities (II, II + III, III, IV, and citrate synthase), but the ratio of the activities of the complexes and citrate synthase activity was unchanged. The activity of citrate synthase per mitochondrion is believed to be constant. Accordingly, it is commonly used as a housekeeping protein or quantitative enzyme marker in studies of mitochondrial dysfunction. Thus, these data suggest a decrease in the total mitochondrial mass or fewer mitochondria per cell rather than decreased mitochondrial function *per se*. This finding is consistent with the observation of decreased mtDNA at follow-up in the simvastatin group only, which may reflect decreased skeletal muscle mitochondrial mass.

Finally, data from Chapter 5 are also consistent. There was a trend toward lower mtDNA content in the simvastatin users with SIR (N = 5) compared to controls (N = 8) (P = 0.09). In addition, when the 5 simvastatin-treated patients with SIR were analyzed separately, ubiquinone levels were decreased in the affected group compared to controls, as was the case with mtDNA.
Unfortunately, a comparison between the simvastatin- and atorvastatin-treated patients in this study is of little value given the small number of atorvastatin-treated patients ($N = 3$).

All these data are consistent with our finding of greater reductions in mtDNA in simvastatin users than in atorvastatin users. This would suggest that simvastatin and atorvastatin differ in their effect on muscle metabolism. Although the specific properties of these drugs that lead to different effects on muscle mitochondria, specifically mtDNA content, are currently unknown, there are several possible mechanisms.

For example, drug lipophilicity may be involved. The different statins vary markedly in their degree of lipophilicity due to the presence or absence of polar moieties on their predominantly hydrophobic backbones (Table 6.1).
Table 6.1: Pharmacologic characteristics of the statins
Data based on a 40 mg dose except for cerivastatin (0.2 mg). LDL-C = low-density lipoprotein cholesterol; mg = milligrams; logP = logarithm of the ratio of the concentrations of the un-ionized solute in the solvent; uM = micromolar; h = hours. References: De Angelis, 2004; Rosenson, 2004; Jamal et al, 2004; Davidson and Toth, 2004; Bellosta et al, 2004; Schachter, 2004.

<table>
<thead>
<tr>
<th></th>
<th>Simvastatin</th>
<th>Atorvastatin</th>
<th>Cerivastatin</th>
<th>Fluvastatin</th>
<th>Lovastatin</th>
<th>Pravastatin</th>
<th>Rosuvastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>Semi-synthetic</td>
<td>Synthetic</td>
<td>Synthetic</td>
<td>Synthetic</td>
<td>Fungal</td>
<td>Fungal</td>
<td>Synthetic</td>
</tr>
<tr>
<td>Administered form</td>
<td>Pro-drug</td>
<td>Active</td>
<td>Active</td>
<td>Active</td>
<td>Pro-drug</td>
<td>Active</td>
<td>Active</td>
</tr>
<tr>
<td>LDL-C reductions (%)</td>
<td>28 - 48</td>
<td>38 - 54</td>
<td>25 - 44</td>
<td>17 - 33</td>
<td>29 - 48</td>
<td>19 - 40</td>
<td>52 - 63</td>
</tr>
<tr>
<td>Dose range (mg)</td>
<td>10 - 80</td>
<td>10 - 80</td>
<td>0.2 - 0.8</td>
<td>20 - 80</td>
<td>20 - 80</td>
<td>10 - 40</td>
<td>10 - 40</td>
</tr>
<tr>
<td>Lipophilicity (logP)</td>
<td>4.68</td>
<td>4.06</td>
<td>1.47</td>
<td>3.24</td>
<td>4.3</td>
<td>-0.23</td>
<td>-0.15</td>
</tr>
<tr>
<td>Cmax (uM)</td>
<td>0.053</td>
<td>0.083</td>
<td>0.0044</td>
<td>1.09</td>
<td>0.037</td>
<td>0.012</td>
<td>0.077</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>1.3 - 2.4</td>
<td>2.0 - 3.0</td>
<td>2.5</td>
<td>0.5 - 1.0</td>
<td>2.0 - 4.0</td>
<td>0.9 - 1.6</td>
<td>3.0</td>
</tr>
<tr>
<td>Elimination half-life (h)</td>
<td>2.0 - 3.0</td>
<td>15.0 - 30.0</td>
<td>2.0 - 3.0</td>
<td>0.5 - 2.3</td>
<td>2.0 - 3.0</td>
<td>1.3 - 2.8</td>
<td>20</td>
</tr>
<tr>
<td>Absorption fraction (%)</td>
<td>60-80</td>
<td>30</td>
<td>98</td>
<td>98</td>
<td>30</td>
<td>34</td>
<td>Moderate</td>
</tr>
<tr>
<td>Bioavailability (%)</td>
<td>5</td>
<td>12</td>
<td>&gt; 60</td>
<td>19 - 29</td>
<td>0 - 5</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>Cytochrome involved in metabolism</td>
<td>CYP3A4</td>
<td>CYP3A4</td>
<td>CYP3A4, CYP2C8</td>
<td>CYP2C9</td>
<td>CYP3A4</td>
<td>none</td>
<td>CYP2C9 (minimal)</td>
</tr>
</tbody>
</table>
X-ray diffraction studies have shown that lipophilic statins such as simvastatin penetrate more deeply into membranes, whereas hydrophilic statins such as pravastatin tend to remain localized with the polar membrane surface (Mason et al, 2005).

