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Prevalence of *SLCO1B1* single nucleotide variations, and their association with statin intolerance in hypercholesterolaemic patients in Gauteng, South Africa

Dissertation submitted in fulfillment of the requirements for the degree,
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Candidate
René de Beer
15048323
Pharmacology
Faculty of Health Sciences
University of Pretoria

Supervisor
Prof Prashilla Soma
Anatomy
Faculty of Health Sciences
University of Pretoria
prashilla.soma@up.ac.za

Co-supervisor
Prof Kim Outhoff
Pharmacology
Faculty of Health Sciences
University of Pretoria
kim.outhoff@up.ac.za

Co-supervisor
Prof Alisa Phulukdaree
Physiology
Faculty of Health Sciences
University of Pretoria
alisa.phulukdaree@up.ac.za

Summary

Statins, the standard treatment for hypercholesterolaemia, have been associated with side effects, including statin intolerance. This study determined the prevalence of *SLCO1B1* single nucleotide variations (SNVs) and possible associations between *SLCO1B1* SNVs, statin intolerance and creatine kinase (CK) in hypercholesterolemic patients on statin therapy.

One hundred and eighty one healthy controls and 100 hypercholesterolaemic patients receiving either simvastatin or atorvastatin were recruited. A questionnaire was used to assess the risk of statin intolerance. Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) was used to identify the presence of *SLCO1B1* SNVs (*rs4149056, rs2306283* and *rs4363657*) and enzyme-linked immunosorbent assay (ELISA) was used to quantify serum creatine kinase (CK) levels.

Of the 100 hypercholesterolaemic patients, 15% presented with high risk, 49% with moderate risk and 36% with low risk to statin intolerance. The prevalence of the *rs4149056* variant was 16% for the control group and 20% for the test group, while the *rs2306283* variant present in 31.5% of the control group compared to only 10.5% in the test group. The prevalence of *rs4363657* variant was similar in each group.

A comparison of genotype frequencies based on calculated statin intolerance risk, i.e. low risk versus moderate to high risk, showed no significant association between any of the SNVs and the either low risk or moderate to high risk statin intolerant presentation. CK levels in patients on simvastatin were significantly higher compared to patients on atorvastatin.

The prevalence of the *SLCO1B1* SNVs in this population is a novel finding. No association between the presence of any one of the SNVs and the statin intolerance severity risk score or CK elevation was found.

Keywords:

SLCO1B1, Statins, Atorvastatin, Simvastatin, Hypercholesterolemia, Statin Intolerance, Single Nucleotide Variations, Muscle pain, Myalgia, Statin-induced

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"Here's to those who inspire you and don't even know it." —Anonymous

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Declaration of Originality

Full names of student: René de Beer

Student number: 15048323

Topic of work: Prevalence of SLCO1B1 single nucleotide variations, and their association with statin intolerance in hypercholesterolaemic patients in Gauteng, South Africa

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Abstract

Background: Hypercholesterolaemia, defined by high circulating levels of total cholesterol and/or low-density lipoprotein (LDL), contributes significantly to the increasing burden of cardiovascular morbidity and mortality in South Africa and globally. Effectively reducing levels of LDL, decreases cardiovascular risk. Statins, inhibitors of the rate limiting enzyme of cholesterol synthesis, HMG CoA reductase, are the standard therapy for hypercholesterolaemia. Statins, however, have been associated with side effects, especially statin intolerance. Statin intolerance refers to all statin-induced muscle-related adverse effects and presents in individuals depending on the dose, efficient uptake and metabolism of the drug. The wide range and severity of symptoms may be attributed to individual genetic variation in the drug transporter (OATP) and metabolizing cytochrome P450 (CYP450) isoenzymes. The aim of this study was to determine the prevalence of *SLCO1B1* single nucleotide variations (SNVs) in a randomly selected sample of the general population in Gauteng, South Africa, and possible associations between *SLCO1B1* SNVs, statin intolerance and creatine kinase (a muscle injury biomarker) in hypercholesterolemic patients on statin therapy.

Methodology: Two hundred and eighty-one participants between the ages of 19 and 75 were included in the study. Of these, 181 were healthy volunteers (control group) and 100 were hypercholesterolaemic patients (test group) receiving either simvastatin (71%) or atorvastatin (29%). A questionnaire adapted from the American College of Cardiology's (ACC) Statin Intolerance Application was used to assess the risk of statin intolerance for each patient. Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) was used to identify the presence of three *SLCO1B1* SNVs (*rs4149056, rs2306283* and *rs4363657*) and enzyme-linked immunosorbent assay (ELISA) was used to quantify serum creatine kinase (CK) levels.

Results: Of the 100 hypercholesterolaemic patients included in the study, 15% presented with high risk for statin intolerance, 49% presented with moderate risk to stain intolerance and 36% present with low risk to statin intolerance. The genotype

distribution of the test and control group conformed with the Hardy-Weinberg equilibrium (HWE), except for *SCLO1B1 rs4149056* and *rs2306283* in the control group. The prevalence of the *rs4149056* variant was 16% for the control group and 20% for the test group. (Odds ratio (OR)=1.324; 95% confidence interval (CI)=0.8430 to 2.078; *p*=0.2405). The *rs2306283* was present in 31.5% of the control group compared to only 10.5% in the test group (OR=0,2552 95% CI=0.1542 to 0.4223, *p*< 0.0001). The prevalence of the *rs4363657* variant was similar in both the test and control group. (OR=1.345, 95% CI=0.8492 to 2.129, *p*=0.2380).

A comparison of genotype frequencies based on calculated statin intolerance risk (i.e. low risk versus moderate to high risk) showed no significant association between any of the SNVs and the either low risk or moderate to high risk statin intolerant presentation, (*rs4149056*, OR=0.7857, 95% CI=0.2115 to 2.919, relative risk (RR)= 0.8800, 95% CI= 0.4433 to 1.747, p=0.7496, *rs2306283*, OR=0.4911, 95% CI=0.1234 to 1.954, RR=0.9659, 95% CI 0.4888 to 1.909, p=0.4877 and *rs4363657*, OR= 0.9375, 95% CI= 0.2634 to 3.3337, RR=0.6984, 95% CI=0.3609 to 1.352, *p*=1.0000). CK levels in patients on simvastatin were significantly higher compared to patients on atorvastatin (*p*=0.0418). No association between the presence of any one SNVs and the statin intolerance severity risk score or CK elevation was found.

Conclusion: This is the first study to report on the incidence of all three SNVs in a South African population. In line with other research, this study also showed that patients on simvastatin therapy have a higher risk of developing statin intolerance compared to patients on atorvastatin therapy. These findings will allow for a better understanding of variation in drug uptake between patients thereby providing a more personalized approach to statin therapy.

