Mitochondrial DNA Alterations and Statin-Induced Myopathy

by

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MASTER OF SCIENCE

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Abstract

**Background/Objectives:** Statins are widely used to treat hyperlipidemia and lower cardiovascular disease risk. While statins are generally well tolerated, ~10-15% of patients experience statin-induced myopathy (SIM), a potentially fatal complication. Statin treatment has been associated with mitochondrial dysfunction. High-dose simvastatin treatment has been associated with skeletal muscle mitochondrial DNA (mtDNA) depletion. The contribution of mitochondrial dysfunction to the development and exacerbation of SIM may be important. The goal of this project was to examine the effects of statins on mtDNA to provide further insight into the etiology and severity of mitochondrial myotoxicity in SIM.

**Methods/Results:** Two studies were performed. PCR quantification of mtDNA and nuclear DNA was used to measure mtDNA content. Long-template PCR was used to amplify the mitochondrial genome and score mtDNA deletion burden. In an *in vitro* study, rhabdomyosarcoma cells were exposed to simvastatin and atorvastatin for over 70 days. Both mtDNA content and deletion burden were measured longitudinally and remained unchanged amongst statin treated cells. In an *in vivo* study, skeletal muscle biopsies from patients diagnosed with SIM (n=24) and comparators showing no pathologic findings (n=23) were retrospectively reviewed from stored clinical samples. The pathologic features and degree of pathology within each biopsy were scored. mtDNA content and deletion score was compared between groups. Two genotypes that are associated with changes in statin response and SIM risk, apolipoprotein E and SLCO1B1, were examined. No difference in genotype frequency between groups was detected.

Controlling for age, gender, biopsy year and apolipoprotein E genotype, SIM subject mtDNA/nDNA (mean±SD, 2036±1146) was significantly lower than the comparators (3220±1594) (p=0.042). No difference was observed in mtDNA deletion score (0-200) between SIM subjects (21.2±19.2) and comparators (19.4±30.0). There was an inverse correlation between mtDNA content and degree of pathology (p=0.006 r=-0.399).
Conclusions: We found decreased in vivo skeletal muscle mtDNA content in association with SIM. How this relates to the pathogenesis of SIM remains unclear. As the mtDNA deletion score was not associated with SIM, quantitative rather than qualitative mtDNA alterations are suggested. MtDNA content should be further investigated as a potential marker of statin drug myotoxicity.
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<td>ACC</td>
<td>American College of Cardiology</td>
</tr>
<tr>
<td>AFCAPS/TEXCAPS</td>
<td>Air Force/Texas Coronary Atherosclerosis Prevention Study</td>
</tr>
<tr>
<td>AHA</td>
<td>American Heart Association</td>
</tr>
<tr>
<td>ALLIANCE</td>
<td>Aggressive Lipid-Lowering Initiation Abates New Cardiac Events</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine Transaminase</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>Analysis of Covariance</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>ASPG</td>
<td>Polymerase γ Accessory Subunit</td>
</tr>
<tr>
<td>AST</td>
<td>Aspatate Transaminase</td>
</tr>
<tr>
<td>AZT</td>
<td>Azidothymidine</td>
</tr>
<tr>
<td>CARE</td>
<td>Cholesterol and Recurrent Events</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine Kinase</td>
</tr>
<tr>
<td>CMA</td>
<td>Canadian Medical Association</td>
</tr>
<tr>
<td>COQ2</td>
<td>Coenzyme Q2 Homolog Prenylation gene</td>
</tr>
<tr>
<td>COX</td>
<td>Cytochrome-c Oxidase</td>
</tr>
<tr>
<td>CPT II</td>
<td>Carnitine Palmitoyl Transferase II</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Cytochrome-p450 3A4</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>eNO</td>
<td>Endothelial Nitric Oxide</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methyl-glutaryl Coenzyme A</td>
</tr>
<tr>
<td>HPS</td>
<td>Heart Protection Study</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile Range</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LDLc</td>
<td>Low density lipoprotein cholesterol</td>
</tr>
<tr>
<td>LDLR</td>
<td>Low Density Lipoprotein Receptor</td>
</tr>
<tr>
<td>LIPID</td>
<td>Long Term Intervention with Pravastatin in Ischemic Disease</td>
</tr>
<tr>
<td>LIPS</td>
<td>Lescol Intervention Prevention Study</td>
</tr>
<tr>
<td>MACE</td>
<td>Major Adverse Cardiac Event</td>
</tr>
</tbody>
</table>
MELAS
Mitochondrial Myopathy, Encephalopathy, Lactic Acid and Stroke-like Syndrome

MGT
Modified Gomori Trichrome

MI
Myocardial Infarction

mtDNA
Mitochondrial DNA

nDNA
Nuclear DNA

NHLBI
National Heart, Lung and Blood Institute

NIH
National Institute of Health

NRTIs
Nucleoside Reverse Transcriptase Inhibitors

OXPHOS
Oxidative Phosphorylation

PBS
Phosphate Buffered Saline

PCI
Percutaneous Coronary Intervention

PCR
Polymerase Chain Reaction

PDT
Population Doubling Time

POLG
DNA Polymerase γ

PP
Pyrophosphate

RCF
Relative Centrifugal Force

ROS
Reactive Oxygen Species

rRNA
Ribosomal RNA

S4
Scandinavian Simvastatin Survival Study

SDH
Succinate Dehydrogenase

Sec-tRNA
Selenocysteine tRNA

SIM
Statin-Induced Myopathy

SLCO1B1
Solute Carrier Organic Anion Transporter Family, Member 1B1

SNP
Single Nucleotide Polymorphism

SPH
St. Paul's Hospital

TC
Total Cholesterol

TG
Triglyceride

tRNA
Transfer RNA

ULN
Upper Limit of Normal

VGH
Vancouver General Hospital

VLDL
Very Low Density Lipoprotein

WOSCOPS
West of Scotland Coronary Prevention Study
Acknowledgements

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Lastly I am grateful to the patients whose muscle biopsies were used in this study.
Dedication

To my family and friends


1 Chapter 1: Introduction

1.1 Overview

Three-hydroxyl-3-methyl-glutaryl Coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, are used to treat dyslipidemia and reduce cardiovascular disease (CVD) risk. Statin-induced myopathy (SIM), an adverse effect of statins, lowers patient quality of life and in rare circumstances can be fatal. While the mechanism of SIM is unknown, mitochondrial dysfunction has been implicated and may be important in the development of SIM. Two studies will be discussed in this paper: an in vitro cell culture experiment and a retrospective study of human skeletal muscle biopsies. The focus of this thesis is statin-induced myopathy and mitochondrial dysfunction, particularly changes in mitochondrial DNA.

1.2 Cardiovascular Disease

Cardiovascular disease is the single largest cause of death throughout the world. While other ailments occupy the public consciousness, CVD is estimated to be responsible for ~17 million deaths a year (30% of global deaths) [1]. In 1998 the economic costs of CVD in Canada were estimated at $18.4 billion [2]. Additionally, CVD negatively impacts the quality of life of millions. It is responsible for 10% of lost disability-adjusted life years in low and middle-income countries and 18% in high-income countries [1]. CVD is often thought of as a disease of the developed world but this is not accurate. The incidence of CVD in developing countries is increasing, 80% of CVD deaths and 87% of CVD-related disabilities occur in low or middle income nations [3]. Most CVD risk factors, such as smoking, diabetes, and obesity, are increasing in developing countries, as a consequence of greater urbanization, economic development, the adoption of less healthy diets and a sedentary lifestyle [4].

While CVD is still the leading cause of death, the treatment options and prognosis for patients are improving. As recently as 60 years ago, many types of cardiovascular events such as myocardial infarction and stroke were considered unpreventable. With today’s modern
treatment however, high-risk CVD patients can experience a 75% reduction in risk [5]. A better understanding of CVD pathogenesis and risk factors has facilitated the development of new medications, uncovered CVD protective effects of existing medications (e.g. aspirin) and facilitated more effective lifestyle modification.

1.2.1 Cholesterol

Over 300 risk factors have been associated with the development and risk of CVD including age, hypertension, hypercholesterolemia, obesity, tobacco use, diabetes and others [1, 6]. As early as the 19th century, Rudolf Virchow identified cholesterol in atherosclerotic plaques and theorized that it was linked to atherogenesis. It took until the Framingham Heart Study of the 1950s for a definitive correlation between blood cholesterol levels and CVD risk to be made. The Framingham Heart Study longitudinally examined heart disease over 60 years encompassing three generations of patients, and was one of the first large scale epidemiological studies associating high cholesterol and CVD mortality [7, 8]. The Seven Country Study solidified the correlation between cholesterol and CVD mortality throughout the world [9].

Additional studies established that low density lipoprotein (LDL) cholesterol is largely responsible for the association between cholesterol and CVD; high density lipoprotein (HDL) cholesterol, on the other hand is inversely related to CVD risk [6, 10]. This led to the largely accepted paradigm that LDL and HDL are respectively atherogenic and atheroprotective particles.

The finding of an association between cholesterol, LDL and CVD led to the development of the lipid hypothesis. The lipid hypothesis proposes that elevations in LDL are a main risk factor for atherosclerosis and that reducing LDL levels will lower the risk of coronary events. The lipid hypothesis was accompanied by a strong debate and had many critics [11]. Concern was raised as to whether cholesterol reduction would actually have an effect on CVD, as this had not been shown conclusively up until this date. Subsequently, the Coronary Primary
Prevention Trial addressed these concerns in a double-blind trial using cholestyramine, a bile acid sequestrant, in 3,600 men with high-CVD risk [12, 13]. Treatment lowered cholesterol levels by 9% and cardiovascular events by 19%. The evidence proved convincing, and in 1984 the National Institute of Health (NIH) adopted the lipid hypothesis [14]. The Canadian Medical Association (CMA) and other health authorities quickly followed suit.

Concerns then shifted as to how, when, and to what degree cholesterol should be lowered. Critics noted that while CVD incidence decreased with treatment, overall mortality remained steady, with an apparent increase in non-CVD mortality [15-20]. Also, most trials had focused on high-risk middle-aged men and the efficacy of treatment in women or the elderly was unclear. It took until the large statin trials of the 1990’s to settle the debate amongst the scientific community; these will be discussed in section 1.3.4.

1.2.2 Pharmacologic Treatment of CVD

With the advent of the lipid hypothesis, a major target for CVD prevention became to minimize dyslipidemia by lowering LDL and / or the raising of HDL. Dietary changes that limit fat and cholesterol intake and produce sufficient changes in lipid levels are associated with poor patient compliance. The dietary changes that most patients will actually adhere to produce only minor changes in LDL cholesterol (LDLc) [21, 22]. Lifestyle modification has been and will continue to be an important part of the war against CVD, however, for high CVD risk individuals, dietary changes alone are usually insufficient [23]. In these high-risk patients, pharmaceutical treatment is needed.

There are numerous medications apart from the statin drugs that are used for the treatment and prevention of CVD. These include fibrates, nicotinic acid and bile sequestrants. Fibrates, such as fenofibrate or gemfibrozil, are a class of peroxisome proliferator-activated receptor α agonists that decrease total triglyceride (TG) levels while increasing HDL [24]. However, their LDL-lowering effect is less than that of statins.
Nicotinic acid (a.k.a. niacin, vitamin B₃) is a common vitamin that when taken in high quantities [2000-6000 mg/day (over 30x recommended daily allowance)] lowers levels of very low density lipoprotein (VLDL). Nicotinic acid lowers blood VLDL levels by, amongst other effects, inhibiting hydrolysis of adipose tissue triglycerides and thus the return of free fatty acids to the liver. The high doses needed and the considerable side effects, particularly skin flushing, are drawbacks of niacin treatment [25]. Bile acid sequestrants such as cholestyramine are effective inhibitors of intestinal bile acid reabsorption. As bile acids are biosynthesized from cholesterol, a reduction in bile acid reabsorption can lower LDL. Bile sequestrants can have disappointing patient compliance due to discomfort when ingesting them. Hence statins have been increasingly used.

1.3 HMG-CoA Reductase Inhibitors (Statins)

Approximately 80% of cholesterol is endogenously produced in our bodies through a steroidal biosynthetic pathway. HMG-CoA reductase inhibitors (statins) directly inhibit the endogenous production of cholesterol and can produce dramatic decreases in LDL. Over the past 35 years many statins have been developed and they play a central role in the modern treatment of CVD risk.

1.3.1 Endogenous Cholesterol Synthesis Pathway

Endogenous cholesterol synthesis occurs in every cell throughout the body, except mature red blood cells. The highest cholesterol production occurs in liver cells (hepatocytes), followed by the intestines [26]. It involves a complex series of enzymes and substrates starting with acetyl-CoA and resulting in the final production of cholesterol (Figure 1). The rate-limiting step of cholesterol synthesis is the reduction of HMG-CoA into mevalonate, facilitated by the enzyme HMG-CoA reductase. Statins competitively inhibit HMG-CoA reductase through steric hindrance [27]. This results in decreased mevalonate and thus decreased cholesterol production. Mevalonate is an important precursor not only for cholesterol production but for a
variety of other compounds involved in mitochondrial function, selenoprotein synthesis, as well as protein prenylation [7, 28, 29].
Figure 1: The Cholesterol Biosynthesis Pathway

Cholesterol is produced from Acetyl-CoA via a complex pathway. Statins inhibit HMG-CoA reductase, the enzyme required for mevalonate production. The resulting reduction in mevalonate decreases the production of a variety of compounds, including cholesterol, that are involved in many cellular functions.

Abbreviations: HMG-CoA = 3-hydroxy-3-methyl-glutaryl Coenzyme A, PP = pyrophosphate, Sec-tRNA = Selenocysteine-tRNA [7, 28, 29]
1.3.2 Statin Mechanism of Action

Statins comprise a large and varied class of drugs, but share a common mechanism. Statins inhibit HMG-CoA reductase by sterically binding to the same enzymatic domain as HMG-CoA, albeit with a much stronger binding affinity [27]. This results in a reduction of intracellular mevalonate production, leading to reduced cholesterol synthesis. Hepatocytes, which are responsible for most endogenous cholesterol production, respond to this drop in intracellular cholesterol by increasing expression of the low density lipoprotein receptor (LDLR) and other proteins such as HMG-CoA reductase and squalene synthase [30]. LDLR is expressed on the cell surface and binds to the apolipoproteins of LDL (apolipoprotein B-100) and VLDL remnants [apolipoprotein E (ApoE)] which are then taken up into the cell by endocytosis. The LDL and VLDL remnants are subsequently broken down within the liver. This results in a reduction of circulating LDL levels and a corresponding reduction in CVD risk (Figure 2).

Figure 2: Statin Mechanism of Action
Statins inhibit HMG-CoA reductase activity leading to a decrease in hepatocyte intracellular cholesterol. Hepatocytes respond by increasing expression of LDLR which transports plasma LDL into the liver. This causes a decrease in circulating LDL levels and CVD risk. [30]
1.3.3 Development

The first statin, compactin, was discovered in 1976 by Dr. Akira Endo at Sankyo Co. (Tokyo) from the fungus *Penicillium citrinum* [31, 32]. Compactin lowered cholesterol in several animal models [33, 34] and was shown by Sankyo to be effective in reducing LDLc levels in patients with familial hypercholesterolemia [35]. Despite a promising start, clinical trials with compactin were halted in 1980 over concerns of carcinogenicity [36]. In 1978 Merck & Co. (NJ, USA) successfully isolated another HMG-CoA reductase, lovastatin, this time from *Aspergillus terreus* [37]. During the 1980’s lovastatin was found to be a safe and effective way to lower LDLc levels in high-risk patients, such as those with familial hypercholesterolemia [38]. Later phase II and III trials confirmed the effectiveness of statins amongst high-risk patients while also showing that healthy patients experienced a similar reduction in LDLc [39, 40]. In 1987, Lovastatin became the first statin drug approved by the United States Food and Drug Administration (FDA), and was marketed under the name Mevacor [7].