The ability of a statin to penetrate through plasma membranes and into peripheral tissues such as skeletal muscle may enhance its myotoxic potential. Indeed, several studies have suggested that lipophilic statins are more likely to generate muscle problems due to their increased ability to passively diffuse into peripheral tissue such as skeletal muscle. For example, \textit{in vitro} studies of isolated skeletal muscle cells indicate that simvastatin and lovastatin are more likely than hydrophilic pravastatin to inhibit protein synthesis, reduce ATP levels (Masters et al, 1995) and decrease cell viability (Gadbut et al, 1995). Simvastatin and lovastatin have also been shown to be more likely to increase CK and cause myopathy in rat (Masters et al, 1995; Reijneveld et al, 1996) and rabbit (Nakahara et al, 1998) models than pravastatin. An \textit{in vitro} study of mouse myoblasts found pravastatin had poor cell membrane penetration (Gadbut et al, 1995). Cerivastatin, a highly lipophilic statin, was found to considerably disturb calcium ions in mouse and rat skeletal muscle fibres, whereas pravastatin had only a minor effect (Inoue et al, 2003). Hydrophilic statins such as pravastatin are able to get inside non-hepatic cells via passive diffusion, but to a much lesser extent than lipophilic statins (Rosenson, 2004).

In the clinical setting, pravastatin therapy is associated with a low myopathy rate (Shepherd et al, 2002; Staffa et al, 2002; Pfeffer et al, 2002) and is well-tolerated when used with gemfibrozil, a fibrate medication often associated with SIM when co-administered with a statin (Wiklund et al, 1993; Athyros et al, 1997; Iliadis, 1999). Cerivastatin was the most lipophilic statin on the market prior to its withdrawal due to an unacceptably high rate of muscle adverse effects (McTaggart et al, 2001).

However, in relation to the other statins, simvastatin and atorvastatin are comparably lipophilic (\textbf{Table 6.1 above}). Simvastatin is currently the most lipophilic statin commercially
available and is slightly more lipophilic than atorvastatin, but only by a small margin. Therefore, it is likely that properties of these drugs other than lipophilicity account for their different effects on muscle mtDNA as observed in this research.

Another possibility is that simvastatin has a higher tendency than atorvastatin to penetrate plasma membranes due to some sort of active uptake mechanism through the action of carrier proteins. Statins, both lipophilic and hydrophilic, are also able to move through membranes via active transport processes, through proteins such as organic anion transporting polypeptide-C (Hamelin and Turgeon, 1998; McTaggart et al, 2001) and multidrug resistance protein 2 (MRP2) (Thompson et al, 2003; Owczarek et al, 2005). Takeda et al (2004) recently described the mediation of statin transport by human organic ion transporter (OAT) 1 and OAT3, which are located in the membrane of human skeletal muscle. Pravastatin, fluvastatin, and simvastatin were studied and found to inhibit OAT activity, suggesting that these transporters may play a role in SIM and also possibly in our observed effect of simvastatin on mtDNA quantity; unfortunately, atorvastatin was not included in this study.

Other pharmacologic properties may offer an explanation for the varying effects on mtDNA content of atorvastatin and simvastatin observed herein, as there is considerable variation within the statin class in terms of solubility, excretion, plasma protein binding, absorption, LDL-C-lowering potency, among others (Vaughan and Gotto, 2003).

Statin metabolism and elimination does play a role in SIM, but it appears that these two drugs are processed similarly and thus this is unlikely to account for their varying effect in muscle. The cytochrome P450 (CYP) enzyme system is a major metabolism and elimination route for many drugs, including all of the statins except pravastatin (Christians et al, 1998; Schachter, 2001). It has been implicated in statin-drug interactions and SIM (Neuvonen and Jalava, 1996; Neuvonen et al, 1998; Kantola et al, 1998; Schachter, 2001). Cyclosporine co-medication increases the risk for myopathy, and it inhibits CYP3A4’s activity, thus increasing
the concentration of those statins metabolized by it (Schachter, 2001; Pasternak et al., 2002). There are various isoforms of the CYP system (Evans and Rees, 2002). The most abundant isoform is 3A4, which accounts for up to 50% of total CYP protein, and is the predominant isoform that metabolizes both simvastatin and atorvastatin (Prueksaritanont et al., 1997; Zhou et al., 1995). Thus it seems unlikely that the metabolic pathway accounts for the varied muscle effect observed in this research.