Contents

List of Figures

List of Tables

List of Abbreviations

ABC	ATP-Binding Cassette
ACC	American College of Cardiology
AE	Adverse events
AHA	American Heart Association
ALT	Alanine transaminase
aPKC	Atypical Protein Kinase C
APOB	Apolipoprotein B
AST	Aspartate transaminase
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
Bp	Base pairs
BCRP	Breast Cancer Resistant Protein
BMI	Body Mass Index
BSEP	Bile Salt Export Pump
CCWG	Canadian Consensus Working Group
CHD	Coronary heart disease
CK	Creatine Kinase
Cmax	Maximum Concentration
CVD	Cardiovascular Disease
CYP	Cytochrome P450
DHB	6, 7- dihydroxybergamottin
DNA	Deoxyribonucleic acid
ER	Endoplasmic Reticulum
Farnesyl-PP	Farnesyl pyrophosphate
Geranyl-PP	Geranyl Pyrophosphate
GGPP	Geranyl-Geranyl-PP
GG-Rab	Geranyl geranylated GTPase
GLUT4	Glucose Transporter 4
GWAS	Genome Wide Association Study
HWE	Hardy-Weinberg equilibrium

HDL	High Density Lipoprotein
HMG-CoA	3-hydroxy-3-methyl-glutaryl Coenzyme A
IDL	Intermediate Density Lipoprotein
ILEP	International Lipid Expert Panel
IRS-1	Insulin Receptor Substrate
LDL	Low-Density Lipoprotein
LDL-R	Low-Density Lipoprotein Receptor
LDLRAP1	Low-density lipoprotein receptor adapter protein 1
MMP	Mitochondrial Membrane Potential
MPT	Mitochondrial Permeability Transition
MDR	Multi-drug Resistant
MRP	Multi-drug Resistant Protein
NLA	National Lipid Association
NE	New England
NOD	New Onset Diabetes Mellitus
NTCP	Sodium ion/taurocholate cotransporter
OATP1B1	Organic Anion Transporter Polypeptide 1B1
OCT	Organic Cation Transporter
PCR	Polymerase Chain Reaction
PCSK9	Proprotein Convertase Subtilisin-Kexin Type 9
PDK-1	Phosphoinositide-dependent kinase-1
P-gp	P-glycoprotein
PI3K	Phosphatidylinositol 3’ kinase
PKB/ Akt	Protein Kinase B
PM	Plasma Membrane
SLCO1B1	Solute Carrier Organic Anion Transporter Family Member 1B1
SNV	Single Nucleotide Variation
T2DM	Type 2 Diabetes Mellitus
Tmax	Time taken to reach the maximum concentration
UGT	Uridine 5'-diphospho-glucuronosyltransferase
UNL	Upper Normal Limit
VD	Volume of Distribution
VLDL	Very Low-Density Lipoprotein

$t\frac{1}{2}$	Half-life

Chapter 1: Introduction and literature review

Research on the treatment of hypercholesterolaemia has become one of the most important topics in modern medicine, due to the high burden elevated low-density lipoprotein (LDL) levels pose on cardiovascular risk. Increased LDL levels increase the risk for cardiovascular disease (CVD) substantially and extensive research shows that reducing LDL decreases this risk significantly, both in patients presenting with hypercholesterolaemia and in those with comparatively normal levels of LDL. (1, 2)

Bile-acid binding resins, fibrates, nicotinic acid, cholesterol absorption inhibitors, and hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors (3) are the current drug classes used to lower LDL levels. HMG-CoA reductase inhibitors, commonly known as statins, are the most well-known and universally prescribed lipid-lowering therapy. (4, 5)

At tertiary healthcare institutions in South Africa, the HMG-CoA reductase inhibitors, simvastatin and atorvastatin, are prescribed most frequently. They are used in combination with dietary and lifestyle changes in order to ensure effective lowering of LDL and elevation of high-density lipoprotein (HDL). When prescribing a statin, the question of whether or not the benefit outweighs the risk sways towards the beneficial. (6) However, statins may be associated with side effects, especially statin intolerance, a term used to encompass all statin-induced muscle-related adverse effects. (7, 8) Various associations and authorities differ in their definition of statin intolerance. The National Lipid Association (NLA), International Lipid Expert Panel (ILEP) and the Canadian Consensus Working Group (CCWG) agree that statin intolerance is a clinical syndrome, which manifests as the inability to tolerate at least two statins, (one of which is at its lowest daily dose), due to symptoms and signs related to statin treatment, e.g. increase in laboratory markers and / or myopathy. These symptoms and signs usually resolve upon withdrawal of statin therapy. (9-12)

Various factors, such as age, gender, drug-drug interactions, and genetic variations in genes encoding for drug transporters are implicated in the pathogenesis of statin induced myopathy. (13) Genetic variations, such as single nucleotide variations (SNVs), may affect the pharmacokinetics (absorption, distribution, metabolism, and

excretion) and hence plasma concentrations and total exposure to a drug. (14-16) Muscle related adverse events associated with statins may be due to inadequate metabolism and clearance of statins resulting in increased plasma concentrations and / or prolonged exposure, leading to toxicity. It is hypothesized that variations (*rs4149056*, *rs2306283* and *rs4363657*) in the gene which encodes for the hepatic drug transporter, Organic Anion Transporter Polypeptide 1B1 (OATP1B1), *Solute Carrier Organic Anion Transporter Family Member 1B1* (*SLCO1B1)*, diminishes statin uptake and metabolism and may be associated with statin intolerance.

This review highlights the pharmacology of statins, different mechanisms that may explain the symptoms associated with statin intolerance and genetic variations that may be associated with increased plasma concentrations of statins leading to toxicity.

1.1 Physiology of cholesterol

The human body requires cholesterol to produce and maintain cell membranes, to synthesize steroid hormones and to synthesize products that aid in fat metabolism. Cholesterol is mostly synthesized *de novo* by liver hepatocytes but is also obtained from dietary intake. Once absorbed, or manufactured, lipophilic cholesterol enters the circulation for distribution where it relies on specialized transporter molecules because plasma is hydrophilic. These transporter lipoproteins are a combination of lipids, most commonly triacylglycerol and cholesteryl ester, and proteins. (17) Lipoproteins, which differ in density, can either form building blocks of cellular plasma membranes for structural integrity or attach to outer plasma membranes. Standard nomenclature depends on the density of the lipoprotein, which is also a reflection of lipoprotein function. (Table 1; Table 2) (18)

Table 1: Functions of lipoproteins (18)	
Lipoprotein	**Function**
Chylomicrons	Transport lipids from the liver to peripheral tissue
VLDL	Transport lipids from the intestinal tract to peripheral tissue
IDL	As lipids are removed from the liver, an alteration in the density of lipoprotein occurs, resulting in the transformation of VLDL to IDL
LDL	This is the ultimate stage of transformation of VLDL; LDL transports lipids from the liver
HDL	Important for the transport of cholesterol back to the liver during reverse cholesterol transport. Contains two subclasses; higher-density lipoprotein 3 (HDL3) and high-density lipoprotein 2 (HDL2), which is less dense and more lipid-filled
VLDL Very low-density lipoprotein, **IDL** Intermediate density lipoprotein, **LDL** Low-density lipoprotein, **HDL** High-density lipoprotein	

Table 2: Density and composition of lipoproteins (18)					
Lipoprotein Fraction	**Density (g/mL)**	**Cholesterol (%)**	**Triglyceride (%)**	**Total lipid (%)**	**Protein (%)**
Chylomicron	<0.960	4	88	8-99	1
VLDL	0.960 – 1.006	23	56	90-93	8
IDL	1.006-1.019	43	29	89	11
LDL	1.019-1.063	58	13	79	21
HDL					
HDL2	1.063-1.125	41	16	67	33
HDL3	1.125-1.210	35	13	43	57

1.2 Pathophysiology – hypercholesterolaemia

Elevated levels of plasma cholesterol, or hypercholesterolaemia, is caused by excessive dietary intake, hepatic overproduction and/or inadequate usage and clearance of LDL from blood and lymphatic circulations. (19) Hypercholesterolaemia is defined as a total cholesterol of > 5.18 mmol/L, LDL of > 3.37 mmol/L and / or an HDL of < 0.91 mmol/L. (Table 3) (20)