Several other statin drugs have since entered the market including simvastatin (Zocor) (1988), pravastatin (Pravachol) (1991), fluvastatin (Lescol) (1994), atorvastatin (Lipitor) (1997), cerivastatin (Baycol) (1998), and rosuvastatin (Crestor) (2003). Pitavastatin (Livalo) has been used in Asia since 2003 and is currently under review in North America and Europe. While all statin drugs operate through the same mechanism of action, they differ widely in terms of their chemical attributes, production origin (synthetic vs natural) and LDL-lowering ability (Table 1).
### Table 1: Pharmacological Properties of Statin Drugs

<table>
<thead>
<tr>
<th>Atorvastatin</th>
<th>Cerivastatin</th>
<th>Fluvastatin</th>
<th>Lovastatin</th>
<th>Pitavastatin</th>
<th>Pravastatin</th>
<th>Rosuvastatin</th>
<th>Simvastatin</th>
</tr>
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<tr>
<td>Trade Name</td>
<td>Lipitor</td>
<td>Baycol ‡</td>
<td>Lescol</td>
<td>Mevacor Generic</td>
<td>Livalo</td>
<td>Pravachol Generic</td>
<td>Crestor</td>
</tr>
<tr>
<td>Dose (mg)</td>
<td>10 – 80</td>
<td>0.2 – 0.8</td>
<td>20 – 80</td>
<td>20 – 80</td>
<td>2 - 4</td>
<td>10 – 40</td>
<td>10 – 40</td>
</tr>
<tr>
<td>% LDL Reduction</td>
<td>50</td>
<td>36</td>
<td>23</td>
<td>30</td>
<td>42</td>
<td>34</td>
<td>57</td>
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<td>Lipophilicity</td>
<td>Lipophilic</td>
<td>Lipophilic</td>
<td>Lipophilic</td>
<td>Lipophilic</td>
<td>Lipophilic</td>
<td>Hydrophilic</td>
<td>Hydrophilic</td>
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<td>Origin</td>
<td>Synthetic</td>
<td>Synthetic</td>
<td>Synthetic</td>
<td>Organic</td>
<td>Synthetic</td>
<td>Organic</td>
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<td>CYP450 Metabolism</td>
<td>CYP3A4</td>
<td>CYP3A4</td>
<td>CYP2C9 CYP3A4 †</td>
<td>CYP3A4</td>
<td>CYP2C9 †</td>
<td>Negligible</td>
<td>CYP2C9 †</td>
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<tr>
<td>Half Life (hours)</td>
<td>12 – 30</td>
<td>2.0 – 3.2</td>
<td>0.5 – 2.4</td>
<td>2.0 – 3.0</td>
<td>13</td>
<td>1.3 – 2.8</td>
<td>20</td>
</tr>
<tr>
<td>% of 2006 USA Market Share</td>
<td>42.8</td>
<td>0</td>
<td>1.8</td>
<td>9.4</td>
<td>0</td>
<td>5.9</td>
<td>8.6</td>
</tr>
</tbody>
</table>

The pharmacokinetic properties of each statin are given based upon a 40 mg dose (apart from cerivastatin and pitavastatin which use 0.4mg and 2mg doses respectively). Pitavastatin is currently used in Japan, Korea, and Thailand and is currently under review in Europe and North America. Percent market share includes simvastatin/ezetimibe combination therapy. Total market share is higher than 100% due to rounding. † only minor involvement, ‡ cerivastatin was withdrawn from the market in 2001. [41-43]
1.3.4 Large Statin Trials

While statins were shown to be effective medications for lowering LDL, there was debate as to whether statins had a net beneficial effect. It was not until the mid 1990’s that studies showed a conclusive association between statins and decreased all-cause mortality. A series of large randomized controlled trials showed a conclusive drop in CVD rates among patients treated with statin treatment compared to controls (Table 2).

**Table 2: Large Randomized Clinical Statin Trials**

<table>
<thead>
<tr>
<th>Study</th>
<th>Sample Size</th>
<th>Treatment Dose (mg)</th>
<th>Patient Population</th>
<th>Demonstrated</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>4S</td>
<td>4,444</td>
<td>Simvastatin 5 – 40</td>
<td>CVD, High LDL</td>
<td>↓ TC, ↓ LDL, ↑ HDL ↓ MI, ↓ Death</td>
<td>1994</td>
</tr>
<tr>
<td>WOSCOPS</td>
<td>6,595</td>
<td>Pravastatin 40</td>
<td>No CVD, High LDL</td>
<td>↓ TC, ↓ LDL, ↑ HDL ↓ MI, ↓ Death</td>
<td>1995</td>
</tr>
<tr>
<td>CARE</td>
<td>4,159</td>
<td>Pravastatin 40</td>
<td>CVD, Normal LDL</td>
<td>↓ TC, ↓ LDL, ↑ HDL ↓ MI, ↓ Death</td>
<td>1996</td>
</tr>
<tr>
<td>LIPID</td>
<td>9,014</td>
<td>Pravastatin 40</td>
<td>CVD, Normal LDL</td>
<td>↓ LDL, ↑ HDL ↓ MI, ↓ Death</td>
<td>1998</td>
</tr>
<tr>
<td>AFCAPS/TEXCAPS</td>
<td>6,605</td>
<td>Lovastatin 20 – 40</td>
<td>No CVD, Normal LDL</td>
<td>↓ TC, ↓ LDL, ↑ HDL ↓ MI, ↓ Death</td>
<td>1998</td>
</tr>
<tr>
<td>LIPS</td>
<td>1,677</td>
<td>Fluvastatin 80</td>
<td>CVD, Normal LDL</td>
<td>↓ LDL, ↑ HDL ↓ PCI, ↓ MACE</td>
<td>2002</td>
</tr>
<tr>
<td>HPS</td>
<td>20,536</td>
<td>Simvastatin 40</td>
<td>High CVD Risk</td>
<td>↓ LDL, ↓ MI, ↓ Death</td>
<td>2002</td>
</tr>
<tr>
<td>ALLIANCE</td>
<td>2,442</td>
<td>Atorvastatin 80</td>
<td>CVD, High LDL</td>
<td>↓ TC, ↓ LDL ↓ MI</td>
<td>2004</td>
</tr>
</tbody>
</table>

An overview of several large statin trials that demonstrated the beneficial effects of statins [44]. Studies include the Scandinavian Simvastatin Survival Study (S4) [45], West of Scotland Coronary Prevention Study (WOSCOPS) [46], Cholesterol and Recurrent Events (CARE) study [47], Long Term Intervention with Pravastatin in Ischemic Disease (LIPID) study [48], Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TEXCAPS) [49], Lescol Intervention Prevention Study (LIPS) [50], Heart Protection Study (HPS) [51] Aggressive Lipid-Lowering Initiation Abates New Cardiac Events (ALLIANCE) [52]. TC = Total Cholesterol, MI = Myocardial Infarction, PCI = Percutaneous Coronary Intervention, MACE = Major Adverse Cardiac Event
The first of these large studies was the Scandinavian Simvastatin Survival Study (4S), published in 1994 [45]. The 4S study examined 4,444 patients with high serum cholesterol (>5.5mmol / L) and either angina or past MI. Patients were randomly assigned to simvastatin (20-40mg /day) or placebo treatment for an average of 5.4 years. Changes in total cholesterol (TC) and LDL levels were negligible in placebo (+1%, +1%) but substantial amongst simvastatin patients (-25% / -35%). The simvastatin group had decreases in myocardial infarctions (MI) (34%), coronary death (42%), as well as in all causes of death (30%), of which 60% were due to coronary events.

The next large statin study, the West of Scotland Coronary Prevention Study (WOSCOPS) observed that hypercholesterolemic men with no history of CVD would benefit from statin treatment [46]. This raised the question that if hypercholesterolemic patients with no prior CVD benefitted from statins, what about patients with CVD and normal cholesterol levels? The “Long Term Intervention with Pravastatin in Ischemic Disease” study (LIPID) found that patients with CVD and normal cholesterol levels still derived a benefit from statins [48].

Other studies showed similar benefits with statins in a variety of different conditions and treatments [47, 49, 50, 52]. The largest study, the Heart Protection Study (HPS) in particular requires mention. 20,536 adults with, or at high risk of, coronary disease, were randomly given simvastatin (40mg) or placebo and followed for five years [51]. A significant decline in both coronary and all-cause deaths as well as a 24% decrease in first time vascular events was seen with simvastatin treatment compared to placebo. While HPS largely confirmed what previous studies had shown it was notable for several reasons. Its vast subject size and open recruiting criteria allowed the beneficial results of statins to be confirmed in less often studied patient groups such as the elderly, women and diabetics. The HPS also showed beneficial effect in patients with average or even below average LDL levels.

The results of the large statin trials proved decisive. Long time sceptics such as Dr. Michael Oliver who had once written “The view that raised plasma cholesterol concentration is
"per se a cause of coronary heart disease is untenable" were convinced, stating “Lower patients’ cholesterol now” [11, 53]. The statin controversy was over and cholesterol treatment expanded dramatically.

1.3.5 Pleiotropic Effects of Statins

The primary effect of statins is to lower LDL levels and CVD risk but many other ancillary effects have been noted. These are called “pleiotropic” effects [54]. Some of these pleiotropic effects are anti-atherosclerotic and may account for part of the beneficial CVD risk lowering seen with statins.

Serum levels of C-reactive protein (CRP), an important inflammatory marker, are known to decrease with statins [55-57]. Statins can reduce expression of leukocyte adhesion and migration proteins in the endothelium, (i.e. vascular adhesion molecule-1, monocyte chemotactic protein-1) [58-63]. This hinders the accumulation of proatherogenic inflammatory leukocytes (monocytes, T lymphocytes) in the sub-endothelial space. Endothelial dysfunction is an early sign of atherogenesis. Statins can improve endothelial function by increasing expression of endothelial nitric oxide (eNO) synthase, which leads to increased vascular protective eNO activity [64]. Statins have anti-oxidative effects and can suppress the oxidation of LDL into ox-LDL, a highly atherogenic particle [65, 66]. Inflammation, endothelial dysfunction, and oxidation of LDL are thought to be important parts of the atherosclerotic process, so these pleiotropic effects may work in conjunction with statin LDL lowering to reduce CVD risk. Statins have also been associated with plaque stabilization and in some cases, plaque regression [67-69].

Apart from CVD-lowering benefit, some large statin trials observed a decrease in cancer incidence, leading to hopes that statins could act as anti-cancer agents. Statins could be anti-cancerous by inducing apoptosis within cancerous cells or by inhibiting cellular growth [70, 71]. Promising results have been found with statin treatment [72-75]. However, large trials will be needed to confirm the anti-cancer effects and to determine which subtypes of cancers are
susceptible to statin treatment. It is an interesting and promising line of future inquiry. Many other pleiotropic effects have been suggested affecting conditions from diabetes to delirium. Not all pleiotropic effects of statins may be beneficial however, recently some studies have suggested a possible increased risk of diabetes with statin treatment [76, 77]. Future research will sort out the wheat from the chaff [78, 79].

1.3.6 Current Status of Statins

From “orphan drug” status in the 1980s, statins have become the mainstay of CVD risk treatment and a multi-billion dollar business. In 1992, statins accounted for approximately 50% of the USA lipid-lowering drug market [80]. By 2002, statins had grown to over 90% of the USA market share. Similar growth has been seen worldwide. In 2006 the market for statins reached $22 billion dollars in the USA alone; sales of the market leader, atorvastatin, were $12.9 billion, making it the best selling drug on the planet [81, 82]. In recent years, several statins have lost patent protection and generic versions have become available, lowering prices [82].

The broad safety and efficacy of statin treatment has resulted in more aggressive and widespread treatment. In 2003, target LDLc levels were 2.5mmol / L. In 2006, the Canadian Cardiovascular Society released therapeutic guidelines recommending a target LDLc level <2.0mmol / L for high-CVD risk patients [83]. While statins are effective, these aggressive LDLc goals are frequently not met. In Canada, it is estimated that ~70% of patients on statin monotherapy fail to reach target LDLc levels, and yet most patients are not prescribed the high doses of statins that are available [84]. Even though higher doses would help patients reach target LDLc levels, physicians and patients are often adverse to high-dose statin treatment due to concerns over adverse effects.

1.3.7 The Adverse Effects of Statins

The abandonment of compactin due to side effects raised concern about the safety of endogenous cholesterol inhibition. Almost all medications, no matter the elegance of their therapeutic action, can cause adverse effects and statins are no exception. High-dose statin
treatment in animal studies demonstrated considerable toxicity including hepatic hyperplasia, cataracts, and tumourgenicity [85, 86]. These toxic effects however are seen with very high doses compared to those used therapeutically, and have not been found in humans.

Therapeutic statin treatment has been associated with a variety of adverse effects including muscle toxicity, elevation of liver enzymes, neuropathy, and renal dysfunction. Statin related muscle toxicity is the most common adverse effect and is described separately in section 1.4. One of the most common non-muscle side effects of statins is an elevation of liver enzymes, namely aspartate transaminase (AST) and alanine transaminase (ALT), which affects <1% of patients in clinical practice [42]. This enzyme elevation most often occurs within 6 months of starting statin treatment, is dose-dependent and largely asymptomatic [87]. Elevations in AST and ALT typically resolve with statin withdrawal or with time. The underlying mechanism of liver enzyme elevations is not yet known. It is possible that the increase in liver enzymes is a direct response of the liver to the lipid-lowering effect of statins [42]. Fortunately, severe hepatotoxic effects are seen very rarely and large randomized trials have not found a strong risk of liver damage with statins [51, 88-91]. Though the overall risk is low, liver enzyme elevations are a concern and are often monitored during statin treatment.

Other rarer side effects have been reported. There are scattered case reports of patients developing neuropathy in conjunction with statin use [92, 93]. This peripheral neuropathy develops shortly after the initiation of statin treatment and resolves after the drugs are stopped. The risk of developing neuropathy is very small and has not been confirmed in randomized trials [42]. In case reports, statins have been associated with renal disturbances but the evidence for this is scant and statins to not appear to cause renal dysfunction unless in conjunction with rhabdomyolysis [94]. Renal dysfunction associated with rhabdomyolysis will be discussed later as a complication of statin-induced rhabdomyolysis.

Gastrointestinal effects including abdominal pain, diarrhoea, and general symptoms such as nausea have been reported but the risk with treatment is minimal. Despite its broad
array of side effects, statins are well tolerated amongst the large and diverse population taking the medications. The side effects of statins are generally rare, transient, and non-life threatening. The most common adverse effect of statins is statin-induced myopathy, which can negatively impact patient compliance, quality of life and, in rare circumstances, can lead to death.