It should be mentioned that perhaps the two most important pharmacologic differences between these two statins is that atorvastatin has a markedly higher bioavailability and elimination half-life (Table 6.1 above). The bioavailability of atorvastatin is 2 to 3 times that of simvastatin, meaning that when orally administered a higher fraction of the dose reaches the systemic circulation; in other words, a higher percentage of simvastatin will be lost due to incomplete digestive absorption and/or the first-pass effect (Lennerhas, 2003). The increased bioavailability of atorvastatin is likely due to the fact that it is administered in an active acid form which is highly soluble and permeable and thus very well-absorbed after oral administration (Lennernas, 2003). Simvastatin, comparatively, has a much lower bioavailability as it is administered as an inactive lactone that is hydrolyzed in the digestive system to produce the active agent, simvastatin acid, which is then readily absorbed in the intestine (simvastatin drug monogram; Shitara and Sugiyama, 2006). The elimination half-life of atorvastatin is approximately 10 times that of simvastatin (14 to 19 hours compared to ≤ 3 hours in simvastatin) (Schachter, 2005), meaning that it takes about 10 times as long for the plasma concentration of atorvastatin to decrease by 50%. However, considering that it was simvastatin and not atorvastatin that significantly reduced mtDNA content, it seems that bioavailability and elimination half-life are unlikely to explain this main observation of this masters thesis.

It may be that the reason that 80 mg of simvastatin and not 40 mg of atorvastatin was associated with a significant reduction in mtDNA is simply that simvastatin was given at a
higher dose. It is true that these selected dosages are considered equally potent in terms of their effect on blood concentrations of LDL-C as mentioned above, and indeed this is precisely what was observed in the original Finnish trial (Paiva et al., 2005). However, these dosages are evidently not equally potent in terms of their effect on muscle mtDNA levels. Our results may simply be due to an increased amount of one drug over the other being present in the body. We can presume that 80 mg of simvastatin will lead to higher peak drug plasma concentrations than 40 mg of atorvastatin (Lilja et al., 2000; Mazzu et al., 2000; simvastatin drug monogram; Lennernas, 2003). This is supported by the fact that any factor that increases the amount of statin in the plasma, such as a higher statin dose or co-medication with a drug that is also metabolized by the CYP system, is well-recognized to increases the risk for SIM and, perhaps, muscle mitochondria pathology (Gotto, 2006). The higher dose of simvastatin seems to be the most likely explanation, but in the end, the precise reason(s) for our observed variable effect between the two drugs studied on human skeletal muscle mtDNA content remains unknown, and deserves further study.

What could be, then, the mechanism of action for the observed decrease of muscle mtDNA/nDNA ratio, and ubiquinone, in the simvastatin-treated subjects? In the HIV context, the nucleoside analogue drugs are thought to deplete mtDNA content mainly through their inhibitory action on DNA polymerase-gamma, the enzyme responsible for mtDNA replication. There is no evidence to suggest that statins inhibit this enzyme. The problem of SIM is more likely related to the metabolic effects of statins on the mevalonate pathway than the drug molecule itself.

One possible explanation for this mtDNA-depletion mechanism may be that from the circulation, simvastatin penetrates the plasma membrane of skeletal muscle and decreases intracellular lipid content through its inhibitory action on HMG CoA reductase, which again is thought to be mainly localized in the ER and in peroxisomes (Hogenboom et al., 2002). As a
result, the integrity of the inner and outer mitochondrial membranes, which is where the process of mtDNA replication occurs on a proteinacious structure, is destabilized (Meeusen and Nunnari, 2003). mtDNA replication (and perhaps also mitochondrial function) may thus be disrupted, leading to an eventual decline in mtDNA/nDNA ratio. This notion is supported by the above-mentioned study of simvastatin reducing ergosterol in yeast with an accompanying total loss of mtDNA content (Westermeyer and Macreadie, 2007).

Another possibility which may occur on its own or simultaneously with the above proposed mechanism is that simvastatin reduces intramuscular ubiquinone content through its inhibition of HMG CoA reductase, as ubiquinone is a downstream product of mevalonate. Given the importance of ubiquinone in the electron transport chain and mitochondrial function, a shortage of ubiquinone in myocytes may lead to mitochondrial dysfunction and increased release of ROS. ROS damage the cell, and the cell may react by selectively eliminating the source of ROS, damaged mitochondria, in an autophagic process termed mitoptosis. In the process, mtDNA would presumably also be eliminated. A decrease in ubiquinone may enhance ROS damage to mtDNA, as ubiquinone has antioxidant properties, and this damage is not easily repairable given the nature of mtDNA compared to nDNA as mentioned above (Bentinger et al, 2007).