Table 3: Normal values of lipogram parameters (20)	
Lipid	**Normal range**
Total cholesterol	2.59 – 5.18 mmol/L
Triglyceride	0.57-1.70 mmol/L
HDL - Cholesterol	0.91 – 1.55 mmol/L
Non-HDL Cholesterol	2.07-4.14 mmol/L
LDL – Cholesterol	1.30 – 3.37 mmol/L
Apolipoprotein Al	1.10 – 2.05 g/L
Apolipoprotein B	0.55 – 1.05 g/L
ApoB/ApoAl Ratio	0.05 – 2.00
Lipoprotein (a)	0.01 – 0.30 g/L

Hypercholesterolaemia is most commonly caused by a combination of lifestyle, environmental and genetic factors. Gene mutations of the LDL-receptor (LDL-R), Apolipoprotein B (APO-B), Low-density lipoprotein receptor adapter protein 1 (LDLRAP1) and Proprotein Convertase Subtilisin-Kexin Type 9 (PCSK9) (21) may cause severe hypercholesterolaemia and may all be hereditary. (5, 8)

The most common and well-known hereditary form is familial hypercholesterolaemia which is due to a mutation in the LDL-R gene, which encodes low-density lipoprotein receptor (LDL-R) proteins. Low-density lipoprotein receptors are most commonly found on hepatocytes. High cholesterol-containing LDL binds to these receptors and is subsequently removed from the circulation. Mutations of LDL-R genes result in decreased transcription of these LDL-R proteins, ultimately causing abnormally high levels of plasma LDL. (5) Mutations in the APO-B, LDLRAP1 or PCSK9 genes are the least common forms of familial hypercholesterolemia. (5, 8) The familial defective apolipoprotein B-100 (APO-B) mutation affects the LDL-R binding domain of APO-B, while LDLRAP1 is an autosomal recessive cause of hypercholesterolemia. Meanwhile, the gain of function PCSK9 mutation leads to an increase in the degradation of LDL-R, and thus hypercholesterolaemia.

Unhealthy lifestyle choices that may lead to hypercholesterolemia include dietary, lack of exercise and cigarette or tobacco smoking. Additional important factors that give rise to or contribute to hypercholesterolemia are gender, age, obesity, and type 2

diabetes mellitus (T2DM). (21) When assessing lifestyle factors that play a role in hypercholesterolemia, the most common is poor diet. Increased saturated fat and high-fat diets increase cholesterol levels. Dietary fats are assembled in the epithelial cells into lipid complexes known as chylomicrons which consist of cholesterol, phospholipids, apolipoproteins, and triglycerides. Once these chylomicrons enter the circulation lipoprotein lipases cause chylomicrons to release triglycerides, which is broken down into fatty acids and glycerol, in adipose deposits and muscle, and the chylomicron remnants are taken up by the liver. An increase in dietary intake of fats results in an increase in chylomicron remnants taken up by the liver, which increases products that aid in the production of lipoproteins. These lipoproteins are released into the circulation from the liver and ultimately result in abnormally high levels of lipoproteins in the blood. (22)

After the transformation of VLDL to LDL, by the removal of triglycerides in the circulation, LDL is transported to the peripheral tissue where it has a tendency to clump and cause plaque formation. (18) This reaction is opposed by HDL, which removes cholesterol from the peripheral tissue, by converting cholesterol to cholesterol esters using lecithin–cholesterol acyltransferase (LCAT) activity, and transports it back to the liver to be metabolized and excreted. (17) However, an imbalance of HDL and LDL (i.e. low levels of HDL and high levels of LDL) results in abnormal buildup of LDL in the periphery and subsequent hypercholesterolemia. (17)

Atherosclerosis is initiated when LDL infiltrates the subendothelium of arteries, in the tunica intima. (22) Apolipoprotein B on the outside layer of LDL is oxidized due to an increase in free radicals and oxidant species, resulting in oxidized LDL. (23) Oxidized LDL causes further increase of reactive oxidant species by the activation of the innate- and complement immune systems, which includes the recruitment of macrophages. As seen in Figure 1, macrophages phagocytize these LDLs and form foam cells. Activation of T cells causes the release of cytokines, which eventually cause smooth muscles to proliferate and migrate from the intima to the endothelium. Due to the overwhelming influence of growth factors, these smooth muscle cells produce collagen, which subsequently aids in foam cell production, forming a fatty streak under the endothelium. (22) Increased LDL uptake by macrophages leads to lipotoxicity of

the endoplasmic reticulum, which eventually results in macrophage apoptosis and plaque necrosis. Necrotized plaque leads to further inflammation and chemotaxis of neutrophils. This results in a continuous cycle of inflammation and necrosis as shown in Figure 1. (22, 23)

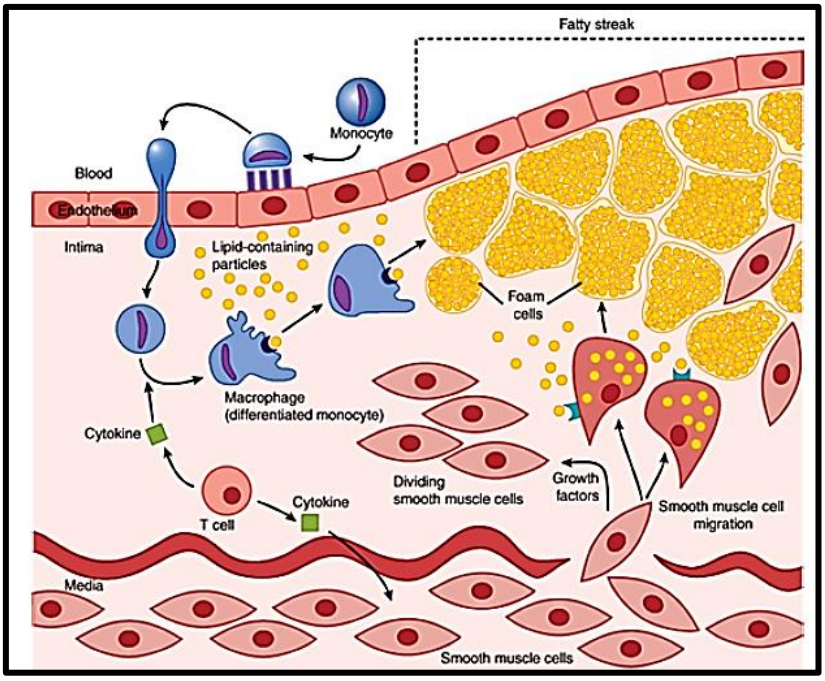

Figure 1: Fatty streak production in atherosclerosis (22)

Increased cholesterol levels are thus primarily associated with atherosclerosis, (3, 7) leading to several important non-communicable diseases, notably coronary heart disease (CHD), type 2 diabetes mellitus (T2DM), peripheral arterial disease and hypertension, all of which are associated with substantially increased morbidity, mortality and treatment costs. (4-6)

1.3 Treatment of hypercholesterolemia

Treatment of hypercholesterolemia includes a healthy diet, physical activity, exercise, and drug therapy. (3) Various drugs are available for the treatment of hypercholesterolemia. These include bile-acid binding resins, fibrates, nicotinic acid, cholesterol absorption inhibitors, and Hydroxymethylglutaryl Coenzyme A (HMG-CoA) reductase inhibitors, also known as statins. (3) Of these marketed drugs, statins are the most frequently prescribed due to their gold-standard efficacy and cost effectiveness. (24) Simvastatin and atorvastatin are currently the two top-selling statins in the public sector. (24, 25) Both of these second generation statins

significantly reduce elevated LDL by an average of 30%, (26) and have been shown to lower mortality of CHD by 28%, cardiovascular death by 25% and all-cause mortality by 18%. (27)