1.4 Statin Induced Myopathy

Statin-induced myopathy is an umbrella term that refers to a spectrum of statin associated muscle symptoms. It is the most serious statin side effect and has resulted in the recall of one drug, cerivastatin. The presentation of SIM varies from mild muscle pain (myalgia) alone in most patients, to muscle weakness and to a potentially fatal breakdown of muscle (rhabdomyolysis). Rhabdomyolysis is the clinical term for substantial muscle breakdown, and is associated with renal failure, myoglobinemia, myoglobinuria and dramatic increases in creatine kinase (CK) [95]. General symptoms of SIM are muscle pain, tenderness, weakness and fatigue [96]. These symptoms are usually proximal and are often, but not always, associated with exertion. Most cases of statin myalgia are associated with normal or slightly elevated levels of creatine kinase.

SIM is a broad term and the field suffers from variations in terminology. Following the withdrawal of cerivastatin, a set of definitions for SIM terminology were agreed upon by various organizations including the American College of Cardiology (ACC), the American Heart Association (AHA) and the National Heart, Lung and Blood Institute (NHLBI) [97] (Table 3).
Table 3: Terminology of Statin-Induced Myopathy

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myopathy</td>
<td>Any muscle disease</td>
</tr>
<tr>
<td>Myalgia</td>
<td>Muscle pain or weakness without elevated CK</td>
</tr>
<tr>
<td>Myositis</td>
<td>Muscle symptoms with elevated CK (&lt;10x ULN)</td>
</tr>
<tr>
<td>Rhabdomyolysis</td>
<td>Muscle symptoms with markedly elevated CK (&gt;10x ULN)</td>
</tr>
</tbody>
</table>

The ACC/AHA/NHLBI defined terminology for statin muscle pathology [97]. ULN = upper limit of normal

This set of definitions is widely used throughout the literature however they are not universally accepted. The National Lipid Association and the FDA use definitions that differ over the role of CK and the definition of rhabdomyolysis [98]. Rhabdomyolysis is a clinical classification and can be quite subjective. Creatine kinase, while a useful marker of general muscle damage, is not an effective measure of SIM. Statin-induced myopathy has been noted in the absence of elevated CK [99]. Throughout this paper SIM will be used as an umbrella term for the myopathic process and rhabdomyolysis will refer to particularly severe myopathy with CK elevations greater than 10 times ULN. While an imperfect system, SIM encompasses a broad clinical spectrum and the focus of this project is the condition as a whole.

1.4.1 Incidence of SIM

The large statin randomized clinical trials failed to detect any association between statins and muscle pain. In a review of 35 statin trials encompassing 74,102 patients, Kashani et al. found no significant difference in muscle symptoms (myalgia, rhabdomyolysis, elevated CK) compared to placebo [100]. In contrast to the large trials, muscle symptoms are seen quite often in the clinical setting and their association with statins is not in doubt. Myopathic symptoms are thought to occur in 10-15% of statin users [101]. Rhabdomyolysis, the most severe and disruptive form of SIM is very rare, affecting approximately 4.4 patients per 100,000 patient years [102]. The apparent disconnect between large, statistically powerful, clinical trials and actual clinical practice is striking and could be explained by several factors. As clinical trial
participants are selected to minimize potentially confounding variables, inherent or intentional biases in the population selection criteria of controlled studies may result in the exclusion of patients at high risk of SIM [103]. In fact most statin trials excluded patients with a prior history of statin intolerance. It is also possible that subjects in clinical trials under-reported experiences of mild or exertional myalgia, attributing the symptoms to other causes. Increased public awareness of statin side effects may have made patients more likely to report SIM and physicians more likely to consider statins as the cause of myopathy. As a result, SIM cases are reported much more commonly in clinical treatment as opposed to trial settings.

SIM symptoms typically appear some time after onset of treatment and resolve after statin cessation. Work by Hansen et al. estimated that SIM symptoms occur on average 6.3 ± 9.3 months after onset of treatment and mean time for resolution of symptoms after statin cessation was 2.3 ± 3.0 months [104]. Patients who experience myopathic symptoms are assessed to determine whether the symptoms are tolerable [96, 105]. If so, the statin is continued with frequent monitoring by the attending physician. If the symptoms are intolerable or rhabdomyolysis is suspected, the medication is discontinued and the patient is followed to see if the symptoms subside and normal CK levels are reached. Once the patient is asymptomatic he or she may be challenged with statins again, either at a lower dose than previously, or with another statin entirely. If statins prove intolerable, other lipid-lowering drugs are considered.

1.4.2 Impact of SIM

The impact of SIM is not to be underestimated. Rhabdomyolysis, the most severe and rare form of SIM, is potentially fatal. Increases in intracellular calcium leads to muscle fibre break down and the release of large amounts of myoglobin and other cellular components into the blood stream [106]. High concentrations of myoglobin can precipitate in the nephron and obstruct tubular flow [95]. The breakdown of intratubular myoglobin produces free iron which catalyzes the production of free radicals and further renal damage. This leads to renal
dysfunction and eventually acute renal failure. In most cases the acute renal failure resolves with appropriate medical management, sometimes requiring temporary hemodialysis. In fatal cases of rhabdomyolysis the cause of death is not the muscle damage but the resulting renal failure. While statin-induced rhabdomyolysis is rare, its effects are not to be underestimated.

Patients with SIM but not rhabdomyolysis can experience a dramatic loss in quality of life as even every day muscle actions become painful [107]. Patients may become unable to work or exercise, further impacting their physical and mental well-being. Even minor, non-debilitating muscle pain can lower patient quality of life. SIM also has an impact on patient compliance to statin treatment [108, 109]. As the benefits of statin therapy are primarily realized with long-term treatment, poor compliance with the drug over an extended period can eliminate the potential therapeutic benefit.

Greater public knowledge of SIM can turn both patients and physicians away from statins. It is estimated that over 60% of patients that would benefit from statin treatment are not on treatment [110]. Many of these patients may not have physicians but some could refuse statins out of concern over side effects. A better understanding of statins and SIM risk factors could increase the number of patients on statins while minimizing the impact of SIM. The number of people on statin therapy is bound to expand in the coming years, as generic drugs lower prices and more aggressive treatment is adopted; including possible over the counter availability of statins [111].

1.4.3 Cerivastatin

The most potent statin in terms of LDL lowering effect, cerivastatin was marketed by Bayer from June 1997 until its recall on August 8th, 2001. After a promising launch, cerivastatin was found to have an incidence of fatal rhabdomyolysis far higher than other statins [112]. Due to this unacceptable risk of rhabdomyolysis and death, cerivastatin was recalled from the market. Fifty-two reported deaths have been linked to cerivastatin [113]. Most of the fatal cases were taking a high dose of cerivastatin (0.8mg / day) and many were also taking a fibrate
(gemfibrozil) which increases statin serum concentration, a major risk factor for SIM [114]. The recall of cerivastatin emphasizes the importance of recognizing muscle effects in patients and the impact of SIM.

1.4.4 SIM Risk Factors

There are a plethora of factors and medications that are known to increase the risk of SIM (Table 4, Table 5). SIM risk increases with statin plasma concentration and as such, factors that inhibit statin clearance or metabolism increase SIM risk. High-dose statin therapy is also a major SIM risk factor [115]. Most statins are metabolized by cytochrome p450 3A4 (CYP3A4). CYP3A4 interacting medications, such as fibrates and verapamil can drive up statin plasma concentration and are the most common pharmacological SIM risk factor [115]. Renal dysfunction or biliary obstruction may also increase statin levels through impairment of excretion. Other medications that are associated with SIM include corticosteroids and beta-blockers.
Table 4: Statin-Induced Myopathy Risk Factors

<table>
<thead>
<tr>
<th>Internal Factors</th>
<th>External Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Statin Dose</td>
<td>Exercise</td>
</tr>
<tr>
<td>Age</td>
<td>Drug Interactions / Polypharmacy</td>
</tr>
<tr>
<td>Female Sex</td>
<td>Excessive grapefruit</td>
</tr>
<tr>
<td>Low Body Mass Index</td>
<td>Major trauma or surgery</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Alcohol Abuse</td>
</tr>
<tr>
<td>Hypertension</td>
<td>Infections</td>
</tr>
<tr>
<td>Hypothyroidism</td>
<td>Drug Abuse</td>
</tr>
<tr>
<td>Renal or Hepatic Dysfunction</td>
<td></td>
</tr>
<tr>
<td>Biliary Obstruction</td>
<td></td>
</tr>
<tr>
<td>McArdle Disease</td>
<td></td>
</tr>
<tr>
<td>Metabolic Muscle Disease</td>
<td></td>
</tr>
<tr>
<td>Myoadenylate deaminase deficiency</td>
<td></td>
</tr>
<tr>
<td>Carnitine palmitoyl transferase II deficiency</td>
<td></td>
</tr>
<tr>
<td>Genetics</td>
<td></td>
</tr>
</tbody>
</table>

A variety of factors are known to increase the risk of statin induced myopathy. [116-119]
Table 5: Medications Known to Increase SIM Risk

<table>
<thead>
<tr>
<th>CYP3A4 Inhibitors</th>
<th>Other Contra-Indicated Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrates</td>
<td>Beta-blockers</td>
</tr>
<tr>
<td>Calcium Channel Blockers (Non-Dihydropyridine)</td>
<td>Cimetidine</td>
</tr>
<tr>
<td>Nicotinic Acid</td>
<td>Corticosteroids</td>
</tr>
<tr>
<td>Cyclosporin</td>
<td></td>
</tr>
<tr>
<td>Warfarin</td>
<td></td>
</tr>
<tr>
<td>Amiodarone</td>
<td></td>
</tr>
<tr>
<td>Nefazodone</td>
<td></td>
</tr>
<tr>
<td>Fluoxetine</td>
<td></td>
</tr>
<tr>
<td>Protease Inhibitors</td>
<td></td>
</tr>
<tr>
<td>Macrolide Antibiotics</td>
<td></td>
</tr>
<tr>
<td>Azole Antifungals</td>
<td></td>
</tr>
<tr>
<td>Grapefruit Juice</td>
<td></td>
</tr>
</tbody>
</table>

A variety of medications increase SIM risk. Medications that inhibit cytochrome p450 3A4 (CYP3A4) lead to an increase in serum statin concentration. [115-118]

Many conditions that stress muscle are SIM risk factors. These include advanced age, small stature (e.g. Asian women), trauma, surgery, infection, and exercise among others [117]. Exercise in particular is a strong physical SIM risk factor and SIM is particularly prevalent amongst professional athletes on statins [120].

1.4.5 Genetics and SIM

While the mechanism of SIM is not yet known, the predilection of some patients to develop SIM suggests genetics may play a role. Possible SIM genetic factors can be placed in two broad categories: those that affect statin pharmacokinetics, and those that affect muscle metabolism. Alterations in statin metabolism can lead to increased statin serum concentration and SIM risk [119].
The solute carrier organic anion transporter family member 1B1 (SLCO1B1) gene has received much attention over its possible role in SIM. SLCO1B1 encodes a protein involved in the transport of statins into hepatocytes. Polymorphisms in SLCO1B1 have been associated with inhibited hepatic uptake of statins, causing a relative increase in serum drug concentration [121-124]. A recent study by the SEARCH Collaborative Group examined 85 SIM patients and 90 statin tolerant controls and identified a nonsynonymous single nucleotide polymorphism (SNP) (rs4149056) in exon 6 of SLCO1B1 during genomic association analysis of patients on high dose simvastatin (80 mg) [125]. The major allele, T, is associated with normal SLCO1B1 function while the minor allele C results in decreased efficiency of statin transport. The C allele is found in approximately 15% of the study subject. The SEARCH Collaborative group observed that the risk of myopathy in CC homozygotes and CT heterozygotes was 16.9 and 4.5 times more likely than TT homozygotes respectively [125]. Over 60% of SIM cases in this study were explained by the rs4149056 SNP.

Genetic disorders of the muscle can decrease the ability of muscles to adapt to the effects of statins, thus increasing the risk of SIM. Patients with genetic deficiencies of carnitine palmitoyl transferase II (CPT II) and myoadenylate deaminase as well as patients with McArdle’s disease all have increased SIM risk [126]. A cross-sectional study of 110 SIM patients demonstrated increased rates of heterozygous CPT II and McArdle’s disease carriers (13 and 20 times increased) compared to 248 controls [127]. SNPs in the coenzyme Q2 homolog prenylation (COQ2) gene, part of the ubiquinone synthesis pathway, have also been associated with statin intolerance. Oh et al. examined 291 subjects for COQ2 SNPs, 133 who had SIM and 158 who tolerated statins for over a year [128]. Two COQ2 SNPs and a COQ2 2-SNP haplotype were each significantly associated with SIM risk [128]. It is possible that in asymptomatic muscle with underlying dysfunction, statins could further stress this damaged muscle causing a “myopathic threshold” to be reached.
Patient lipid levels, as well as response to statin treatment, have been associated with apolipoprotein E genotype, though the overall implications of this are debated [129-131]. ApoE4 alleles are associated with higher levels of LDL compared to ApoE3; while patients with ApoE2 generally have lower LDL levels [132, 133]. In terms of statin treatment, when compared to the E3 allele, the E2 allele is associated with a greater response to therapy; while the E4 allele is associated with a weaker response [130, 134, 135]. The Genetics of Diabetes Audit and Research in Tayside, Scotland study examined 1,383 diabetics and found a significant linear association between ApoE genotype and both initial LDL levels and responses to statin treatment [136]. E2 homozygotes achieved greater reductions in LDL compared to E4 homozygotes. All E2 homozygotes were able to achieve target LDLc levels; while only 68% percent of E4 homozygotes were successful. ApoE accumulates in skeletal muscle inclusion body myositis and may have a role in other skeletal muscle pathology [137]. ApoE genotype plays a role in lipid balance, as well as statin treatment. While ApoE genotype may not directly affect SIM development, it may influence the statin dosage prescribed to patients, and higher doses could be associated with higher risk of SIM.

Many genetic factors have been associated with statins either by directly affecting muscle or by altering statin pharmacokinetics and efficacy. It is foreseeable that in the future, SNP analysis may play a role in determining statin dosage, as a form of “personalized medicine”.

1.4.6 Theories of SIM Aetiology

Even though statin-induced myopathy has been extensively studied, the mechanism of SIM is still unknown. It is most likely that SIM is related to the inhibition of HMG CoA reductase and thus depletion of mevalonate and its downstream products. Mevalonic aciduria, a rare autosomal recessive disorder, is caused by a deficiency in mevalonate kinase and blocks the creation of downstream compounds from mevalonate. This condition presents with a variety of symptoms (i.e. hypotonia, developmental delay) and is associated with skeletal myopathy [138-
Mevalonate supplementation has been observed to eliminate or prevent statin toxicity in cell culture [141-143]. If depletion of mevalonate is related to SIM pathogenesis then the question becomes “which mevalonate product pathway is responsible?”

As cholesterol is involved in maintaining proper cellular membrane fluidity, one of the first theories brought forward was that SIM is related to the cholesterol depletion caused by statins. Statins have been associated with electrical myotonia in rabbit skeletal muscle, an effect thought to be dependent on membrane cholesterol [144]. Similar effects are seen with cholesterol lowering fibrates [145].