The term mitoptosis was coined in 1998 to describe the programmed death of the mitochondrion as a natural mechanism in place to remove malfunctioning mitochondria that produce ROS (Skulachev, 1998). mtDNA turnover occurs every 7 to 31 days, organism-, tissue-, and cell type-dependent (Gross et al, 1969). It is a natural process that serves to rid the cell of damaged and/or mutated mtDNA molecules (Kopsidas et al, 2002). It may be that those cells experiencing mitochondrial problems that are able to remove their mitochondria are more viable, as mitochondria can generate pro-apoptotic proteins in addition to ROS (Lyamzaev et al, 2004; Skulachev, 2006). The selective elimination of mitochondria has been described in apoptotic
cells (Xue et al, 2001) and in cells exposed to inhibitors of mitochondrial function (Lyamzaev et al, 2004).

It is possible that in our research, high-dose simvastatin treatment stimulated skeletal muscle mitoptosis, which would explain the reduced levels of mtDNA. It may also be the case that severe losses of mitochondria and/or mtDNA leads to cell death, or just chronic reduced mtDNA levels and accompanying defective mitochondria and reduced function of the respiratory chain; both options could play a role in the development of SIM. If this is the case, then reduced mtDNA may function as a marker of mitochondrial damage or toxicity in the pathogenesis of SIM, rather than being a primary event of statin treatment. On the issue of cell loss due to mtDNA damage, the accumulation of mtDNA mutations is a process that occurs naturally as an individual ages and it is accompanied by increasingly dysfunctional mitochondria. Eventually, a point is reached whereby mitochondria are lost from the organ in a very gradual and slow process (Kopsidas et al, 2000). In human skeletal muscle and other post-mitotic tissue this type of cell loss is minimal prior to age 40 but accelerates after that to an approximate loss of 5% per decade (Aoyagi and Shephard, 1992; Brooks and Faulkner, 1994; Frischknecht, 1998). This process may partially explain why the elderly are at an increased risk for SIM, although co-medications, concomitant illnesses, and frailty are also more common in the elderly and are also recognized risk factors for SIM (Thompson et al, 2006).

A key feature of the Chapter 2 study is that no subjects reported muscle weakness or pain or had elevated serum CK at follow-up, despite considerable decreases in muscle mtDNA and ubiquinone in the simvastatin group; 7/14 lost over 50% of their muscle mtDNA in just 8 weeks of treatment. This would be consistent with the “threshold effect” observed in mtDNA depletion disease, whereby clinical muscle symptoms appear only when mtDNA levels fall below approximately 25 to 30% of normal (Blake et al, 1999; de Mendoza et al, 2004).
In addition, we observed that individuals in the Chapter 2 study with a high level of mtDNA at the beginning of the study lost a larger percentage of their mtDNA (Table 2.4). We can speculate that such individuals had a pre-existing metabolic condition, whereby their mitochondria were not functioning effectively, and thus the muscle cells attempted to compensate for this by making more mitochondria in order to meet the energy demands of the cell, and thus more mtDNA. This is supported by a study of muscle biopsies collected from 300 individuals with mitochondrial disease which observed a positive correlation between mtDNA proliferation and ragged-red fibres, a hallmark indicator of defective mitochondria (no R or P values were provided) (Bai et al, 2004). When these subjects initiated high-dose statin therapy, the cellular energy deficit already in place was “pushed over the edge,” so to speak, into a more serious situation due to the statin’s above-mentioned possible metabolic intracellular effect in skeletal muscle. The result may be increasing dysfunctional mitochondria to the point that these organelles may cause more harm than good through excessive release of ROS, and thus the cell initiated mitoptosis to eliminate the mitochondria from the cell in an attempt to rescue the cell from apoptosis.

Interestingly, in the atorvastatin group in Chapter 2, subjects with low mtDNA content at baseline actually had an increased amount of mtDNA at the 8-week follow-up (Table 2.4). Again, to speculate, it may be that at baseline these individuals had fewer but well-functioning mitochondria. Exposure to 40 mg atorvastatin daily for 8 weeks may have had a mild negative effect on this function, and stimulated the cell to increase the number of mitochondria (and mtDNA copies) in an attempt to compensate for the decline in mitochondrial ATP production. Such speculation can also be applied to the observation that although baseline mtDNA did not correlate with age, the older people in the study did tend to lose more mtDNA between baseline and follow-up (data not shown). Larger controlled studies are necessary to verify these hypotheses.
As an aside, few reports exist in the literature concerning the relationship between changes in amount of mtDNA and the amount of mitochondria inside a cell, but several publications from the same group of investigators are suggestive of a direct relationship (Lee et al, 2000; Wei et al, 2001; Lee and Wei, 2005). Data from Chapter 4 of this thesis makes a modest contribution to this relationship. Of the 10 SIR biopsies, 3 were determined by the examining pathologist to have evidence for increased mitochondrial content. Two of these 3 had the two highest mtDNA/nDNA ratios of all 18 samples analyzed by our laboratory, the third ranked 8th out of 18 (data not shown). However, further studies are necessary to confirm this relationship.