1.3.1 Statins mechanism of action

Statins work by competitively inhibiting HMG-CoA reductase, an enzyme that plays an essential role in the *de novo* synthesis of cholesterol as shown in Figure 2. In the absence of statins, HMG-CoA is converted to mevalonate which is converted to farnesyl pyrophosphate (Farnesyl-PP). Farnesyl-PP yields three products, one of which is cholesterol. However, in the presence of statins, HMG-CoA reductase is inhibited and cannot catalyze the conversion of HMG-CoA to mevalonate, which is the rate-limiting step in cholesterol biosynthesis. Statins sterically inhibit substrates from binding to the enzyme. To accommodate the rigid hydrophobic rings of statins, the substrate-binding sites on the enzyme undergo conformational changes. This competitive inhibition results in decreased cholesterol synthesis in hepatocytes and subsequently decreased LDL levels. (4) Decreased intracellular LDL induces membranous expression of hepatocyte LDL-R, which facilitates extraction of plasma LDL, thus further reducing circulating LDL concentrations. Statins are also effective in lowering triglyceride and increasing HDL concentrations. (4)

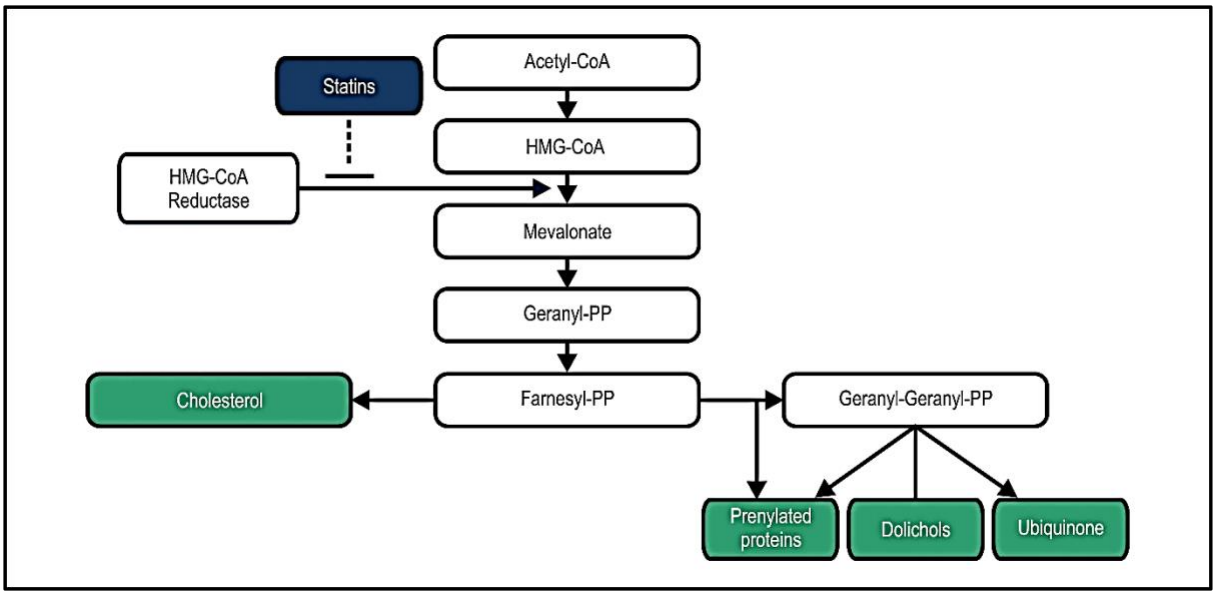

Figure 2: Mechanism of action of statins (3)

HMG-CoA reductase Hydroxymethylglutaryl coenzyme A reductase, **Geranyl–PP** Geranyl pyrophosphate, **Farnesyl–PP** Farnesyl pyrophosphate, **Geranyl–Geranyl–PP** Geranyl – Geranyl pyrophosphate

1.3.2 Pharmacokinetics of statins

Statins are complex agents. Simvastatin is a lactone prodrug (16) and has to be hydrolyzed by carboxyesterases (17) *in vivo* to its active metabolite, β-hydroxy-acid, in order to achieve pharmacological activity. Atorvastatin acid is pharmacologically active, and its two metabolites, 2-hydroxy- and 4-hydroxy-atorvastatin acid, are pharmacologically active too. (18) However, atorvastatin and its acid metabolites appear to exist in equilibrium with their inactive lactone forms *in-vivo*, which introduces several layers of complexity when assessing clinically relevant pharmacokinetic parameters. (28)

The pharmacokinetics of parent statins follow the usual “ADME” processes: After oral administration, they are absorbed in the small and large intestine and distributed in the plasma. This is followed by their hepatic uptake utilizing the important transporter, OATP1B1, which is located on the sinusoidal surface of hepatocytes and is responsible for the hepatic uptake of many endogenous and exogenous substrates. (29) Once metabolized in the liver, statins are eliminated, either via the biliary tract or directly from the systemic circulation, into the small intestine.

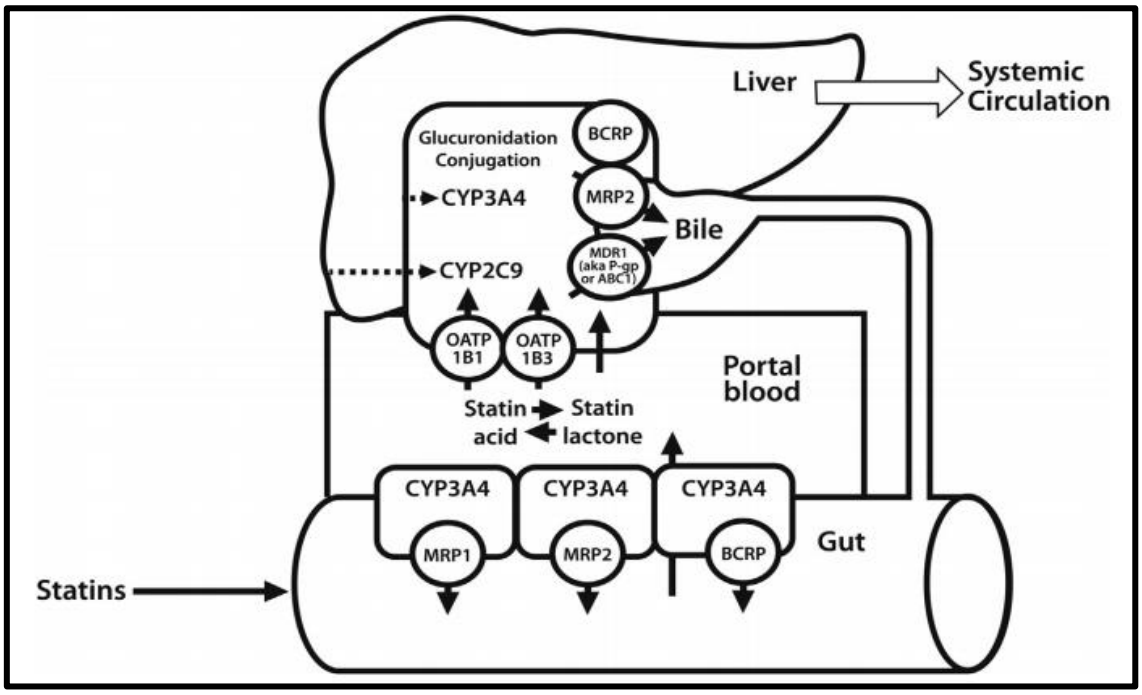

Figure 3: Metabolic pathway of statins (30) Permission for the re-use of image was obtained from Elsevier. License number: 4938311467201