However, more recent studies have cast serious doubt on the connection between cholesterol depletion and SIM. If the depletion of endogenous cholesterol production by statins was linked to SIM then genetic defects in cholesterol biosynthesis would presumably produce similar phenotypes. This is not the case. Genetic defects in stero-δ-7-reductase and 3-β-hydroxysterol-δ-24-reductase, which result in Smith-Lemli-Opitz syndrome and desmosterolosis [146, 147], disrupt the terminal steps of cholesterol production. These and similar mutations present as a collection of symptoms such as microcephaly and cardiac abnormalities; however, they are not associated with skeletal muscle myopathy [146-150]. The link between cholesterol depletion and SIM is also weakened by observations that further cholesterol reduction, in conjunction with statins, can actually ameliorate myopathy. Squalene production is the committed step towards cholesterol production, and is inhibited by squalene synthase inhibitors. Work by Tomoyuki Nishimoto has shown that a squalene synthase inhibitor prevented cerivastatin-induced myotoxicity in guinea pigs [151] and attenuated statin-myotoxicity in cultured human skeletal muscle [141].

The depletion of ubiquinone, also known as coenzyme Q10, may be involved in SIM. Ubiquinone is a product of the mevalonate biosynthesis pathway and interacts with complexes I, II, and III, of the electron transport chain (ETC). Ubiquinone is necessary for proper mitochondrial function. Statins have been shown extensively to lower serum ubiquinone levels
Ubiquinone is carried in the blood by lipoproteins and, once the LDL lowering effect of statins is taken into account, the actual reduction in serum ubiquinone levels is negligible [157-159]. Within skeletal muscle, changes in ubiquinone levels with statins appear variable, suggesting that statins may not reduce skeletal muscle ubiquinone. One study has shown a significant increase in skeletal muscle ubiquinone in simvastatin treated hypercholesterolemic patients [160] while another study measured a decrease [161] and yet another no change [162, 163].

Skeletal muscle mitochondrial dysfunction and pathology (ragged red fibres etc.) has been associated with SIM and ubiquinone depletion is a plausible link between the two. Skeletal muscle mitochondrial pathology and the link to SIM will be discussed later in this paper.

Studies evaluating ubiquinone supplementation as a treatment for SIM have yielded mixed results. In a double-blind randomized trial, Cato et al. randomly assigned patients with SIM who were taking a variety of statins either ubiquinone supplements or vitamin E [164]. After 30 days the severity of muscle pain was significantly decreased within the ubiquinone but not the vitamin E group. In contrast to this, a study by Young et al. randomly assigned 44 hypercholesterolemic patients with a history of SIM to take ubiquinone supplements or placebo. After 12 weeks of simvastatin treatment there was no difference in myalgia score between the ubiquinone or placebo groups. Research on ubiquinone depletion as a mechanism of SIM has produced conflicting results and needs further study.

Ubiquinone and cholesterol are just two of the many compounds produced from mevalonate (Figure 1). Production of complete selenocysteine tRNA (Sec-tRNA) requires isopentenylation from mevalonate-derived isopentenyl pyrophosphate. The isopentenylation of Sec-tRNA can be disrupted by statins which can lead to premature termination during translation of selenoproteins [165]. Selenoprotein N is thought to be involved in both myogenesis and myoregeneration amongst other functions; mutations in this selenoprotein
have been linked to juvenile myopathy [166, 167]. Human selenium deficiency causes skeletal myalgia and elevated CK though not rhabdomyolysis [29, 168].

Statins can reduce the prenylation of many compounds that may explain statin myotoxicity. Small G proteins such as Ras and Rho G are intracellular regulators that require prenylation to function. Depletion of these compounds can produce muscle dysfunction, atrophy and apoptosis [169]. Lovastatin treatment has been associated with induction in atrogin-1, a gene that leads to muscle atrophy in human skeletal muscle [170]. Cao et al. demonstrated that lovastatin induced atrogin-1 expression and cell damage in mouse myotubes and zebrafish skeletal muscle and that this was caused by deficient geranylgeranylation (a type of prenylation) [171]. Statins also impair N-linked glycosylation by depleting dolichol pyrophosphate production. This can result in impairment of insulin-like growth factor 1 receptor and possibly α-dystroglycan production [28].

While the exact mechanism of SIM remains unclear, evidence points to the depletion of mevalonate pathway by-products as the responsible factor. SIM may “unmask” underlying deficiencies in cellular metabolism that are asymptomatic until challenged by mevalonate depletion. More research is needed to understand the mechanism of SIM as no single by-product stands out. A multi-factorial pathogenesis involving a general deficiency in prenylation may be at play.

1.5 Mitochondria

1.5.1 Overview

The mitochondrion is an intracellular organelle found in almost all eukaryotes. While often thought of as static centres of cellular respiration, mitochondria are highly dynamic and complex organelles responsible for a variety of physiological functions. In humans, mitochondria typically have a sausage-like, oval shape, approximately 3 - 4µm in length, 1µm in diameter, and can move at speeds up to 0.5µm / s [172]. The number of mitochondria in a cell
varies by 4 orders of magnitude between cell types. For example, platelets have ~5 mitochondria, hepatocytes have on average ~800 mitochondria and oocytes can have in excess of 100,000 mitochondria [172]. The number, size, and position of mitochondria change in response to the specific needs of the cell. In skeletal muscle, mitochondria are anchored along the z lines of the sarcomere but are evenly spaced throughout the cell so that an efficient supply of ATP is available for contraction [172].

Mitochondria contain their own genome. The mitochondrial genome is a single circular loop of 16,569 base pairs that is found in the mitochondrial matrix and encodes 37 genes (22 transfer RNA (tRNA), 2 ribosomal RNA (rRNA) and 13 proteins). The mitochondrial genome has no introns, making it one of the most gene rich sections of the genetic code. All of the 13 proteins encoded by the mitochondrial DNA (mtDNA) are subunits of electron transport chain complexes. The other ~1200 proteins that are known to be involved in mitochondrial function are encoded by nuclear DNA (nDNA) and are transported into the mitochondria from the cytoplasm [173]. There are approximately 2 to 10 copies of mtDNA per mitochondrion and numerous copies per cell [174]. Several mtDNA copies are packaged within a nucleoid structure along with protein factors such as mitochondrial transcription factor A and mitochondrial singe-strand binding protein [175]. The mtDNA nucleoids are anchored to the inner mitochondrial membrane. It has been suggested that mtDNA nucleoids have a layered structure in which replication and transcription occurs in the core while translation occurs at the edges [176].

Mitochondria are the main site of intracellular energy production, being responsible for both the tricarboxylic acid cycle and oxidative phosphorylation (OXPHOS), via the electron transport chain (Figure 3). The proteins complexes that form the ETC are comprised of many subunits that are encoded by a mixture of nuclear and mitochondrial genes (Table 6). Mitochondria also play a central regulatory role in the induction of apoptosis by releasing pro-
apoptotic compounds such as cytochrome c, apoptosis-inducing factor, and endonuclease G into the cytosol [177].

**Table 6:** Complexes Forming the Electron Transport Chain

<table>
<thead>
<tr>
<th>Complex Name</th>
<th>#</th>
<th>mtDNA Encoded</th>
<th>nDNA Encoded</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH Ubiquinone Reductase</td>
<td>I</td>
<td>7</td>
<td>35</td>
<td>45</td>
</tr>
<tr>
<td>Succinate Dehydrogenase</td>
<td>II</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Ubiquinol Cytochrome c Reductase</td>
<td>III</td>
<td>1</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>IV</td>
<td>3</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>ATP Synthase</td>
<td>V</td>
<td>2</td>
<td>12</td>
<td>14</td>
</tr>
</tbody>
</table>

The complexes of the electron chain consist of many different subunits, some of which are encoded by mitochondrial DNA (mtDNA) and some by nuclear DNA (nDNA). [172]
Figure 3: An Overview of the Electron Transport Chain

The electron transport chain generates a transmembrane proton gradient by transporting protons from the mitochondrial matrix into the inter-membrane space. Reactive oxygen species (ROS) are a potential by-product of the electron transport chain. A variety of complexes are involved in this process with complexes I, III, and IV transporting protons directly. Simultaneously, electrons are transported (dashed line) from complexes I, and II to complex III via ubiquinone. Complex III then transports electrons to complex IV via cytochrome c. Complex V generates ATP from ADP by transporting protons back into the mitochondrial matrix, thus releasing the energy of the chemiosmotic gradient.
Mitochondria are produced and recycled through countervailing processes of biogenesis and mitophagy in response to cellular demands [178]. Mitophagy is the selective autophagy of mitochondria. MtDNA replication occurs independently of nDNA replication and is not linked to the cell cycle [172, 179]. DNA polymerase γ (POLG) is the polymerase responsible for mtDNA replication. This occurs in conjunction with many other compounds such as TWINKLE, a mitochondrial helicase [180]. POLG has a 3-5' exonuclease activity, however compared to nuclear polymerases it is error-prone [181]. MtDNA is also exposed to the reactive oxygen species (ROS) produced at the inner mitochondrial membrane by the ETC. The mtDNA is not protected by histones, though some protective anti-oxidant proteins have been identified [182]. With poor DNA repair ability, exposure to ROS and limited protection from damage, mtDNA is prone to oxidative damage and mutations [183-186].

While the mitochondrion has the repair mechanisms to deal with some DNA damage, it is sometimes unable to compensate for sustained stress. In a study by Yakes et al. human fibroblasts treated with hydrogen peroxide for 15 minutes developed 2-3 times more DNA damage in mtDNA compared to nDNA [187]. All of the induced DNA damage was repaired after 1.5 hours. Longer hydrogen peroxide treatment however resulted in sustained mtDNA damage. Cells were treated with hydrogen peroxide for 1 hour, and analyzed after 24 hours. While the nDNA damage was repaired, the mitochondrial damage persisted, with cells undergoing apoptosis. Mitochondria with damaged mtDNA could become dysfunctional, producing more ROS and leading to further mtDNA damage in a self-perpetuating vicious cycle [188].

Apart from ROS production, mitochondrial dysfunction leads to changes in membrane potential, ATP production and Ca\(^{2+}\) balance. Mitophagy can act as a safety mechanism against dysfunctional mitochondria [189, 190]. Damaged mitochondria can be selectively targeted for degradation, sparing the cell further ROS damage [191]. Mitochondrial fusion and fission can also play a role in the elimination of dysfunctional mitochondria. A damaged mitochondrion
could be “rescued” by fusion with a healthy organelle [192]. Alternatively, damaged mtDNA could be isolated by fission and targeted to undergo mitophagy [192, 193].

It has been well described that mtDNA deletions and point mutations accumulate with age [184, 188, 194-196]. This is associated with a decline in mitochondrial function including OXPHOS and ATP synthesis capacity. These deletions are primarily found in non-actively dividing tissue such as skeletal muscle whereas cells in high turn-over tissues, such as blood, have few detectable mtDNA deletions. It may be possible that in high turnover tissues, cells with impaired mitochondria are eliminated before significant mitochondrial deletion accumulates. As mtDNA damage slowly accumulates, ETC complexes may become inefficient or ineffective thus leading to accelerated ROS production [196]. ROS production can in turn lead to greater mtDNA damage forming a “vicious circle” of mitochondrial dysfunction [185]. Progressive mitochondrial dysfunction is a major theory of aging and falls in line with the “vicious circle” model of mitochondrial dysfunction.

1.5.2 Mitochondrial Disease

Mitochondrial dysfunction has been associated with many conditions including, diabetes, cancer, myopathy and neurological diseases [179, 197-200]. Since each cell contains hundreds of copies of mtDNA, a single cell can have several different mtDNA genotypes simultaneously (heteroplasm). The effect of an mtDNA mutation is dependent upon the portion of the mtDNA within a cell that is mutated and is sometimes asymptomatic until a threshold of dysfunctional mtDNA is reached [201, 202]. The pathogenesis of disease from mtDNA mutations is complex and many factors appear to play a role [179]. Mitochondrial depletion is a form of mitochondrial disease. Most mitochondrial depletion syndromes arise from mutations in the nDNA involved in mitochondrial function [203].

Mitochondria can be damaged by many compounds and may be responsible for drug side effects; for example, amiodarone, an antiarrhythmic drug disrupts the ETC [204, 205]. Even common medications like nonsteroidal anti-inflammatory drugs (i.e. aspirin) have a slight
decoupling effect on the mitochondrial proton gradient [206]. As mtDNA levels can change in response to cellular conditions or stresses, mtDNA content has been suggested as a marker of drug toxicity [207]. Nucleoside reverse transcriptase inhibitors (NRTIs) such as azidothymidine (AZT) or stavudine are a part of human immunodeficiency virus (HIV) treatment and interfere with viral reverse transcriptase. These NRTIs can also inhibit POLG leading to disrupted mitochondrial function and changes in mtDNA [208].

1.5.3 Skeletal Muscle Mitochondrial Pathologies

In skeletal muscle, mitochondrial dysfunction has particular histological features, some of which require special stains to be revealed [209] (Figure 4). Of these, ragged red fibres, succinate dehydrogenase (SDH) staining, Sudan black staining and cytochrome c oxidase (COX) staining bear mention. Modified Gomori trichrome (MGT) staining can highlight ragged red fibres, which have a distinctive “cracking” appearance (Figure 4, Image 1). These cells contain a massive accumulation of mitochondria typically in the subsarcolemmal region and usually have >80% mutant mtDNA [210]. SDH staining is another method of detecting mitochondrial proliferation, heavily stained fibres are described as “ragged blue” (Figure 4, Image 2). All the subunits of SDH are encoded by nDNA and SDH deficiency is rare [209]. SDH is a more sensitive stain than MGT for identifying mitochondrial proliferation and dysfunction. As mitochondria are necessary for β-oxidation of fatty acids, mitochondrial dysfunction can lead to an accumulation of intracellular lipids. This lipid accumulation can be detected by Sudan black staining and is often seen in ragged red fibres. However, lipid accumulation is not a specific sign of mitochondrial pathology [209].

Though several types of skeletal muscle fibres exist, they can be broadly split into two groups, type I and type II. Type I fibres are “slow twitch” cells that are designed for long term endurance activity; accordingly, type I fibres rely heavily upon mitochondrial aerobic respiration. Type II fibres are designed for short term yet strong contractions and rely largely on anaerobic respiration. Most skeletal muscles contain varying proportions of the fibre types. COX, also
called complex IV of the ETC, staining can expose a loss of mitochondrial function [209]. With COX staining, type I muscle fibres stain more darkly than type II in line with their energetic needs, but in normal biopsies, almost all fibres will exhibit some degree of COX staining. COX negative cells are a sign of decreased mitochondrial function (Figure 4, Image 3). Staining for SDH and COX in serial sections can identify cells that stain strongly for SDH but are COX negative. These cells are indicative of mitochondrial toxicity. These features of mitochondrial muscle pathology accumulate with age and are in line with the mitochondrial theory of aging. The sight of a few ragged red fibres in an elderly person is not unusual.
Figure 4: Skeletal Muscle Mitochondrial Pathology:

Image 1: Ragged red fibre demonstrated in a modified Gomori trichrome stain section (200x magnification)

Image 2: Ragged blue muscle fibre in a SDH stain showing proliferation of mitochondrial with increased staining (100x magnification)
Figure 4: Skeletal Muscle Mitochondrial Pathology:

Ragged red fibres and other pathologies that accumulate with age do not do so uniformly within a muscle. A mosaic pattern is often seen with only some cells displaying pathology [211]). This suggests that the accumulation of age-related mitochondrial dysfunction may be a stochastic process [212, 213].