The cell culture experiments described in Chapter 4 give contrary evidence to the Chapter 2 observation of reduced mtDNA in the simvastatin group. In general, there was a trend toward an increase in the mtDNA/nDNA ratio in those skeletal myotubes exposed to simvastatin. This may be due to timing. Soon after statin exposure, the cell may attempt to compensate for defective mitochondrial function by increasing the number of mitochondria and presumably mtDNA copies. This may be the approximate time period when the cell cultures were harvested. In the clinical study, subjects had experienced 8 weeks of statin exposure. In some subjects, mtDNA levels may have initially increased upon statin treatment initiation, but then gradually declined as speculated above. That we observed cell death in those cells exposed to the higher doses of statin treatment is not surprising as statins are known to be cytotoxic and/or pro-apoptotic in numerous cell types, possibly due to statin-induced inhibition of protein isoprenylation (Agarwal et al, 2002).

In the Chapter 5 research, there was no statistically significant difference between the SIR group (N = 10) and the controls (N = 8) in terms of mtDNA content (P = 0.96) or mtDNA expression (P = 0.76). This puzzling finding suggests that mtDNA quantification is not a valuable tool to evaluate muscle tissue for the presence of rhabdomyolysis in statin users. The
majority of the other laboratory tests conducted on these biopsy specimens were likewise unable to discern a statistically significant difference between the two groups of samples, including: histopathology; fatty acid oxidation response to statin treatment of cultured myocytes; mitochondrial respiratory chain enzyme activities including citrate synthase; succinate dehydrogenase, and COX; ubiquinone; and levels of intramuscular cholesterol, lathosterol, and desmosterol. The other methods were able to distinguish between normal and SIR muscle samples: levels of sitosterol, sitosterol/cholesterol, and campesterol; changes in the gene expression of atrogin-1 and in the prenylated protein Ras.

However, the sub-analysis of the 5 simvastatin-treated SIR patients compared to the 8 controls revealed a trend toward decreased mtDNA content in the former group (P = 0.09). This did not reach statistical significance, but the group size was very small. A much larger decrease in mtDNA content would be necessary in order to reach 95% confidence in such a small patient population, especially considering the great heterogeneity of the participants as discussed below.

While there was no difference in ubiquinone level between the SIR (N = 10) and control (N = 8) groups, even when ubiquione was indexed to mitochondrial respiratory chain enzyme activities, there was a trend toward reduced muscle ubiquinone in the 5 simvastatin-SIR patients. This data is consistent with previous (Paiva et al, 2005) and the Chapter 2 data. Also consistent is the finding of reduced mitochondrial enzymes in the 5 SIR-simvastatin subjects compared to the 8 controls (citrate synthase, succinic dehydrogenase, COX).

Thus, although mtDNA levels were not severely reduced in the muscle biopsies from statin users with SIM, as we believed may be the case due to the Chapter 2 data and the possibility of a “threshold effect” at work, at least there is some consistency between the two clinical data sets (Chapters 2 and 5) in terms of the effect of simvastatin on nucle mitochondria. However, comparisons between the two studies are problematic due to the fact that the biopsies from the Chapter 5 study originated from patients with the rare condition of severe muscle
breakdown, not just muscle pains, due to statin therapy. Also, the samples were processed differently as described above. For interest’s sake, ‘untreated’ mtDNA/nDNA ratios were similar between the two studies: the baseline mtDNA/nDNA ratio was approximately 2000 in the Chapter 2 study (N = 43) and approximately 1750 in the 8 statin-naïve controls in the Chapter 5 study. That ratio was approximately 900 at follow-up in the Chapter 2 simvastatin-subjects (N = 14), and about 1350 in the 5 simvastatin-subjects in the Chapter 5 study.

6.2 LIMITATIONS

There are several limitations to the research described in Chapter 2. First, exercise was not taken into account, and evidence suggests that SIM is more frequent in subjects who exercise as exercise may induce mitochondrial changes (Sinzinger and O’Grady, 2004). This is a potential confounding factor that was not taken into account in the patient selection or study design, and may have had a consequence on our final results. Exercise should be considered in future study design.

Second, there was a higher proportion of males in the atorvastatin group (80%) compared to the simvastatin and placebo groups (57% in each). This difference in gender proportion was not statistically significant (P = 0.32; Table 2.1), but that may be mostly due to the small sample size. Differences in mitochondrial functioning between the genders may exist (Borras et al, 2007) and could have influenced the results of our study.