OATP organic anion transporter protein, **BCRP** Breast cancer resistant protein, **MRP 1 & 2** Multi-drug resistant associated protein 2, **MDR1** Multi-drug resistant/ **P-gp** P-glycoprotein/ **ABC1** ATP-binding cassette, **CYP** Cytochrome P450 isoenzymes, e.g. CYP3A4, CYP2C9

Absorption

Both atorvastatin and simvastatin are administered orally with gastrointestinal absorption percentages ranging from 30–80% (31), which is higher for simvastatin (60%-80%) compared to atorvastatin. Rapid absorption occurs from the small to the large intestine, due to the functional specialization of the cells lining the intestinal mucosa, as well as the high solubility and permeability of the parent compounds, as shown in Figure 3. (30, 32, 33) The rate and extent of atorvastatin absorption is decreased by food intake, because of physiological and physicochemical interactions. Atorvastatin is seen as a case 1 (high solubility, high permeability) drug according to the biopharmaceutic drug classes, which means that the rate of absorption of the drug is dependent on gastric emptying and there is no correlation with the dissolution rate. Thus, when administered to fasting patients, the absorption is rapid due to atorvastatin's high solubility and high intestinal permeability. (28, 34) which substantiates the recommendation that most statins be taken at night. (4)

Absorbed simvastatin or atorvastatin are partially metabolized by oxidation to their active metabolites by intestinal enterocyte CYP450 isoenzymes, in particular CYP3A4, which is the predominant catalyzing enzyme in the gut wall. This high gut wall extraction of simvastatin and atorvastatin may be aided by P-glycoprotein (p-gp), an efflux transporter that is extensively distributed along the epithelial lining of the small intestine. (30). From the gut wall, the remaining simvastatin or atorvastatin travel to the liver via the hepatic portal vein, where they undergo further first-pass metabolism by CYP450 enzymes.

This extensive presystemic metabolism in the gut and liver, results in substantially decreased parent drug concentrations reaching the systemic circulation. Due partly to this large first-pass effect, the maximum systemic plasma concentration (Cmax) of 20 mg simvastatin is only 10–34 ng/mL compared to the 27–66 ng/mL of 20 mg atorvastatin, giving low bioavailabilities of < 5% and 12%, respectively. (4, 31) It takes approximately 2-4 hours (Tmax) to reach these maximum plasma concentrations (Cmax) (16) (Table 4)

The systemic plasma concentrations of simvastatin lactone and atorvastatin acid are thus relatively low. However, the extensive presystemic metabolism of these statins, particularly in the liver, produces pharmacologically active metabolites. These may exert therapeutic effects preventing *de novo* hepatocyte synthesis of cholesterol, be excreted via bile, or may enter the systemic circulation via the three hepatic veins and inferior vena cava, from where they may be distributed to many tissues, including the liver, to exert both therapeutic and adverse effects, provided they are in acid (rather than lactone) form. The relatively low systemic plasma concentrations of atorvastatin acid and simvastatin lactone may thus prove inconsequential as liver concentrations of statins and their active metabolites (i.e. total active HMG CO-A reductase inhibitors) might better correlate with pharmacological effects on plasma LDL as the liver is their primary site of action.

Table 4: Basic pharmacokinetic profile of simvastatin and atorvastatin (4, 31, 35)		
	Simvastatin	**Atorvastatin**
Solubility	Lipophilic	Lipophilic
IC_{50} HMG-CoA reductase (nM)	1 – 2 (active metabolite)	1.16
Oral absorption (%)(31)	60 – 85 food has no effect	30 decreased by food intake
Bioavailability (%)	< 5	12
Cmax (ng/mL)	10 – 34	27 – 66
Tmax (hours) (35)	2-4	2-4
Liver extraction (%)	≥ 80	70
Protein binding (%)	> 95	> 98
Half-life ($t\frac{1}{2}$) (h)	2 - 5	7 - 20
Volume of distribution (VD) (L/kg)	-	~5.4
Metabolism (CYP450)	CYP 3A4	CYP 3A4
Metabolites	Active	Active
Hepatic extraction (%)	78 - 87	>70
Transporter involved	OATP1B1	OAT1B1
Lipophilicity (*C* log *P*) (octanol/water)	4.68 (47.860)	4.6 (1.482)
Standard daily dose (mg)	10 – 40	10 – 80
Clearance (L/hr/kg)	0.45	0.25

Distribution

After statins and their metabolites enter the systemic circulation where they are bound largely to plasma proteins, they are distributed to body tissues. The volume of distribution (Vd) is one of the most frequently used variables when accessing the distribution and tissue binding of a specific drug. The volume of distribution is determined by variables such as protein binding, tissue binding and membrane permeability. (32) It is a measure of how much statin is tissue-bound, rather than in circulation. Drugs with very high volumes of distribution have much higher concentrations in extravascular tissue than in the vascular compartment. This is evident in the Vd seen with simvastatin and atorvastatin, respectively, of approximately

232.57 ± 132.54 L/kg (*232.57 L/kg x 70 kg (weight of the average person)= 16279.9 L)* (36) and 381 L/kg (*381 L/kg x 70 kg (weight of the average person)= 26670 L*)(determined after administration of 5 mg of drug as intravenous infusion). (28)

Distribution of statins is affected by the high percentage bound to plasma proteins, especially albumin. (32) Only free unbound drug is able to diffuse across membranes into the extravascular space or tissues, exert an effect, be metabolized or excreted. More than 90% of both atorvastatin and simvastatin are protein bound. Thus, concentrations of unbound, free, pharmacologically active drug are low. This contributes to their long elimination half-lives ($t\frac{1}{2}$) of 15 – 30 hours. (35)

Cell membranes consist of lipid bilayers which influence their permeability and hence the trans-cellular uptake of drugs. For drugs to move passively across both apical and basolateral membranes, they require specific physiochemical properties. These include lipophilicity, charge, size, and hydrogen bond potential. (33) Since simvastatin and atorvastatin are both lipophilic, and have favourable charge, size and hydrogen bond potential, they are theoretically able to diffuse across membranes with ease, leaving the vascular compartment in favour of tissues, and accounting for their relatively high volume of distribution. (28, 37)

Once statins have entered the tissues, they are able to exert their lipid lowering effects. Their efficacy in lowering cholesterol is related to inhibiting hepatic cholesterol synthesis, yet theoretically, this may occur in any other cell that synthesizes cholesterol due to their ability to inhibit the production of mevalonate which is the main component in the production of molecules such as geranyl – geranyl pyrophosphate (GGPP), the precursor for GG-Rab which plays a role in many steps of membrane trafficking, vesicle movement, vesicle formation and membrane fusion. (38, 39)