1.5.4 Links Between SIM and Mitochondria

There are numerous links between mitochondrial dysfunction and statin-induced myopathy, though their exact relation is as yet unknown. In 1993, Chartio et al. described a 63-year old woman who developed MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like syndrome) following simvastatin-induced rhabdomyolysis, hyperCKemia, and ubiquinone depletion [214]. Ragged red fibres and pleiomorphic mitochondria were seen within her muscle biopsy and a MELAS associated mtDNA mutation (3243A-G) was found. Neale et al. presented a case of statin-induced lactic acidosis in 2004; an 82 year-old woman with thiamine deficiency developed lactic acidosis while taking
atorvastatin (10mg / day) [215]. Upon stopping statin treatment her condition immediately improved and her acid/base balance returned to normal. Another case report describes a 56-year old man for whom simvastatin and pravastatin both caused severe myalgia [216]. Muscle biopsies displayed ragged red fibres and increased numbers of mitochondria within the subsarcolemmal region. The statins were deemed to have uncovered an underlying, previously asymptomatic mitochondrial myopathy. These cases and others suggest that SIM is linked to skeletal muscle mitochondrial dysfunction [217-220]. This assertion has been supported by several clinical studies.

In a double-blind, crossover study of 4 patients with SIM but with normal (or near normal) CK levels, muscle biopsies displayed pathological features of mitochondrial dysfunction [99]. Symptoms and biopsy pathology resolved after statin cessation. Statin muscle symptoms have also been seen in the absence of mitochondrial abnormalities. A cross-sectional study of 110 SIM patients detected biochemical abnormalities in only 52% of muscle biopsies [127]. Other studies have also seen associations between SIM and mitochondrial dysfunction [221-224].

Statins can impact mitochondrial function in patients with no myalgia, and it may be that SIM presents only once mitochondrial dysfunction reaches a certain level. A study by Paiva et al. examined the effects of high-dose statins on muscle metabolism by randomly assigning patients to atorvastatin (n = 15), simvastatin (n = 15), and placebo (n = 14) [161]. Statin doses were chosen to have equipotent LDL lowering effect (40mg / day atorvastatin = 80mg / day simvastatin). Quadriceps biopsies were collected from each subject at baseline and after 8 weeks of treatment. In the simvastatin group, skeletal muscle ubiquinone levels and respiratory chain enzyme activity were decreased compared to placebo. Similar effects were not found with atorvastatin. These findings sparked an interest in the effects of statins on mtDNA. Brian Schick, a previous graduate student in the Côté lab, collaborated with the authors of Paiva et al. to examine the effects of statins on skeletal muscle mtDNA content [225]. Using a ratio of
mtDNA/nDNA as a measure of mtDNA quantity (see section 2.2.6), the change in mtDNA levels over 8 weeks was measured. A 47% decline in skeletal muscle mtDNA content between baseline and 8 weeks was measured in the simvastatin group but not in placebo or atorvastatin groups (Figure 5). No subjects reported muscle pain and none had elevated CK levels. Would mtDNA quantity have continued to decline with longer treatment, eventually leading to SIM?

Figure 5: Changes in Skeletal Muscle MtDNA/nDNA over an 8 Week Statin Treatment
This is a representation of data from Schick et al. [225]. A significant decrease in mtDNA/nDNA was observed with simvastatin treatment compared to placebo. Statin treatments are equipotent in terms of LDL lowering effect.
While the mechanism is unknown, statin treatment and mitochondrial dysfunction appear to be linked. The connection between mtDNA depletion and SIM however remains unresolved. Is mtDNA depleted within SIM patients? Is mtDNA deleted in SIM patients?

This aim of this masters project was to examine the potential effects of statin-induced myopathy on skeletal muscle mtDNA. Both mtDNA quantity and deletion burden were examined. The underlying hypothesis was that statin-induced toxicity would be associated with mtDNA depletion and changes in mtDNA deletion burden. Two experiments were performed; an *in vitro* cell culture experiment in which a rhabdomyosarcoma cells were treated with statins (Chapter, 2) and a retrospective *in vivo* study examining skeletal muscle biopsies of SIM patients (Chapter 3).
2 Chapter 2: Cell Culture Study

2.1 Introduction

The connections between statins and changes in mitochondria are numerous. Work by Shick et al. demonstrated a decline in mtDNA amongst high-dose simvastatin users [225]. Could this decline be reproduced in an *in vitro* model? In contrast, increases in mtDNA have been observed in primary myocytes after 12 days of simvastatin treatment but only at cytotoxic doses [226]. The effect of longer, sub-cytoxic statin treatment on the mtDNA in cells is unknown. This project sought to examine this by exposing rhabdomyosarcoma cells to statin treatment over a longer period of time.

The experimental plan was to expose cultured cells to various concentrations of statins over several months. Cell pellets would be collected longitudinally throughout the course of the experiment, DNA extracted, and mtDNA content examined.

2.1.1 Hypothesis

The hypothesis of this study was that rhabdomyosarcoma cell mtDNA levels would be altered in response to statins in a dose dependent manner.

2.2 Methods and Materials

2.2.1 Cell Line

An immortalized rhabdomyosarcoma cell line (RMS-13) was used in this experiment. This cell line has not been used previously in the context of statin toxicity. While primary myocytes might have been a more representative model, differentiated myocytes become senescent after ~12 days and thus were not effective for long term exposure. RMS-13 is an immortalized cell line derived from the bone marrow of a 17 year old male. These cells were acquired from the American Type Culture Collection (CRL-2061™, ATCC, Manassas, VA, USA). Other designations for the cell line are CRL-2061, RC13, and SJRH30. As per the
ATCCs established guidelines, cells were cultured at 37°C in RPMI-1640 medium supplemented with 10% fetal bovine serum (ATCC).

2.2.2 Treatments

Cells were treated with either atorvastatin or simvastatin at one of four different concentrations (20µM, 5µM, 1µM and 0.1µM). These concentrations were chosen to represent 400, 100, 20, and 2 times the therapeutic Cmax concentrations of roughly equivalent simvastatin and atorvastatin doses (40mg simvastatin or 20mg atorvastatin). Cerivastatin was not used in this study as a supply could not be found. Atorvastatin and simvastatin were chosen as they are the most widely used statin drugs. Salts of acid forms of simvastatin-sodium and atorvastatin-calcium were obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA). The chemicals were each dissolved in dimethyl sulfoxide (DMSO) and added to cell media to reach the desired statin concentration. The final DMSO concentration in each treatment was less than 0.1%.

The negative control in this experiment was media supplemented with 0.1% DMSO. As a positive control, a nucleoside analog reverse transcriptase inhibitor, AZT, was used at two concentrations (800µM, and 40µM). AZT is typically used as an HIV antiretroviral drug and has been shown to affect mtDNA quantity in lymphoblastoid cell culture [227]. All cell treatments were run in duplicate.

Cells were grown in 25cm² t-flasks and incubated in ~5mL of media at 37°C and 5% CO₂. Approximately every 48 hours, the media was aspirated, discarded and fresh media introduced. An inverted microscope was used to visually estimate cell culture confluence. General materials used for tissue culture were acquired from Gibco (Invitrogen, Carlsbad, CA, USA) and Falcon BD (BD Biosciences, Franklin Lakes, NJ, USA).
2.2.3 Subculturing Technique

Subculturing was performed once a cell culture flask exceeded 80% confluence. The following subculturing procedure was used:

1. The flask media was removed and placed into tubes.
2. The media was centrifuged for 8 minutes at 420 relative centrifugal force (RCF). 1mL of the resulting supernatant was stored at -80°C.
3. The flasks were then washed with 2mL of phosphate buffered saline (PBS) (Gibco).
4. One and a half millilitres of TrypLE Express (Gibco), a cell dissociating agent similar to trypsin that detaches adherent cells, was added to cell culture flasks. Flasks were then incubated at 37°C for 10 minutes to facilitate resuspension of cells.
5. Two millilitres of fresh media was added to the flask to neutralize TrypLE. The resulting solution was then pipetted over the adherent cells to ensure resuspension of the cells from the flask surface.
6. The harvested cells were placed in a 15mL conical tube (Falcon BD) and set aside.
7. The flask was then examined under an inverted microscope (Nikon) to confirm that the cells were harvested.
8. A small amount of the harvested suspension (10µL) was mixed with an equal volume of trypan blue (Sigma), placed on a haemocytometer (Hausser Scientific, Horsham, PA, USA) and the number of cells (viable and dead) were counted with a light microscope.
9. Fresh T25 flasks were filled with 5mL of media and then seeded from the harvested solution at a density of 1.2 * 10^4 cells per cm^2.
10. The remaining cell mixture was split into two, 2mL vials (Sarstedt, Newton, NC, USA) for cell pellet collection. The vials were centrifuged at 300 RCF for 6 minutes and the supernatant removed.
11. The resulting pellets were stored at -80°C for future DNA extraction.
At the end of the study, cells from each treatment were cryopreserved in 10% DMSO/growth medium and stored in liquid nitrogen.

2.2.4 Population Doubling Time

After each subculturing, the population doubling time (PDT) was calculated using the final cell number within the flask, the initial number of cells that were seeded in the flask, and the number of days since the flask was seeded. The following formula was used:

\[ PDT = \left( \frac{\log 2}{\log \frac{N}{N_0}} \right) \times d \]

**Figure 6:** Population Doubling Time Equation
PDT = Population Doubling Time; N = final cell number; \( N_0 \) = initially seeded cell number; d = days since seeding

2.2.5 DNA Extraction

Total DNA and RNA extraction was performed using a QIAGEN ALLPrep DNA/RNA Mini Kit following the manufacturer’s procedure for DNA / RNA extraction of pelleted cells (QIAGEN, Mississauga, ON, Canada) with the following minor changes. Cells were homogenized using QIAShredder (QIAGEN) tubes, and the DNA was eluted using AE buffer (QIAGEN) as opposed to provided EB buffer (QIAGEN).

2.2.6 Real-Time PCR Gene Quantification

MtDNA quantification was performed using a quantitative real-time polymerase chain reaction (PCR) technique that has been used extensively in our laboratory [207, 225, 227] (Figure 7). A FastStart fluorescent probe kit and Lightcycler® 480 thermocycler platform (Roche, Laval, Quebec, Canada) were used to quantify a mitochondrial gene (COX) and a single copy nuclear gene [polymerase γ accessory subunit (ASPG)]. The quantified ratio of COX / ASPG provides a measure of mtDNA/nDNA or mitochondrial genome quantity per nuclear genome, allowing comparison between samples containing different cell numbers.
Fluorescent probes specific for the target genes were used (Table 7). Each PCR reaction contained 5mM magnesium\(^{2+}\), 1\(\mu\)M of each PCR primer, 0.2\(\mu\)M fluorescein probe, 0.4\(\mu\)M LC Red 640 probe, and 2\(\mu\)L extracted DNA. The PCR conditions consisted of 95°C for 10 minutes followed by 45 cycles of 95°C/5s, 60°C/10s, and 72°C/5s. Fluorescence measurements were taken automatically after each annealing step. Standard curves for quantification were generated using known dilutions of ASPG and COX containing plasmid DNA. The maximum second derivative of each reaction was compared to the standard curve in order to determine sample gene copy number. Both genes were quantified on the same PCR plate in duplicate; both internal and negative controls were also included.
Using a Lightcycler® 480 on isolated sample DNA, real-time PCR quantification of a mitochondrial (COX) and nuclear (ASPG) gene is performed. The ratio of COX/ASPG provides a measure of the ratio of mtDNA to nDNA in a sample. As the nDNA content per cell is assumed to remain constant, changes in the ratio reflect changes in mtDNA content.

### Table 7: List of Primers and Probes Used in MtDNA Quantification Technique

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CCO1</strong></td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>5’-TTCGCCGACCGTTGACTATT-3’</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5’-AAGATTATTACAAATGCATGGGC-3’</td>
</tr>
<tr>
<td>Fluorescein Probe</td>
<td>5’-GCCAGCCAGGCAACCTTAGG-Fl-3’</td>
</tr>
<tr>
<td>Red640 Probe</td>
<td>5’-LCRed640-AACGACCACATCTACAACGTTATCGTCAC-P-3’</td>
</tr>
<tr>
<td><strong>ASPG</strong></td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>5’-GAGCTGTGACGAAGGAGAG-3’</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5’-CAGAAGAGATCCCGCTAAG-3’</td>
</tr>
<tr>
<td>Fluorescein Probe</td>
<td>5’-GAGGCGCTGTAGAGATCTCGAGA-Fl-3’</td>
</tr>
<tr>
<td>Red640 Probe</td>
<td>5’-LCRed640-GGCATTTCCTAAGTGGAAGCAAGCA-P-3’</td>
</tr>
</tbody>
</table>

P = Phosphate, Fl = Fluorescein, LCRed640 = Lightcycler Fluorophore Red 640
2.2.7 Long-Template PCR

PCR amplification of the mitochondrial genome was performed to detect the presence of mtDNA deletions. Due to the size of the mtDNA genome, long template PCR was necessary to amplify the entire mtDNA genome. PCR amplification of the mtDNA genome in two fragments was performed using an Expand Long Template PCR Kit (Roche) (Figure 8). PCR reactions contained 0.35mM dNTP, 0.3µM of each primer, 2.5 units of enzyme and 5µL of template DNA in 50µL total PCR reaction mix. PCR primers were obtained from IDTDNA (IDTDNA, Coralville, IA, USA) (Table 8). The PCR amplification was performed using a MyCycler thermocycler (Bio-Rad, Hercules, CA, USA) under the following reaction conditions: 93°C for 2 minutes followed by 35 cycles of 93°C/10s, 58°C/30s, and 68°C/6 min. Beginning on cycle 11, 20 seconds of additional time was added to the extension step during each cycle. After amplification, the PCR products were separated by electrophoresis on a 0.75% agarose (Invitrogen, Carlsbad, CA, USA) gel containing 5µL of 10mg/mL ethidium bromide (SIGMA) per 100mL of TAE buffer gel solution. An image of the gel was taken under UV light using a digital camera (Canon Inc., Tokyo, Japan). The mtDNA deletions within each sample were visualized and estimated subjectively to determine whether there was a difference in mtDNA deletion amounts between samples. The analysis of the deletion amount was subjective as, at the time of the experiment, a quantitative method of analysis had not been implemented in our laboratory.
Figure 8: Overview of Long Template PCR Amplification of MtDNA
The mitochondrial genome is amplified in two fragments and the resulting products are separated by size through electrophoresis. The bands at the top of the gel are full length products while other bands farther down represent smaller fragments suggestive of mtDNA deletions. The deletion burden was estimated subjectively as the amount of deletion bands compared to full length product. The gel picture provided is an example of various deletion amounts and was generated in a separate experiment in our laboratory. No deletions were observed in this experiment and an alternative gel picture is shown in this figure to demonstrate the assay.