Third, there was insufficient tissue to perform histology or EM examinations. This would have been very interesting, to investigate a possible correlation between the number of mitochondria and the mtDNA/nDNA ratio.

Fourth, we were unable to quantify blood mtDNA due to the fact that there were no leftover samples available to us. It would be very interesting to compare mtDNA/nDNA ratios
in the two different tissues, blood and skeletal muscle, in this statin-myopathy context. Another tissue worth analyzing would be oral epithelial cells, or a cheek swab, as this could lead to the development of an inexpensive, non-invasive and painless clinical diagnostic test.

The cell culture experiments were limited by the possibility that cholesterol present in the cell culture medium may have invalidated an eventual toxic effect of statins through reduced cellular cholesterol availability. Also, markers of apoptosis, such as caspase-3, were not assessed.

There are numerous limitations of the research presented in Chapter 5. First, there was considerable heterogeneity in the affected group in terms of type of statin type and dose range: 5 patients were on simvastatin (dose range 20 to 40 mg), 3 atorvastatin (dose range 20 to 80 mg), 1 lovastatin (unspecified dose), and 1 pravastatin 20 mg. The duration of statin therapy also makes the interpretation of these data problematic, as it was unspecified in 6/11 SIR patients; furthermore, it was 1 month, 2 months, 18 months, 36 months, and ‘years,’ in the 5 in which it was specified.

Importantly, the number of days between the last statin dose and the muscle biopsy was highly variable: 6 of 10 were biopsied within 7 dates of statin discontinuation, but the average number of days was 20 ± 24, with a range of 68 days. Analyzing muscle tissue collected one or two months after statin cessation may not accurately reflect what was going on in the muscle during the myopathic episode. Muscle tissue, and mitochondria especially given their dynamic nature, may be ‘rebounding’ from the statin insult by this point. This may help to explain the observations, as most (4 of 5) of the simvastatin-treated SIR subjects were biopsied soon after statin discontinuation (≤ 5 days).

In addition, the sample size is small: the affected SIR group N = 10, and the control group N = 8, and this was a retrospective cross-sectional study. Light microscopy data was also discouraging, as only 4/10 SIR were graded myopathic, along with 3/8 healthy controls.
Another limitation is the method of muscle sampling which pooled intra and intermuscular compounds together. Skeletal muscle consists of two major fibre types: type I fibres which contain more mitochondria and rely more heavily on OXPHOS, and type II fibres which have a greater reliance on glycolysis (Essen et al., 1975). The latter may be more sensitive to statin toxicity (Waclawik et al., 1993; Schaefer et al., 2004; Westwood et al., 2005). Aging, illnesses, or medications may affect the two types differently given the difference between them in terms of reliance on the mitochondrial genome for energy metabolism (Kopsidas et al., 2002). In this study, all muscle fibre types were combined, but the statin-induced toxicity or pathology may be occurring in only one type. Thus, in our testing of overall intramuscular content (not only in Chapter 5) we may be unable to determine important aspects of the distribution of changing levels of muscle mitochondrial nucleic acids in the context of SIM.

Finally, the controls in this study may be inappropriate for the following reasons. First, in the control group there were 6 females and 2 males, compared to 2 females and 8 males in the SIR group. Second, comparing muscle biopsies from patients on statin therapy with acute rhabdomyolysis to statin-naïve patients with no muscle complaints whatsoever is probably inadequate. A second control group, one consisting of statin-users without muscle pain, would strengthen the scientific value of the findings, in particular the issue of causality versus mere association, by better distinguishing between variable changes due to statin therapy versus variable changes due to the condition of rhabdomyolysis itself.

6.3 FUTURE DIRECTIONS

Chapter 2 of this thesis describes significantly decreased skeletal muscle mtDNA in asymptomatic 80 mg simvastatin users at only 8 weeks of treatment. This is an important observation relevant to the long-term safety of statin therapy.
It would be interesting to compare levels of mtDNA in the blood and/or urine of SIM patients to appropriate controls, perhaps to one control group of statin users without muscle pain, and another group of healthy statin-naïve controls. It may be that myocyte death could be detected in this fashion, which is far less invasive, traumatic, time-consuming and costly than muscle biopsy. A comparison of mtDNA levels in SIM patients in various tissues such as skeletal muscle, peripheral blood mononuclear cells, and oral epithelial tissue would be similarly interesting and may contribute to the development of a clinical assay for assessing statin toxicity (provided first that a convincing body of evidence exists proving an association between SIM and mtDNA depletion).

Chapter 5 describes the measurement of mtDNA content and expression in individuals with SIM, which was an important step in the progression of this research project. Had those individuals shown severely reduced mtDNA content, this would have fit nicely with data from the Chapter 2 study of asymptomatic statin users. This was not the case. However, given the numerous limitations of that study described above, especially the small sample size, the variation in statin type, and the time between therapy cessation and biopsy, the finding of no significant difference in mtDNA/nDNA ratio, or mtDNA expression, does not signify the end of the line for this stream of research. Further studies of mtDNA content should be conducted of statin-users with myopathy.