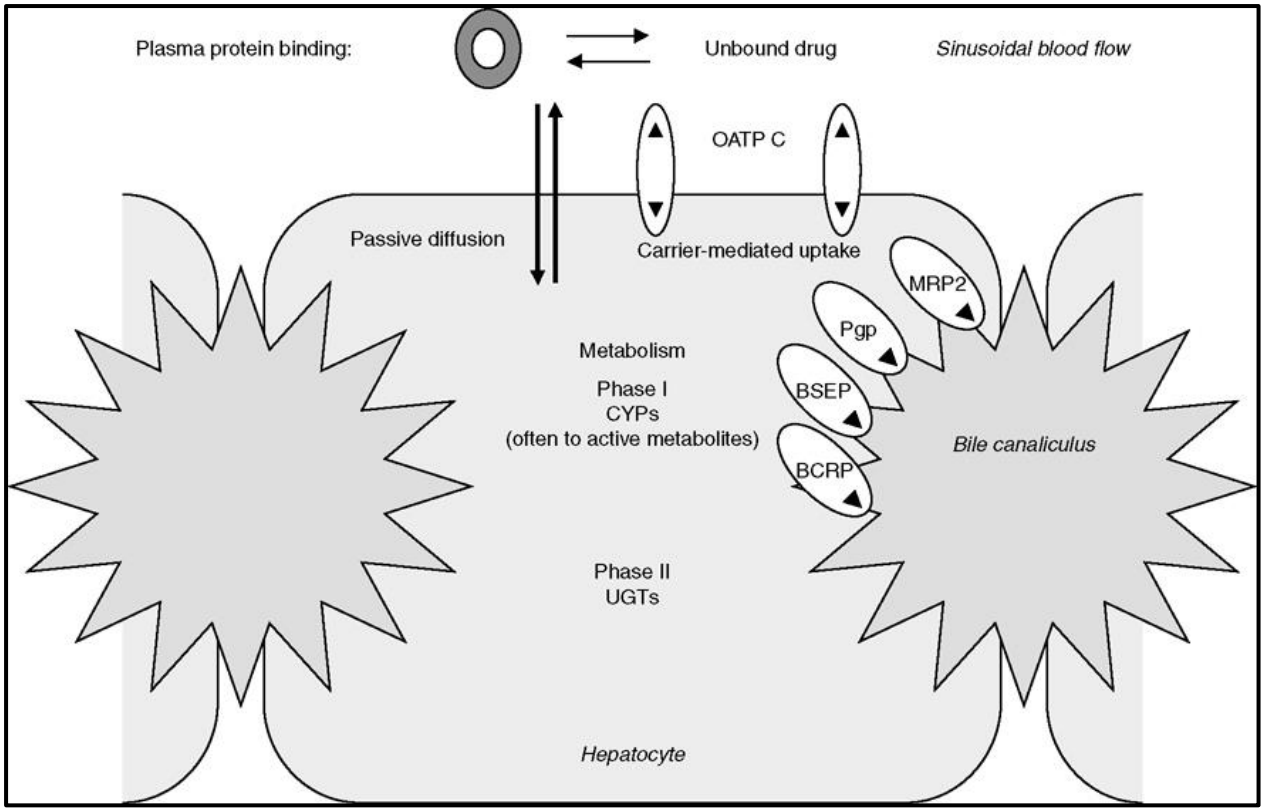

Figure 4: Hepatic extraction of statins and their active metabolites (28) Permission for the re-use of image was obtained from Springer Nature. License number: 4938950088134

BCRP breast cancer resistance protein; **BSEP** bile salt export pump; **MRP2** multidrug resistant protein 2; **Pgp** P-glycoprotein

Hepatic extraction of statins and their active metabolites is therefore critical, not only for their efficacy, but also for their metabolism, either to active metabolites or to inactive excretable byproducts. Hepatocellular uptake mechanisms include both passive diffusion as well as carrier-mediated uptake. Two carriers are important: OATP 1B1, located on sinusoidal surfaces of hepatocytes, aids in the hepatic uptake of statins, initially from the portal circulation, and subsequently from the systemic circulation. (30) The multi-specific OATP C carrier is capable of bidirectional transport across the sinusoidal liver membrane. (28)

Metabolism

Statins are mainly metabolized in the liver by the CYP450 enzymes (phase I metabolism (30), specifically the CYP3A4 isoenzyme (Figure 4), which is responsible for the metabolism of most drugs in humans. (4, 32) CYP3A4 metabolizes atorvastatin

and simvastatin into various active metabolites, which include 2-hydroxy- and 4-hydroxy-atorvastatin acid, and the simvastatin-derived *b*-hydroxy acid and its 6¢-hydroxy, 6¢-hydroxymethyl and 6¢-exomethylene derivatives. (4) These may all undergo further phase II metabolism by uridinediphosphoglucuronyltransferases (UGTs) to inactive compounds that are excreted. (4)

Excretion

The major (75% - 85%) route of elimination for statins is via the bile. Statins and their metabolites may enter the bile canaliculi either directly by diffusion or by utilizing the unidirectional efflux transporters at the bile canalicular membrane, which include breast cancer resistance protein (BCRP), bile salt export pump (BSEP), multidrug resistant protein 2 (MRP2) and P-glycoprotein (Pgp). (Figure 4). (4) The remaining water-soluble metabolites are excreted by the kidneys via the urine. (32)

1.3.3 Statin adverse effects

Statins are normally regarded as safe and well-tolerated. In some cases, adverse effects such as nausea, vomiting, diarrhea, abdominal pain, skin rash, cognitive decline (which includes memory loss and confusion) have been reported. These usually disappear with discontinuation of statin therapy or when changing to another statin regimen. (40) The short half-life ($t_{\frac{1}{2}}$) of most statins, with the exception of atorvastatin, means that there is a low likelihood of drug accumulation in the systemic circulation after continuous once daily dosing, thus decreasing the probability of severe toxic effects. (35) It is noteworthy that the elimination half-lives of statins appear to be relatively short, but that their pharmacological effects can last up to 24 hours. (32, 35)

Adverse event data as reported by pivotal statin safety and efficacy studies are presented in Table 5. Each of the studies included large sample sizes from multiple research centers. Despite significant adverse event reporting, the incidence of the adverse events reported was low. (41-47)

Table 5: General adverse effects associated with simvastatin and atorvastatin				
Author	**Statin**	**Total number of participants**	**Adverse event reported**	**Statin intolerance (Muscle-related adverse events)**
Scandinavian simvastatin survival study (41)	Simvastatin	4444 (Simvastatin = 2221; Placebo = 2223)	70 reported cases of non-fatal cancer in simvastatin group and 67 in placebo group.	1 case of rhabdomyolysis
Newman *et al*. (42)	Atorvastatin, simvastatin, fluvastatin, lovastatin, pravastatin	9416 (Retrospective analysis of pooled data from 44 completed statin trials.)	Arthritis (0.15% in patients who received atorvastatin and 0.21% in other statin groups), Cholecystitis and cholelithiasis (0.18% and 0.17% in atorvastatin group respectively) other hepatic adverse events included: abnormal liver function tests (0.06%), hepatitis (0.05%), cholestatic jaundice (0.02%), enzymatic abnormality (0.01%), and increases in ALT (0.02%) and AST (0.01%). Discontinuations considered related to hepatic and musculoskeletal adverse events were rare (<1%).	Myalgia (0.03% in atorvastatin and 0.02% in other statins)

Jones *et al*. (43) The CURVES study	Atorvastatin, fluvastatin, lovastatin, simvastatin, pravastatin	534 (518 completed the study)	Abdominal pain (3 patients (pts)), diarrhea (3 pts), depression (1 pt), dizziness (2 pt), hypertonia (2 pts), flatulence (2 pts), angina (1 pt *unlikely related to therapy), back pain (1 pt)	Myalgia (1 pt)
Ballantyne *et al*. (44) CHESS study	Simvastatin and atorvastatin	866 (149 patients failed to complete the study)	Diarrhea (simvastatin 1.3%; atorvastatin 3.0%), constipation (simvastatin 1.3%; atorvastatin 1.5%), nausea (simvastatin 1.8%; atorvastatin 0.9%),	Myalgia, arthralgia, muscular weakness, muscular cramp, musculoskeletal stiffness, and body ache (however no patients presented with a Creatinine Kinase (CK) >5)
Jones *et al*. (45) STELLAR study	Simvastatin, atorvastatin, rosuvastatin, pravastatin	2431 (94% completed the trial)	Pain (6%), pharyngitis (5%),	Myalgia (4% overall, highest number (>5%) of patients reporting myalgia were rosuvastatin 80 mg (7.3%), atorvastatin 20 mg (6.4%), atorvastatin 80 mg (5.4%), or pravastatin 20 mg (5.4%)), and headache (3%). Five patients (2 pts on atorvastatin 80 mg; 1 pt on atorvastatin 20 mg; 1 pt on