Table 8: List of Primers Used in Long Template PCR Amplification of MtDNA

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
</tr>
<tr>
<td>MT_16535F Forward Primer</td>
<td>5'- GCCCACACGTTCCTAAATAAGA-3'</td>
</tr>
<tr>
<td>MT_8388R Reverse Primer</td>
<td>5'- CGGTAGTATTTAGTGGGCAATTCCAC-3'</td>
</tr>
<tr>
<td>B</td>
<td></td>
</tr>
<tr>
<td>MT_7988F Forward Primer</td>
<td>5'- CTCCTTGACGTGACAATCGAGT-3'</td>
</tr>
<tr>
<td>MT_709R  Reverse Primer</td>
<td>5'- GGGGATGCTGTGATGTAATCTTAC-3'</td>
</tr>
</tbody>
</table>
2.3 Results

Both of the 1µM atorvastatin treatment flasks were contaminated early in the experiment and were discarded. The 1µM atorvastatin treatments were not restarted due to logistical constraints. The remainder of the treatments were cultured for over 70 days. Cells treated with DMSO, 40µM AZT, and statin treatments below 20µM (0.1µM, 5µM atorvastatin, 0.1µM, 1µM, 5µM simvastatin) remained viable throughout the experiment. Cells treated with the highest doses of drug (20µM simvastatin, 20µM atorvastatin, 800µM AZT) exhibited initial cell toxicity and death. Cells that survived this cytotoxicity appeared to “adapt” to treatment and subsequently thrive. This “adaptation” took place over a long period of static non-proliferation and lasted for ~40 days of statin treatment and ~20 days of AZT treatment.

The population doubling time (PDT) of DMSO cells fluctuated between 1.2 to 1.7 days (Figure 9). No discernible change in PDT was observed with doses of statin below 5µM. An initial, moderate increase in PDT was apparent in 5µM atorvastatin (3.4 days), 5µM simvastatin (~2.3 days) and 40µM AZT (3.2 days) treatments. High-dose treatments had dramatically increased first passage PDT in conjunction with cytotoxicity and static non-proliferation (>10 days).
Figure 9: Population Doubling Times of RMS13 Cell Treatments
Overall PDT fluctuated roughly around 1.5 days. 40µM AZT, 5µM atorvastatin, and 5µM simvastatin had initial increases in PDT. High dose treatments (bottom left graph) displayed increased first passage PDT in conjunction with cell death.
Long-template PCR amplification of selected DMSO, 20µM atorvastatin, 20µM simvastatin, and 800µM AZT samples was performed. No difference in number or intensity of deletions was seen. MtDNA/nDNA ratio was quantified for all treatments (Figure 10). MtDNA/nDNA levels did not vary dramatically over time and spanned the range of 23 to 138. Several treatments, including DMSO, experienced a spike in mtDNA/nDNA at ~17 days, likely a spurious finding. There was an increase in 0.1µM simvastatin mtDNA/nDNA from approximately day 47 to 60.
Figure 10: MtDNA / nDNA Ratio of RMS13 Cell Treatments over Time
A spike in mtDNA/nDNA at ~17 days is present in DMSO, 0.1µM atorvastatin, and 0.1µM simvastatin treatments.
3 Chapter 3: Retrospective Muscle Biopsy Study

3.1 Overview

As described in section 1.5.4, an association has been observed between high-dose simvastatin treatment and depletion of skeletal muscle mtDNA [225]. In that experiment, despite a 47% decline in mtDNA, no patient experienced muscle pain or SIM symptoms. If statins are associated with depletion of skeletal muscle mtDNA with no muscle pain, do SIM patients have reduced mtDNA as well? In order to address this, a retrospective study of muscle biopsies was conducted.

While a carefully controlled, prospective study would be ideal and was initially considered, the cost and time required would have been considerable so we opted to instead do a retrospective study.

3.2 Study Design

This study was a retrospective examination of skeletal muscle biopsies in patients diagnosed with SIM and patients with normal muscle biopsies. The subjects were identified from clinical biopsy databases at Vancouver General Hospital (VGH) and St. Paul’s Hospital (SPH). Clinical information about each subject was collected from pathology reports and referral documents as well as through direct contact with referring physicians. Two subject groups were to be identified: patients whose skeletal muscle pathology diagnosis was of statin-induced myopathy (SIM patients) and patients whose muscle biopsy was normal (comparators). The stored muscle biopsies of each subject were then located, the pathology reviewed, the DNA extracted, and mtDNA content examined.

SIM patients were defined as patients whose pathology was deemed related to statin therapy, and were on statins less than a year prior to the biopsy. The comparator group acted as a control group. It should be noted that the comparators are not true controls as they were
referred for a muscle biopsy by a physician for a reason, though their biopsy was normal. To be included in this study, comparators had to have normal or subclinical muscle pathology. Comparators were excluded if they were hospitalized with debilitating symptoms or if they were on statins. Comparator age also needed to be within two years of SIM subject age range to minimize age-related differences in muscle pathology. Any patient was excluded if they had a family history of muscle dysfunction or myopathy.

3.2.1 Hypothesis

The hypothesis of this study was that patients with SIM symptoms would have significantly decreased skeletal muscle mtDNA quantity and an increased mtDNA deletion burden compared to comparators.

3.3 Materials and Methods

3.3.1 Subject Identification

At VGH there is a large archive of clinical biopsy samples, (over 8000 muscle biopsies) that can be searched using an electronic database. Potential samples were identified by searching the database with various keywords including variations of “statin”, “statin induced myopathy”, as well as statin drug names (both generic and commercial). Potential comparator biopsies were identified using search keywords such as “normal” and “sub-clinical”. SPH has a smaller archive of biopsy samples which uses a different database system. Keyword searching was not possible with the SPH database and as a result a manual technique was used for subject identification. At SPH, the pathology report for every processed muscle biopsy dating back to 2002 was examined manually for similar keywords that were searched at VGH. After initial screening, the pathology reports and referral documents of potential subjects were examined by myself to confirm their eligibility.
3.3.2 Data Collection

A wide variety of clinical information was sought from pathology reports and referral documents. The following parameters were collected: name, age, gender, statin type, statin dose, date of statin cessation, biopsy date, biopsy site, time from start of symptoms to biopsy, concurrent medication, prior medical conditions, past lab results (CK, AST, ALT etc.). In SIM cases with prominent missing data, referring physicians were contacted. Normal CK levels were considered to be between 25 and 190 ng/mL. Statin doses were classified into high dose and non-high dose groups according to their equivalent LDL lowering ability. The following statin doses were considered high doses: ≥80mg simvastatin, ≥40mg atorvastatin, ≥0.8mg cerivastatin, and ≥60mg lovastatin.

Each pathology report was examined in a blinded fashion by a certified neuropathologist (Dr. John Maguire) and the overall degree of pathology scored on a scale of 0 – 3 (0 = normal, 1 = mild, 2 = moderate, 3 = severe). Pathological features were classified into 6 categories and their presence in each report was recorded (Table 9). The 6 pathology categories were: inflammatory, myopathic, neurogenic, mitochondrial, metabolic and muscle injury. In cases where the pathology report contained scant microscopic details the original slides were reviewed again.

Table 9: Pathological Features within Each Pathology Category

<table>
<thead>
<tr>
<th>Inflammatory</th>
<th>Myopathic</th>
<th>Neurogenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammatory Infiltrates</td>
<td>Fibre Hypertrophy</td>
<td>Fibre Type Number</td>
</tr>
<tr>
<td>Rimmed Vacuoles</td>
<td>Fibre Size Variability</td>
<td>Fibre Type Grouping</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fibre Atrophy</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>Metabolic</td>
<td>Muscle Injury</td>
</tr>
<tr>
<td>Ragged Red Fibres</td>
<td>Lipid Accumulation</td>
<td>Regeneration</td>
</tr>
<tr>
<td>Ragged Blue Fibres</td>
<td>Glycogen Accumulation</td>
<td>Degeneration</td>
</tr>
<tr>
<td>COX Negative Fibres</td>
<td></td>
<td>Necrosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Moth Eaten Fibres</td>
</tr>
</tbody>
</table>

The various pathological features in each pathology category
3.3.3 DNA Extraction

Total DNA extraction from frozen muscle biopsies was performed using a Qiagen DNA MiniKit (QIAGEN Mississauga, ON, Canada). A small portion (~30mg) of each muscle biopsy was used for extraction. Extraction was performed once per sample. All extractions and subsequent measurements were done while blinded from the subject group and clinical information of each subject.

3.3.4 Mitochondrial DNA Quantification

Mitochondrial DNA quantification was performed by the same real-time PCR technique that was described previously (section 2.2.6). Due to the high mtDNA / nDNA ratios typically found in skeletal muscle, it was not possible to use undiluted DNA for COX quantification as the results would lie outside the accurate range of the assay. To compensate for this, COX quantification was performed using 100 to 150 fold diluted DNA while undiluted DNA was used for ASPG quantification. Other than this change, the procedure is identical to that described in chapter 2.

3.3.5 Long Template PCR

The mtDNA deletion burden assay performed in this study operates under the same principle as the mtDNA deletion method described in section 2.2.7, however, the technique was refined and a few adjustments were made. Long template PCR was still used to amplify the mtDNA genome and to examine mtDNA deletions. A different kit and set of reaction conditions were used. An Expand Long Range dNTP pack kit (Roche) was used to produce 25µL PCR reactions (0.5mM dNTP, 0.3µM of each primer, 3.5 units of enzyme and 2µL of template DNA). The template DNA was diluted such that each PCR reaction contained 100 copies of ASPG. The same PCR primers were used as before (Table 8).

After amplification, the PCR products were separated by electrophoresis on an agarose gel as described in chapter 2. The resulting digital picture was converted to inverse greyscale and analyzed with UN-SCAN-IT software (Silk Scientific, Orem, UT, USA). The pixel density of
full-length and deleted bands was calculated to determine the deletion burden of each sample (Figure 11). The sum of the deletion burden of each fragment is used in the analyses as a total mtDNA deletion score.
Figure 11: Overview of Long Template PCR Amplification of MtDNA
The mitochondrial genome is amplified in two fragments and the resulting products are separated by size through electrophoresis. The resulting gel is digitized and the pixel density of each band is calculated. The area above and below each band was used to calculate the background pixel density. The deletion burden of each fragment is then calculated. A total deletion score is calculated from the sum of the fragment deletion burdens.

Lanes:  A = DNA Ladder;  B = Sample I Fragment A;  C = Sample I Fragment B;  D = Sample II Fragment A;  E = Sample II Fragment B
3.3.6 SLC01B1 Rs4149056 Genotyping

Extracted DNA was used to amplify and genotype the SLC01B1 rs4149056 SNP with all PCR reactions taking place in a MyCycler thermocycler (Bio-Rad). A portion of DNA surrounding the rs4149056 SNP was amplified. Primers were identified using the National Center for Biotechnology Information website and Primer-Blast (NCBI, Bethesda, MD, USA). Primers were ordered from IDT (Table 10). Each PCR reaction contained 0.2mM dNTP, 0.5mM of each primer, 0.1 units of TAQ polymerase, and 2.5µL of template DNA. The PCR amplification conditions were as follows: 94°C for 30 seconds followed by 35 cycles of 94°C/15s, 51°C/30s, and 72°C/30s and ending with 72°C/ for 7 minutes. In samples that exhibited poor amplification, PCR amplification was performed using 0.15 units of TAQ polymerase with 40 cycles of amplification. The amplified PCR product was then used as the template for dye-terminator sequencing using one primer. Sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA). The sequencing reaction conditions were: 96°C/10s, 50°C/5s, and 60°C/55s for 25 cycles in a MyCycler. Sequence analysis was performed at the BC Centre for Excellence in HIV/AIDS, Vancouver, BC.

Table 10: Primers Used in Rs4149056 SNP Genotyping

<table>
<thead>
<tr>
<th>Rs4149056 Primer Sequence</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Sequencing Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLCO1B1_ex6_PCR_F</td>
<td>5'- TTGTCAAAGTTGCAAAGTG -3'</td>
<td>5'- GGTAAGCCATGGATGGAC -3'</td>
<td>5'- GCCAAGAATGCATGGTTCTT -3'</td>
</tr>
<tr>
<td>SLCO1B1_ex6_PCR_R</td>
<td>5'- TTGTCAAAGTTGCAAAGTG -3'</td>
<td>5'- GGTAAGCCATGGATGGAC -3'</td>
<td>5'- GCCAAGAATGCATGGTTCTT -3'</td>
</tr>
</tbody>
</table>

3.3.7 Apolipoprotein E Genotyping

Apolipoprotein E genotyping was performed at SPH by Dr. Daniel Holmes’ laboratory using a Lightcycler® Apo E Mutation Detection Kit (Roche). This real-time PCR technique measures simultaneously the melting temperatures of two fluorophores covering positions 112 and 158 of the apolipoprotein E gene. The genotype (E2, E3, E4) of the sample can be determined by the resulting fluorophore melting points. For the purposes of data analysis ApoE
genotype assessed as a linear model as described by Donnelly et al. [136]. In this model ApoE genotypes are scored as follows from -2 to 2: E2/E2 = -2, E2/E3 = -1, E2/E4 = 0, E3/E3 = 0, E3/E4 = 1, E4/E4 = 2.

3.3.8 Statistics and Data Analysis

Statistical analysis was performed using Microsoft Excel (Microsoft, Redmond, WA, USA) and SPSS (SPSS Inc., Chicago, IL, USA). Two-tailed p values less than 0.05 were considered significant. Differences in mtDNA/nDNA and mtDNA deletion score between treatment groups were tested by analysis of covariance (ANCOVA) controlling for the following covariates: age, gender, year of biopsy and ApoE genotype. The effects of biopsy site and SLCO1B1 genotype on mtDNA were assessed separately by analysis of variance (ANOVA). Differences between treatment group pathology scores were calculated using a Mann-Whitney U test. A student’s t-test was used to compare mtDNA/nDNA levels amongst high dose and non-high dose SIM patients. A chi-squared test was used to determine differences in frequencies of binary variables between groups. Correlation calculations were performed using Pearson’s correlations.

3.4 Results

3.4.1 Study Population

A keyword search of the VGH muscle biopsy database identified 47 subjects whose pathology reports were then examined. At SPH, the pathology reports of all 321 muscle biopsies since 2002 were manually examined. From the total of 368 pathology reports, 60 potential SIM patients and 54 potential comparator patients were identified. Of the initial group of 60 potential SIM patients, 25 were excluded for various reasons: 22 were diagnosed with a non-SIM muscle condition (e.g. inclusion body myositis) which was judged responsible for the pathology, while 3 patients had not taken statins in over a year prior to biopsy. Twenty of the initial 54 potential comparator patients were excluded for various reasons: 12 had abnormal muscle pathology, 4 patients were taking statins, 2 patients had a family history of debilitating
symptoms, and 2 patients were too young based on the previously defined exclusion criteria (section 3.2). The 4 potential comparators who were found to be taking statins could not be included as SIM patients because their muscle complaints were not deemed related to statin therapy in the pathology reports. Additionally, 22 biopsies (11 in each group) could not be found in hospital freezers. In total, 24 SIM and 23 comparator patients muscle biopsies were enrolled in the study. The reasons for biopsy referral were recorded for each patient and are shown in Table 11. All SIM patients were referred to investigate muscle pain, weakness or fatigue. Twelve comparators were referred for muscle pain and 2 for suspected neuropathy. No reason for referral of 9 comparator subjects could be found.

| Table 11: Reason for Patient Referral for Biopsy |
|-----------------|--------|--------|
|                 | Comparator | SIM |
| N               | 23      | 24    |
| Reason for Biopsy Referral: | | |
| Muscle pain / weakness / fatigue | 12      | 24    |
| Neuropathy      | 2       | 0     |
| Unknown         | 9       | 0     |

Subject age ranged from 47 to 85 years old with the average age being 62 and 61 years old in SIM and comparator groups respectively. A majority of subjects were male, 62.5% (9/24) of SIM subjects and 73.9% (6/23) of comparators. The population characteristics are shown in Table 12. Forty-six percent of SIM patients were taking a medication known to be a SIM risk factor, of which approximately half were medications associated with CYP3A4 inhibition. In line with market share, atorvastatin was the most common statin followed by simvastatin. Statin dosage was known in 15 out of 24 SIM patients; of these, 3 patients were taking clinically high doses.