This is an important future direction of this research: a sufficiently designed and powered study comparing the mtDNA levels in skeletal muscle (and ideally in other tissues as well such as blood and oral epithelial tissue to investigate a possible correlation with the affected tissue) collected from hypercholesterolemic statin users with and without myopathy, and from statin-naïve controls. Other possible SIM markers, such as serum aldolase level, atrogin-1, and Ras could be included. Such a study may be worth doing prior to the continued exploration of
mtDNA content changes in statin-exposed cultured human skeletal muscle cells in an attempt to validate the in vivo studies with in vitro data.

However, future cell culture experiments should be conducted eventually to determine whether cultured cells can offer a valid model for studies of SIM and mitochondria. After identifying an appropriate dose range with one statin as a reference point, such studies could compare all available statins' effect on mtDNA. Fluvastatin, mevastatin and pravastatin, in addition to simvastatin and atorvastatin, are all available commercially (Calbiochem). Rosuvastatin, cerivastatin, lovastatin and pitavastatin may be obtained directly from their manufacturers (AstraZeneca, Bayer, Merck and Zydus-Cadila, respectively), or purified from tablet form. In spite of the fact that its clinical use has been discontinued, cerivastatin especially should be included, perhaps as a positive control, in future studies given its well-recognized myotoxic potential.

A key feature of future cell culture studies of this nature should be a more mechanistic approach. This is necessary to increase the understanding of statins and decreased mtDNA in context of myopathy, and may be accomplished by blocking off certain parts of mevalonate pathway and measuring the activities of various enzymes of this pathway. For example, squalene synthase could be inhibited with zaragozic acid (Bergstrom et al, 1995), and there is evidence that inhibition of squalene synthase dose not cause myotoxicity (Flint et al, 1997). In addition, the effects of ubiquinone and mevalonate supplementation, among other compounds, should be studied. Can these prevent or rescue mtDNA content and/or expression alterations? Such analyses have better potential to contribute to the mechanistic understanding of the myotoxic effect of statins. Similarly, the connection between SIM and mitochondrial-mediated apoptosis should also be investigated. Thus, future in vitro studies could include markers of apoptosis such as caspase-3 activity. Isoprenoids could also be studied as these compounds may be involved in a pro-apoptotic capacity (Mo and Elson, 1999). In addition, other mitochondrial
variables could be included in these cell culture studies, such as assaying the activities of the mitochondrial respiratory chain enzyme complexes, both those containing mtDNA-encoded proteins and those encoded entirely by nDNA, and other markers of mitochondrial potential or respiration.

6.4 CONCLUSIONS

Statins are the most widely prescribed drugs of all time – they have revolutionized the management of hypercholesterolemia, a major risk factor for CVD. They are easy to administer, are the most effective means of reducing LDL-C, and have well-documented efficacy for decreasing the risk for CVD even in diverse patient groups including men and women of all ages, patients with and without previous CVD, children with familial hypercholesterolemia, diabetics, hypertensives, smokers, and the elderly (Shepherd, 2006).

The major side effect of this class of drugs is myopathy which ranges from mild weakness to fatal rhabdomyolysis. Despite this undesirable effect, statin therapy is clearly beneficial to the majority of hypercholesterolemic patients at risk for CVD. Overall, the benefit to risk ratio of statins and adverse side effects is desirable. A recent meta-analysis of statin adverse events in 18 trials, including 71,108 patients (301,374 person-years) found that treating 1000 patients prevented 37 cardiovascular events and caused only 5 adverse events (not all muscle-related); severe myopathies were sporadic only (Silva et al, 2006).

However, SIM is nonetheless a considerable problem in the clinical setting. Fear of myopathy is the main obstacle in the effective use of these highly beneficial drugs, and failure to recognize SIM and cease statin therapy may lead to rhabdomyolysis, myoglobinuria, and acute renal necrosis (Pierce et al, 1990). Muscle toxicity limits aggressive LDL-C-lowering with
statins. Fear of rhabdomyolysis was major a factor in Merck’s decision in the mid-1990s to cease development of a 160 mg gradual-release form of simvastatin (Davidson et al, 1997).

Despite 20 years of clinical use, the mechanism of this side effect remains to be fully elucidated. Until the mechanism of SIM is fully understood, concerns about statin muscle toxicity will remain and may be exaggerated. In addition, at present there is no convenient and reliable marker of statin muscle damage. CK has been widely used but recently has been shown to be an inconsistent and poor marker.

Prevention is the best approach toward SIM, such as using lower statin doses to meet lipid targets and to avoid co-medications known to increase the risk for myopathy. Patient education is also key; patients should be instructed to cease treatment if muscle symptoms arise.