				simvastatin 40mg; and 1 pt on simvastatin 80 mg) had clinically important ALT elevations (>3 times the upper limit of normal). 3 patients (1 pt on rosuvastatin 80 mg; and 2 pts on simvastatin 10 mg) had a clinically important elevation (>10 x upper normal limit (UNL) of CK.
Heart protection study (46)	Simvastatin	20536 (Simvastatin = 10269 and placebo = 10267)	New cancer incidences (7.9% in simvastatin group, 803 in placebo group), non-melanoma skin cancer (2.4% in simvastatin group, 2% in placebo), elevated liver enzymes (0.5% in simvastatin group and 0.3% in placebo group).	Myopathy (32.9% in simvastatin group and 33.2% in placebo group)
Tragni *et al*. (47)	Simvastatin, atorvastatin, pravastatin, cerivastatin, fluvastatin	14120 (first time statin users)	Elevation AST (> 1 x UNL - 1.3%), (> 3 x UNL - 0.1%), elevation of ALT (> 1 x UNL - 3.7%), (> 3 x UNL – 0.1%), CK elevation (> 1 x UNL – 11.3%), (> 3 x UNL – 1.6%), Creatinine elevation (> 1 x UNL – 4.6%), (> 3 x UNL 0.4%), > 10 x UNL of CK and AST/ALT in only 3 and 4 patients, respectively.	

1.4 Statin intolerance

Despite the low incidence of general adverse events associated with statin treatment, according to post-marketing registries and observational studies, ~20% of patients may develop statin intolerance. (12, 48-52) Statin intolerance is a term that loosely embraces all skeletal muscle related adverse events. (7, 8, 12) Statins are likely to be the cause of reported symptoms, if symptoms such as muscle aches and pains, cramps, fatigue and weakness start within 3 months of treatment initiation and resolve once treatment is terminated. (9, 12)

Increased serum creatine kinase (CK) is an indirect biomarker of skeletal muscle damage. Common classifications of statin intolerance therefore rely on CK levels, and range from asymptomatic myopathy to myalgia, myopathy, myositis and rarely, potentially fatal rhabdomyolysis (Table 6). (53-55) Myositis implies that muscles are inflamed. Rhabdomyolysis occurs with rapid destruction of skeletal muscle resulting in release of myoglobin which may cause severe renal damage and failure.

Elevations of serum CK have also been classified into 3 major biochemical categories, namely (i) incipient myopathy (> 3 x ULN < 10) (ii) myopathy (> 10 x ULN < 50) and (iii) rhabdomyolysis (> 50 x ULN). (56, 57)

In some, symptoms of muscle fatigue and muscle weakness are not accompanied by elevated CK levels. (12) Tragni *et al*. (2007) (46) conducted a primary care study in ~14 120 first time statin users and reported that only 3 patients presented with a CK of > 10 x upper normal limit (UNL) and 4 patients with an Aspartate transaminase / Alanine transaminase (AST/ALT) of > 10 x UNL. (46) Similarly, Ballantyne *et al*. (2003) (43) reported on a large variety of muscle associated adverse events which resulted in discontinuation by 3 of 435 patients on simvastatin and in 15 of 464 patients on atorvastatin. These included myalgia, arthralgia, muscular weakness, muscular cramp, musculoskeletal stiffness and body ache. However, none of these symptoms were accompanied by a CK of > 5 x UNL. (43)

Table 6: Types of statin intolerance (53-55)	
Type	**Symptoms**
Asymptomatic myopathy	Elevation of CK levels but no muscle-related symptoms
Myalgia	Muscle-related symptoms (muscle tenderness, muscle weakness, cramps, fatigue) but normal CK levels
Myopathy	Muscle-related symptoms with a CK elevation of < 5 times the upper normal limit
Myositis	Severe muscle-related symptoms with a CK elevation of > 5 times the upper normal limit
Rhabdomyolysis	Severe muscle-related symptoms with a CK elevation of > 10 times the upper normal limit

Generally, muscle pain is mild and is easily managed by lowering the statin dose or by prescribing an alternative statin at an equivalent dose while monitoring CK levels to ensure that the myopathy does not worsen. (Table 7). (7)

Simvastatin is the most widely prescribed statin in the public sector as it is the most affordable. (58, 59) Despite the significant lipid-lowering seen with simvastatin therapy, it is also most frequently associated with statin intolerance; it has been reported that up to 11% of patients may present with statin intolerance, which may possibly be a reflection of its wide use, but may also be due to its lipophilicity, which facilitates its transmembranous transport. (54) When simvastatin is discontinued, e.g. if a patient experiences muscle pain while on simvastatin therapy, the alternative therapy is generally atorvastatin. (60) Partial intolerance implies that a lower dose or alternative statin is tolerated, while complete intolerance is severe and demands the discontinuation of all statins. (38, 82)

Table 7: Dose equivalence of simvastatin- and atorvastatin (60)		
%LDL reduction	**Simvastatin**	**Atorvastatin**
20-30%	10 mg	-
30-40%	20 mg	10 mg
< 40%	40 mg	20 mg

Mechanism of statin intolerance

The mechanism by which statins cause statin intolerance is still unknown due to the difficulty in culturing skeletal muscle cells. (61) There are numerous proposed mechanisms for this common side effect. Sakamoto *et al*. (2013) proposed that reduced production of the products yielded from the *de novo* synthesis pathway of cholesterol may result in myopathy and muscle weakness. (39)

Statins inhibit HMG-CoA reductase resulting in decreased *de novo* synthesis of cholesterol as well as other products yielded from the conversion of HMG-CoA to mevalonate, including geranyl-geranyl pyrophosphate (GGPP) as indicated in Figure 2. Geranyl-geranyl pyrophosphate is the precursor for GG-Rab, which plays a role in many steps of membrane trafficking including vesicle movement, vesicle formation and membrane fusion (Figure 5). (37) Reduced production of GGPP leads to decreased GG-Rab which reduces membrane trafficking and decreases mitochondrial membrane potential. This rapid decrease in the mitochondrial membrane potential could decrease Adenosine Triphosphate (ATP) production and promote eventual muscle weakness and necrosis. (62)

Hanai *et al*. (2007) proposed that the muscle-specific ubiquitin protein ligase, atrogin-1, which plays a key role in protein breakdown and muscle atrophy when expressed, could cause statin-induced myopathy, as shown in Figure 5. To test this theory, real-time PCR was used to determine atrogin-1 levels in 3 groups of patients, i.e. a control group, patients experiencing myopathy unrelated to statins, and patients treated with HMG-CoA reductase inhibitors presenting with myopathy. Significantly higher levels of atrogin-1 were found in patients treated with HMG-CoA reductase inhibitors presenting with muscle pain. This association could mean that increased levels of atrogin-1 may lead to increased muscle atrophy and thus myopathy and muscle weakness. These results were corroborated using cultured myotubules as well as zebra fish embryos. (5, 38, 63)