### 3.4.2 Genotyping

Patient genotyping was performed as described above. The SLCO1B1 SNP (rs4149056) genotype was successfully determined in each patient. Approximately 23 %
patients were heterozygous for T and C alleles [Comparator (22%), SIM (25%)], consistent with other studies [125]. No patients were homozygous for the C allele of the rs4149056 SNP. ApoE genotyping was unsuccessful in 3 subjects, and no patients were homozygous for rare alleles (E2, E4).
Table 12: An Overview of the Study Population Demographics

<table>
<thead>
<tr>
<th></th>
<th>Comparator</th>
<th>SIM</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>23</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Gender (% Male)</td>
<td>74%</td>
<td>63%</td>
<td>0.401</td>
</tr>
<tr>
<td>Age (Mean, [Range])</td>
<td>61 [47 – 82]</td>
<td>62 [47 – 85]</td>
<td>0.609</td>
</tr>
<tr>
<td>Elevated CK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severely Elevated (&gt; 1900ng/mL)</td>
<td>2</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Elevated (&gt; 190ng/mL &lt; 1900ng/ml)</td>
<td>5</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>15</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>SLCO1B1 Genotype:</td>
<td></td>
<td></td>
<td>0.792</td>
</tr>
<tr>
<td>T</td>
<td>18</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Y</td>
<td>5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ApoE Genotype:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-2 (E2/E2)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>-1 (E2/E3)</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>0 (E2/E4, E3/E3)</td>
<td>13</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>1 (E3/E4)</td>
<td>5</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>2 (E4/E4)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>SIM Associated Co-Medications:</td>
<td>1</td>
<td>11</td>
<td>0.001</td>
</tr>
<tr>
<td>CYP3A4 Inhibiting</td>
<td>1</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Non-CYP3A4</td>
<td>0</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Muscle Pain Associated Medical Conditions:</td>
<td>5</td>
<td>11</td>
<td>0.081</td>
</tr>
<tr>
<td>Diabetes</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>3</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Hypothyroidism</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Myoglobinuria</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Statins (High Dose / Non-High Dose / Unknown):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>1 / 7 / 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simvastatin</td>
<td>1 / 3 / 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lovastatin</td>
<td>1 / 1 / 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerivastatin</td>
<td>0 / 1 / 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>0 / 0 / 2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.4.3 Biopsy Characteristics

Muscle biopsies of each subject were located, the DNA extracted, and mtDNA content analyzed. The muscle biopsy data is shown in Table 13. The most common biopsy site was the quadriceps followed by the biceps. Scoring of biopsy pathology and a listing of pathological features present was performed for each biopsy by a certified neuropathologist in a blinded fashion (Table 14). In 5 cases (1 SIM, 4 comparators) the pathology report contained too little information to score the biopsy so the original slides were examined. Minor pathological features were seen within the comparator group, these were within the range of normal for each subject’s age. Amongst the SIM patients the most common features were indicative of muscle injury.

Table 13: An Overview of the Subject Biopsy Information

<table>
<thead>
<tr>
<th></th>
<th>Comparator</th>
<th>SIM</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>23</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Biopsy Site:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biceps</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Deltoid</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Quadricep</td>
<td>10</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Vastus Lateralis</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Pathology Score</td>
<td>0.30 ± 0.47</td>
<td>2.08 ± 0.72</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>mtDNA/nDNA</td>
<td>3220 ±1594</td>
<td>2036 ± 1146</td>
<td>0.006 †</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.042 ‡</td>
<td></td>
</tr>
<tr>
<td>mtDNA Deletion Score</td>
<td>19.35 ± 29.96</td>
<td>21.17 ± 19.58</td>
<td>0.805 †</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.749 ‡</td>
<td></td>
</tr>
<tr>
<td>Biopsy Year:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2000</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2000 – 2002</td>
<td>3</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>2003 – 2005</td>
<td>17</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>2006 – 2008</td>
<td>3</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

† = Student’s t-Test; ‡ = ANCOVA, controlling for age, gender, year of biopsy, and ApoE genotype
Table 14: Pathology Features within Subject Biopsies

<table>
<thead>
<tr>
<th>Type of Pathology Features</th>
<th>Comparator</th>
<th>SIM</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>23</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Inflammatory</td>
<td>1</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Myopathic</td>
<td>1</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Neurogenic</td>
<td>3</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>5</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Metabolic</td>
<td>0</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Muscle Injury</td>
<td>3</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

Amongst SIM subjects, the frequency of pathology features within each ApoE genotype was explored for possible trends (Table 15). There was no apparent association between type of pathology feature and ApoE genotype.

Table 15: Pathology Features within SIM Subjects of Known ApoE Genotype

<table>
<thead>
<tr>
<th>E2/E3 (n = 1)</th>
<th>E3/E3 (n = 14)</th>
<th>E4/E3 (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammatory</td>
<td>1 / 100%</td>
<td>Inflammatory</td>
</tr>
<tr>
<td>Myopathic</td>
<td>0</td>
<td>Myopathic</td>
</tr>
<tr>
<td>Neurogenic</td>
<td>0</td>
<td>Neurogenic</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>0</td>
<td>Mitochondrial</td>
</tr>
<tr>
<td>Metabolic</td>
<td>0</td>
<td>Metabolic</td>
</tr>
<tr>
<td>Muscle Injury</td>
<td>1 / 100%</td>
<td>Muscle Injury</td>
</tr>
</tbody>
</table>

One SIM subject for whom ApoE genotype could not be determined is not included in this table.

MtDNA/nDNA ratio quantification was successful for all samples using the previously described real-time PCR technique. The results of statistical analyses are summarized in table 16. There were no significant differences between the muscle types in mean mtDNA/nDNA ratios ($p > 0.250$). Mean mtDNA/nDNA levels were significantly lower amongst SIM patients ($n = 23$, mean ± standard deviation, $2036 ± 1146$) compared to comparators ($n = 24$, $3220 ± 1594$).
(p = 0.006) (Table 16, Figure 12). Controlling for the effects of age, gender, year of biopsy and ApoE genotype, mean mtDNA/nDNA levels remained significantly lower amongst SIM patients compared to comparators (p = 0.042).

In a subanalysis of the 15 SIM patients for whom statin dosage was known, the mean mtDNA/nDNA of patients on high-dose statins (n = 3) was significantly higher compared to patients on lower doses (n = 12) (p = 0.012). SIM contra-indicated medication and statin type did not have a significant effect on mtDNA/nDNA. Within the SIM group, mtDNA/nDNA was higher amongst patients on simvastatin (2746 ± 1160) than patients on atorvastatin (1790 ± 1214) however this difference did not reach statistical significance (p = 0.130).

Controlling for age, gender, year of biopsy, and ApoE genotype, no difference was observed in mtDNA deletion scores between groups (p > 0.5) (Figure 12). No significant interaction was found between various variables and mtDNA deletion score including: high-dose statins, biopsy site, statin type and SIM contra-indicated medication (Table 16).

There was a significant interaction between SLCO1B1 genotype and mtDNA/nDNA between groups, controlling for age, gender, year of biopsy, and ApoE genotype (p = 0.036). Follow-up revealed that the mean mtDNA/nDNA ratio of the comparator group was significantly higher than the mean of the SIM group, but only amongst T genotype patients. The same analysis examining SLCO1B1 genotype and mtDNA deletion score found no significant interaction (p = 0.051)
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t-Test = Student’s t-Test; ANOVA = Analysis of Variance
ANCOVA = Analysis of Covariance, controlling for age, gender, year of biopsy, and ApoE genotype
Figure 12: MtDNA/nDNA Ratios and MtDNA Deletion Scores within the Study Groups

Box plots of mtDNA/nDNA ratio and mtDNA deletion score between comparator and SIM patient groups. The line represents the median, the cross represents the mean, the box represents the interquartile range (IQR), and the dashed lines encompass non-outlier values. Mean SIM mtDNA/nDNA ratio was significantly lower than the comparator group ($p = 0.042$). There was no significant difference in mtDNA deletion score between groups.
A significant, overall correlation was found between mtDNA content and biopsy pathology score ($p = 0.006, r = -0.399$) (Figure 13). This association was also found when only examining the SIM subgroup ($p = 0.017, r = -0.481$).

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**Figure 13:** Correlation between MtDNA/nDNA Ratio and Pathology score

A scatterplot of mtDNA/nDNA ratio vs. pathology score that demonstrates the relationship between the two variables. The line represents the correlation between mtDNA/nDNA ratio and pathology score over all samples.
No correlation was found between mtDNA/nDNA ratio and mtDNA deletion score ($p = 0.397$) (Figure 14). As mtDNA is known to alter with age, possible associations between age and mtDNA/nDNA ratio as well as mtDNA deletion score were explored. No correlations were observed between overall mtDNA/nDNA ratio and age ($p = 0.409$) or between mtDNA deletion score and age ($p = 0.397$) (Figure 15). Analysis of each treatment group separately also showed no correlation.

**Figure 14:** Scatter Plot of MtDNA/nDNA Ratio vs Pathology Score
Figure 15: Scatter Plots of MtDNA Content and MtDNA Deletion Score vs Age
A scatterplot of mtDNA deletion score vs age that demonstrates the relationship between the two variables. The line represents the overall correlation between mtDNA deletion score and age.
4 Chapter 4: Discussion

4.1 Cell Culture Study

This study examined the effects of atorvastatin and simvastatin treatment on an immortalized rhabdomyosarcoma cell line (RMS13). Cell culture is an imperfect but often practical model of a tissue or organ. As described previously (section 2.1), prior work in our lab had determined that mtDNA/nDNA ratios in commercially available human myocytes were ~200 and that human skeletal muscle mtDNA/nDNA was in the thousands [226]. Cultured human myocytes became senescent approximately 12 days after differentiation and as such, long term treatment was not possible; RMS13 cells were chosen because they can be cultured indefinitely and display primitive signs of skeletal muscle differentiation. RMS13 cells have not been previously used to investigate statin-treatment and these cells may not be an appropriate model of statin toxicity. The low mtDNA/nDNA ratios (~40) measured in RMS13 cells suggests that the cell line may have a lesser dependence on mitochondria than in vivo skeletal muscle and thus may be a poor model of skeletal muscle mitochondrial toxicity. A limitation of this study is that mitochondrial activity assays were not performed. Possible mitochondrial activity assays include: citrate synthase activity, ATP production, each of the complexes of the ETC and others. While mtDNA/nDNA provides a measure of mtDNA content, it does not indicate specifically how mitochondrial function may be changing.

The treatment of RMS13 cells with atorvastatin, simvastatin and AZT at various concentrations was associated with varied effects on population doubling time and mtDNA content (mtDNA/nDNA). No toxicity was observed with 40µM AZT and statin doses below 20µM. Dramatic cell death was observed in cells treated with high dose treatments (20µM statins, 800µM AZT). A subset of cells survived the high-dose toxicity, apparently adapted to treatment and subsequently thrived. The population doubling time of cells treated with 40µM AZT, 5µM simvastatin and 5µM atorvastatin was initially increased; the meaning of this is not
known. MtDNA content was increased at ~17 days of treatment in DMSO, 0.1µM atorvastatin and 0.1µM simvastatin but not in higher dose treatments. This spike may have represented a transient change in mtDNA due to DMSO which higher dose treatments could inhibit. However, as the increase is represented by a single datapoint in each treatment, this observation is likely spurious. A repeated study would be needed to confirm the impact of this single increased mtDNA/nDNA peak. No change in mtDNA content was detected in AZT treated cells, suggesting that AZT is not an effective positive control for drug induced mtDNA toxicity in RMS13 cells. No difference in mtDNA deletions was observed between treatments.

Other than the determining that 20µM atorvastatin, 20µM simvastatin and 800µM AZT treatment was toxic to RMS13 cells, the results from this study were inconclusive. It is possible that any mitochondrial changes in the high dose treated cells would have been obscured or lost with the dramatic cell death. The initial increase in PDT with 5µM statin treatment may have represented an anti-proliferative effect but that is entirely speculative. Statins have been associated with anti-proliferative effects in cultured human myoblasts [228]. Due to the low mtDNA/nDNA level observed in RMS13 cells, it is likely that this cell line is a poor cell model for skeletal muscle mitochondrial toxicity. Other potential cell models include different rhabdomyosarcoma cell lines, rat L6 myoblasts and others. Human skeletal muscle cell lines cannot be cultured stably but may be a potential platform for short experiments.

In future work it will be important to measure mtDNA alterations in tandem with cellular function. Work recently done in our laboratory demonstrates the benefits of this approach [227]. Papp et al. treated lymphoblastoid cells with anti-retroviral treatment (AZT and stavudine) over 74 days and observed an initial, dose dependent rise in mtDNA/nDNA compared to control [227]. MtDNA content returned to control levels after 16 days; shortly afterwards, increases in COX mtRNA and multidrug-resistance protein gene RNA were observed. A biphasic response was theorized wherein the initial mtDNA increase represented mitochondrial proliferation in response to acute stress and the subsequent changes in gene expression were a cellular
adaptation to prolonged treatment. Similar changes could be occurring in RMS13 statin treated cells and gene expression will be important to examine in future work.

It is possible that, in a nutrient rich environment, the impact of mitochondrial dysfunction is minimized as the cell can turn to other, non-mitochondrial, sources of energy production. One laboratory has observed that *Candida* yeast grown in rich media with fermentable glucose exhibited minor growth inhibition when treated with statins [229]. However, when the media was changed to replace glucose with non-fermentable ethanol as the primary carbon source, statin treatment strongly inhibited growth. The RMS13 cells in the present study were grown in rich media and it is possible that the availability of fermentable energy sources could have affected the response to statin treatment.

Cell culture is an attractive platform for drug toxicity research. Other cell models have been used in the context of statin-induced toxicity including primary human myoblasts, smooth muscle, rodent myoblasts, yeast and RD human rhabdomyosarcoma [142, 230-233]. The results however are varied and statin response appears to be cell-type specific.

### 4.2 Retrospective Skeletal Muscle Biopsy Study

The main finding of this retrospective skeletal muscle biopsy study was that patients with statin induced myopathy had lower skeletal muscle mtDNA content compared to comparator patients with normal biopsies and not on statins. However, the comparator patients were not true controls. Each comparator was originally referred for a biopsy in order to investigate a muscle complaint, most commonly muscle pain, weakness or fatigue. While a neuropathologist determined that these biopsies showed no diagnostic pathologic findings (within the range of “normal”), it is possible that underlying mtDNA defects were present but undetected in some patients. It would have been ideal to compare patient history of myocardial infarctions, degree of CVD, or other pathology within this study, however insufficient clinical data was available to do so. This is a limitation of the study’s retrospective design. Of the variables that were examined, there were gaps in the clinical data where information could not be found from
hospital records or referring physicians. In particular, information on statin dosage and creatine kinase levels were often missing hence could not be included in the statistical analyses. Additionally, previous work in our laboratory showed considerable inter-individual variation in skeletal muscle mtDNA content which may affect results since this study is cross-sectional in nature. Despite these limitations, several findings can be derived from this study.