However, the problem of SIM remains and is expected to increase due to mounting clinical trials evidence which supports the notion that “lower is better” in terms of blood concentration of LDL-C and CVD risk (Grundy et al, 2004; LaRosa et al, 2005). Given the clear benefit of reducing cholesterol levels, aggressive statin-LDL-C-lowering is anticipated, and therefore research into the mechanism of SIM is well-warranted. In addition, the identification of a consistent clinical marker would greatly assist in the diagnosis of this adverse effect and help to identify those patients at higher risk for prolonged muscle damage. It could also contribute to the design of novel, less toxic statins.

This masters thesis is comprised of several different but related studies of the toxic effect of the cholesterol-lowering drugs statins on human skeletal muscle mtDNA. The main finding was significantly reduced levels of muscle mtDNA in mildly hypercholesterolemic individuals after only 8 weeks of treatment with 80 mg of simvastatin, a clinically approved dose. None of these patients reported muscle complaints or had increased serum CK. Given that statin therapy is often life-long, the large observed decrease (median - 47%) raises concern about the potential long-term effect of statins on mtDNA levels and skeletal muscle mitochondria. Although our
findings do not demonstrate a direct causal relationship between statin-associated muscle mtDNA depletion and SIM, they point to differences between statins. Drug actions and properties relevant to muscle mitochondria should be further evaluated in larger controlled studies. This is especially true given that cholesterol targets have recently been lowered in many guidelines, leading to an increased prevalence of high-dose statin therapy.

This research has contributed the first exploratory step toward the possibility of utilizing mtDNA levels as a clinical diagnostic tool to assess those individuals at greater risk for developing myopathy on statin treatment, and to using mtDNA as a method of evaluating and/or monitoring the severity of statin muscle mitochondrial toxicity, which is believed to play a role in SIM. We have shown that statin treatment can alter mtDNA in the affected tissue, skeletal muscle, an important initial step in this line of research. Additionally, we have shown that simvastatin has a greater tendency to reduce mtDNA levels than atorvastatin, in two separate studies with different patient populations.

This research also emphasizes the fact that statins can have considerable sub-clinical effects, as highlighted by a recent study which observed ultrastructural damage in various skeletal muscles of statin users on various statins at varying doses, all without muscle complaints (Draeger et al, 2006). It shows that these drugs may be causing previously unnoticed or undetected cellular damage, and thus calls into question the long-term safety of these drugs. Statin users should be monitored closely, especially those on high-dose therapy, and a sensitive and reliable clinical marker of statin toxicity should be identified.

Importantly, data from Chapters 2 and 5, in addition to a previous related study (Paiva et al, 2005) suggests that there may be a difference in the muscle reactions to different statins, probably relating to their physiochemical and pharmacokinetic properties. This was underscored by the withdrawal of cerivastatin from the market and is a key aspect of the future of statin
muscle toxicity research and also important in the clinical setting in terms of managing patients with poor statin tolerance.

Finally, this research has contributed to the understanding of the mechanism of SIM. It supports those studies that demonstrate that mitochondrial pathology plays a role in the aetiology of SIM. It suggests that statins may be causing a decrease in the mtDNA content in human cells, which may in turn result in these cells having mitochondrial defects including reduced respiratory function, possibly due to statin-induced decreased mitochondrial membrane content. We hope that the research described here will stimulate future studies in this area.
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Certificate of Expedited Approval
Clinical Research Ethics Board Official Notification

<table>
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<tr>
<th>PRINCIPAL INVESTIGATOR</th>
<th>DEPARTMENT</th>
<th>NUMBER</th>
</tr>
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<tbody>
<tr>
<td>Frohlich, J.</td>
<td>Pathology &amp; Laboratory Med</td>
<td>C06-0358</td>
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INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT
Vancouver Coastal Health Authority

CO-INVESTIGATORS:
Cote, Helene, Pathology & Laboratory Med; Maguire, John, Pathology & Laboratory Med; Schick, Brian, Pathology & Laboratory Med

SPONSORING AGENCIES
Unfunded Research

TITLE:
Mitochondrial DNA Levels in Skeletal Muscle Biopsies of Patients with Statin-Induced Myopathy

APPROVAL DATE: 24 July 2006
TERM (YEARS): 1
DOCUMENTS INCLUDED IN THIS APPROVAL: Protocol version date 01 June 2006

CERTIFICATION:
In respect of clinical trials:
1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The documentation included for the above-named project has been reviewed by the Chair of the UBC CREB, and the research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved by the UBC CREB.

The CREB approval for this study expires one year from the approval date.

Approval of the Clinical Research Ethics Board by one of:
Dr. Gail Bellward, Chair
Dr. James McCormack, Associate Chair
Dr. John Russell, Associate Chair
Dr. Caron Strahlendorf, Associate Chair