Schick et al. observed a decrease in skeletal muscle mtDNA levels in patients treated with high-dose simvastatin, but not atorvastatin, for 8 weeks when compared to placebo [225]. None of those patients presented with muscle pain or elevated CK.

This study sought to examine whether mtDNA depletion such as that reported by Shick et al. presented in patients with SIM. While an association between mtDNA depletion and SIM was observed, it is not possible to distinguish whether this is related to SIM or to statin treatment alone. However, the observation that skeletal muscle mtDNA/nDNA ratio is significantly lower in SIM patients supports the association of statin treatment and mtDNA depletion potentially leading to SIM. Furthermore the notion that decreased mtDNA is indicative of pathology is strengthened by the inverse correlation observed between mtDNA/nDNA ratio and the degree of pathology score.

Patients on high dose statins had significantly higher mtDNA/nDNA ratios compared to other SIM patients. However, as there were only 3 subjects on high-dose treatment, the impact of this finding is questionable. A larger sample size would be needed to confirm this. The effect of statin type (atorvastatin vs simvastatin) on mtDNA/nDNA levels was not significant but the calculated effect size (partial $\eta^2$) was high (0.137). Partial $\eta^2$ is a statistical measure that represents to what degree the dependent variable (mtDNA/nDNA) is explained by independent variable, in this case statin type. There was a trend for lower mtDNA/nDNA levels amongst patients on atorvastatin compared to those on simvastatin. While not statistically significant, the effect size was considerable (13.7%) and statin type may be important. This seemingly contradicts Shick et al. but supports the notion that statin mtDNA depletion is a class effect.
The effect of statin type and dosage on mtDNA/nDNA cannot be determined from these results and further enquiry is recommended.

There was no difference in mtDNA deletion score between groups, nor was there a correlation between mtDNA/nDNA ratio and mtDNA deletion score. This could suggest that quantitative rather than qualitative mtDNA changes were occurring. The range of mtDNA deletion was broad; the subject with the highest mtDNA deletion score, a 58 year old comparator with an unremarkable quadriceps biopsy, exhibited striking mtDNA deletion with little full length fragment B remaining. The SIM patient with the highest mtDNA deletion score was the youngest patient in the study and had substantial skeletal muscle mitochondrial pathological features. Either of these patients could have an underlying mitochondrial disorder related to mtDNA deletions which may be a factor in their muscle symptoms. These subjects were not examined further to identify any specific mtDNA mutations that are associated with mitochondrial disease as this was considered beyond the scope of the study.

There was no overall correlation between mtDNA deletion score and subject age. This is inconsistent with the well accepted concept that mtDNA deletions and mutations accumulate with age. Several things may account for this discrepancy. The mtDNA deletion assay does not quantify point mutations and may not have been sensitive enough to detect age-related mtDNA mutations in humans. Also, the SIM subjects were investigated for drug-induced mitotoxicity and the comparators had muscle complaints. Therefore the composition of the subject groups could have obscured any relationship between mtDNA deletions and age. These effects, and the small study sample size, could account for the lack of correlation between mtDNA deletion and subject age.

The primary comparisons in this study were the mtDNA content and mtDNA deletion score between groups. Other analyses were post-hoc in nature and were intended to be hypothesis generating. A wide variety of pathological findings were observed in SIM muscle biopsies but no apparent trend appeared between type of pathology and mtDNA content or
deletion score. There was a greater frequency of pathology features within the SIM group. This was expected given that the comparators were selected to have little or no pathology. The pathology features were determined by Dr. John Maguire who examined the existing pathology reports. In 5 cases, the pathology report did not contain enough information to evaluate the biopsy and so the original slides were examined. The original slides were available for most, if not all of the subjects but were not all examined due to the time that would be required to evaluate 47 sets of slides. Doing so though could standardize the procedure and strengthen future analyses of the pathology feature data.

There was no difference in SLCO1B1 and ApoE genotype between groups which is unsurprising given the small sample size. A larger sample size would be needed to enable a meaningful comparison in genotype frequency. There was however, a significant interaction between SLCO1B1 genotype and mtDNA content. Comparator subjects with the common SLCO1B1 allele (T) had significantly higher mtDNA content compared to Y comparators, and all SIM patients. Apart from statins, SLCO1B1 transports a variety of drugs and other compounds. It is possible that comparators with the Y allele were taking a medication that is transported by SLCO1B1 and has a dose-dependent mitotoxic effect. The interaction between SLCO1B1 genotype and mtDNA deletion score was non-significant, but barely so (p = 0.051). Given the small sample size in this study, the interactions between SLCO1B1 genotype and mtDNA content were possibly driven by the presence of outliers, particularly within mtDNA deletion score. No association was found between ApoE genotype and mtDNA content. ApoE genotype did not appear to be associated with pathology type as a similar distribution of pathology features was seen amongst E3/E3 and E4/E3 SIM patients. Currently, there is no clear evidence in the literature of a relationship between ApoE genotype and SIM. A larger sample size would be needed to properly examine genetic factors, and SLCO1B1 is an interesting candidate for continued research.
While elevated CK was common within SIM patients, CK data was missing for the majority of comparators and comparisons between groups was not possible. Significantly more SIM patients (46%) than comparators (4%) were taking medications known to increase SIM risk. The proportion of SIM patients that were on a SIM risk medication is striking and is higher than what studies have observed in clinical practice. In an analysis of 5.6 million patients in the IMG Health prescription database it was found that nearly 19% of patients on statins were prescribed an interacting or contra-indicated medication [234]. The high rate of SIM risk medication amongst SIM subjects in this study suggests that some of these patients’ symptoms may have been avoided with a change in non-statin medication. However, statistical analyses detected no relationship between SIM risk medication and mtDNA content or deletion score.

The observation that SIM patients have lower mtDNA/nDNA levels than comparators is in line with evidence that points towards statins and mitochondrial dysfunction. In vivo, statins have been shown to increase both the respiratory exchange and lactate/pyruvate ratios [223, 224]. Mitochondrial pathology has been observed in studies of skeletal muscle biopsies from SIM patients [99, 221]. Mitochondrial dysfunction has also been described in case reports of statin myopathy [214-220]. In the yeast model, Candida glabrata, simvastatin can induce a complete loss of mtDNA resulting in petite colonies; no data on mtDNA deletions was reported [232]. The same group has stated that atorvastatin also produces petite colonies though no data is given [229]. Previously, mtDNA depletion and statin treatment has been associated, in vivo, with high-dose simvastatin [225]. This study has shown that mtDNA may be lowered in SIM patients taking a variety of statins.

What could the mechanism of statin-induced skeletal muscle mtDNA depletion be? MtDNA depletion may be indicative of mitochondrial dysfunction in general as selective mitophagy targets damaged mitochondria in an attempt to preserve the cell. How could statins cause mitochondrial dysfunction? Mevalonate depletion is the most likely cause, but several products downstream from mevalonate could be responsible (Figure 1, Figure 16). Cholesterol
depletion could disrupt the fluidity of the mitochondrial membranes leading to disruption of mtDNA replication as well as ROS production [232, 235]. Ubiquinone depletion is a logical source of mitochondrial dysfunction as it is part of the ETC. Ubiquinone depletion could cause disruptions in the ETC leading to increased ROS production and mtDNA damage. Inhibition of selenoprotein synthesis can negatively impact antioxidant defence as well as myocyte regeneration. Mitochondrial depletion has been associated with selenium deficiency and similar dysfunction could occur with statins [236]. A reduction in protein prenylation can impact small G proteins such as atrogin-1 or RhoA and lead to autophagy.

![Diagram](image)

**Figure 16:** Possible Method of Statin Induced Mitochondrial Dysfunction
Statins inhibit mevalonate production leading to a depletion of mevalonate downstream products (i.e. cholesterol, ubiquinone) as well as inhibition of protein prenylation and selenoprotein synthesis. Through an unknown mechanism, this leads to a vicious cycle of mitochondrial dysfunction, increased ROS production, and mtDNA damage. The cycle of mitochondrial dysfunction results in mtDNA depletion.
While skeletal muscle mtDNA depletion is seemingly associated with statins, the connection to SIM is not yet understood. It is plausible that statin-induced mitochondrial dysfunction develops into SIM once a threshold of dysfunction is reached. MtDNA levels have been suggested as a possible marker for drug toxicity in the context of HIV medication and may have use in a statin setting [237]. Though the available data has not validated mtDNA as a biomarker of SIM, it is a promising avenue to research. The elderly, who have generally accumulated mtDNA deletions, as well as patients with underlying asymptomatic mitochondrial conditions, are at increased risk of SIM. MtDNA levels may be a potential marker of sub-threshold mitochondrial damage. The utility of skeletal muscle mtDNA levels as a biomarker would be quite limited due to the difficulty, cost, and discomfort involved in muscle biopsy collection. In order to be a practical marker, mtDNA changes in the skeletal muscle would need to be related to mtDNA changes in an accessible tissue such as blood, or oral epithelium. Currently, creatine kinase is used as a test for muscle damage but it is a poor marker of SIM. The development of a more effective SIM biomarker would be a great benefit to the treatment of statin patients.

4.3 Future Directions

The association between SIM and mtDNA depletion, the primary finding of this thesis, sets the ground for some interesting future work. This could examine whether long term statin treatment leads to progressive mtDNA depletion and whether SIM develops once a certain level of mtDNA depletion takes place.

Ideally a prospective study of statin patients would be performed. Subject groups would include those with SIM, patients who tolerate statins, and matched placebo controls. Muscle biopsies as well as other tissues such as mouth swabs and blood would be collected before, during, and after treatment. Changes in mtDNA, mitochondrial function and morphology could be examined. Since it is not possible to identify patients who will develop SIM, subjects could be recruited who have SIM and are about to stop statin treatment. SIM mostly resolves after
statin cessation and the changes in skeletal muscle could be examined before and after SIM resolution. Blood and mouth swabs could also be collected to determine the universality of mtDNA changes. The main difficulty in a study such as this would be subject recruitment as muscle biopsies are an invasive and uncomfortable procedure. Needle biopsy techniques could be used to minimize discomfort, however subject recruitment would remain an obstacle [210].

While an association between statin treatment and mtDNA depletion has been demonstrated, a mechanistic link has not. If a proper model can be found, cell culture experiments could be used to examine in vitro statin induced mitochondrial changes. While RMS13 cells did not appear to be a good model, there are many other potential cell models including RD rhabdomyosarcoma, rat L6 myoblasts, and Candida glabrata. The effects of statins on mtDNA content and deletions should be examined in conjunction with markers for autophagy (e.g. atrogin-1), apoptosis (e.g. caspases 3/7/9) and mitochondrial function (mitochondrial membrane transition, complex IV activity, ATP production etc.) as statins may affect these [169, 170, 230, 231, 238]. Cells could be grown in media with limited fermentable carbon sources to promote reliance on OXPHOS and prevent cells from switching to anaerobic respiration pathways. Once a suitable cell line is found, each portion of the mevalonate pathway could be examined in turn by inhibition or supplementation to determine their contribution, if any, to statin-induced mitochondrial dysfunction. Cell culture work could potentially help elucidate the mechanism of statin mitochondrial dysfunction. Supplementation of mevalonate pathway compounds could also be investigated to see if statin toxicity can be reversed or prevented while maintaining the cholesterol depletory effects of statins.

4.4 Conclusion

Statins play a large and expanding role in the treatment of CVD. With increasingly aggressive lipid lowering guidelines and the upcoming patent expiration of several statins, statin use is likely to become even more ubiquitous. Despite the beneficial effects of statins, legitimate concerns about side effects remain. The most common side effect, myopathy, leapt
into the public eye with the recall of cerivastatin and the death of many patients. Concerns about side effects may lead physicians and patients to reject potentially life-saving treatment. The benefit/risk relationship of statin treatment is overwhelmingly positive and the benefits of statins should be publicized [239]. A better understanding of SIM could have an impact on the treatment of millions. With the development of informative biomarkers (mtDNA or others), as well as a better understanding of SIM pathogenesis and risk-factors, patients could be switched to lower statin dosages before symptoms arise or high risk patients could be identified before treatment. Additionally a better public understanding of both the benefits of statins and the risks of SIM could help reduce the approximately 60% of patients who would benefit from, but are not receiving, statin treatment [110].

The goal of this thesis was to examine the relationship(s) between SIM, statins and mtDNA. Two studies were undertaken; the first study was an in vitro exposure of cultured human rhabdomyosarcomal cells to atorvastatin and simvastatin. Cells were exposed to statins for over two months and no definite changes were observed in mtDNA quantity or quality.

The next study was a retrospective examination of banked clinical skeletal muscle biopsies. MtDNA depletion was associated with SIM while controlling for several variables including statin type and biopsy site. This study demonstrated that, the skeletal muscle mtDNA depletion previously observed in asymptomatic statin patients can also be measured amongst SIM patients. It remains to be seen whether mtDNA depletion is related to SIM or merely a by-product of statin treatment. If the degree of statin induced skeletal muscle mtDNA depletion is indicative of SIM, then mtDNA in skeletal muscle or other tissues might function as a clinical marker of statin mitochondrial toxicity.

This thesis has attempted to address the effect that statin treatment can have on mitochondrial DNA. The mitochondrion, far from a simple ATP factory, is a complex organelle which may hold the key to cellular dysfunction in the context of drug toxicity as well as other
areas such as aging. Hopefully this research may lead to future enquiry into the relationships between mtDNA and statin treatment.
5 References


49. Downs, J.R., et al., *Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS*. Air

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# 6 Appendix: Ethics Review Board Approval Certificate

The University of British Columbia  
Office of Research Services  
Clinical Research Ethics Board – Room 210, 828 West 10th Avenue,  
Vancouver, BC V5Z 1L8

**ETHICS CERTIFICATE OF EXPEDITED APPROVAL: AMENDMENT**

**PRINCIPAL INVESTIGATOR:** Helene Cote  
**DEPARTMENT:** UBC/Medicine, Faculty of Pathology & Laboratory Medicine  
**UBC CREB NUMBER:** N08-70358

**INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:**

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**Other locations where the research will be conducted:**

Referring physicians may be contacted for relevant information that cannot be found in the pathology reports.

**CO-INVESTIGATOR(S):**

Henry Stringer  
John Maguire  
Jiri Frohlich  
Helene Cote

**SPONSORING AGENCIES:**

- Unfunded Research - "Mitochondrial DNA Levels in Skeletal Muscle Biopsies of Patients with Statin-Induced Myopathy"

**PROJECT TITLE:**

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

**REMARKS:**

The current UBC CREB approval for this study expires: July 11, 2009

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**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 amendment(s) for the above-named project has been reviewed by the Chair of the University of British Columbia Clinical Research Ethics Board and the accompanying documentation was found to be acceptable on ethical grounds for research involving human subjects.

**Approval of the Clinical Research Ethics Board by:**

Dr. Stephen Hopton Cann,  
Associate Chair