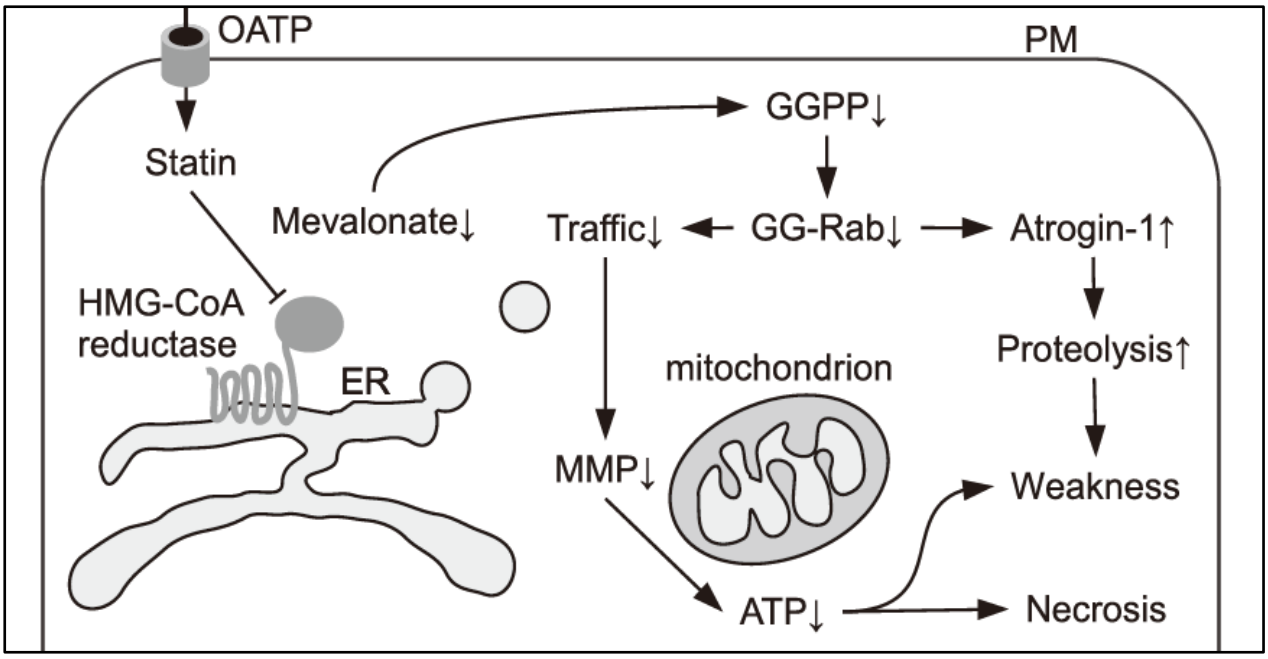

Figure 5: Schematic of the proposed mechanism of statin intolerance (39) Permission for the re-use of image was obtained. License number: 201030-017893 (Submission ID: 1082953)

ER Endoplasmic reticulum, **GG-Rab** Geranyl geranylated Rab GTPase, **MMP** Mitochondrial membrane potential, **GGPP** Geranyl – Geranyl pyrophosphate, **OATP** Organic Anion Transporting polypeptide, **PM** Plasma membrane, **ATP** Adenosine Triphosphate

The possible association between statins and their ability to induce mitochondrial-mediated apoptosis in muscle cells by depleting mevalonate ultimately leading to an increase in caspase-3 and caspase-9 activity (Figure 6), has gained more attention over the past few years. (62) Liao *et al*. (2002) (63) proposed that the depletion of mevalonate due to increased action of statins in the muscle cells leads to a rapid decrease in GGPP and farnesyl-PP. Decreased geranyl geranylation and farnesylation of membrane-associated proteins results in decreased activity. (63) Sacher *et al*. (2005) (64) showed that statins, specifically lipophilic statins like simvastatin, cross the cell membrane spontaneously and increase cytosolic calcium which in turn increases calpain concentration and activity. This initiates mitochondrial permeability transition (MPT) which is a process involved in cell death. (64, 65) Dirks *et al*. (2006) hypothesized that this may be a possible explanation for the muscle weakness in patients with statin intolerance. (62)

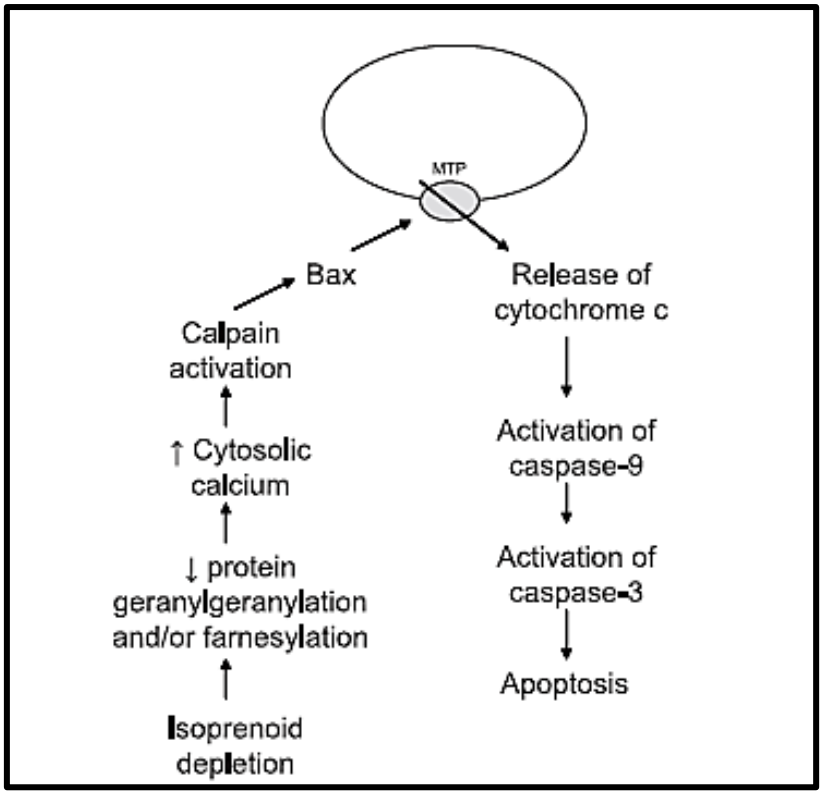

Figure 6: Hypothesized pathway of statin-induced apoptosis proposed by Dirks *et al*. (2006) (62) Permission for the re-use of image was obtained. License number: 501610233

MTP Mitochondrial permeability transition

Risk factors for statin intolerance

Various risk factors such as excessive physical activity, quantified as more than 300 minutes per week (81), hypothyroidism, grapefruit juice (CYP3A4 inhibitor), chronic kidney disease, small body frame and the female gender are all either associated with statin intolerance or play a role in worsening of symptoms. (81) However, the most significant factor in this drug-induced AE is the statin dose, and therefore plasma concentration. (82) Currently, plasma statin concentrations are not monitored. Many factors affect plasma statin concentrations, including dose, interactions with co-administered drugs, metabolizing capacities of hepatic CYP enzymes, and variations in transport mechanisms.

1.4.1 Drug-drug interactions

Drug interactions may either be beneficial or deleterious. (66) Interactions that

increase plasma concentrations of statins may increase cholesterol-lowering efficacy but may pose a greater risk for statin intolerance.

Most drug interactions involving statins take place during their metabolism. These interactions occur when two or more drugs that are metabolized by the same CYP450 isoenzyme are administered concurrently leading to toxicity of one of the drugs. (67) CYP3A4 isoenzymes play a significant role in the metabolism of statins. (68) Lipophilic statins tend to bind to the CYP3A4 isoenzyme with low affinity, which means that drugs that have a stronger binding affinity for CYP3A4 may inhibit their breakdown, leading to increased statin plasma concentrations and thus toxicity. (67) These CYP3A4-inhibiting drugs include erythromycin, itraconazole, ritonavir, cyclosporine and amiodarone (Table 8), all of which may disrupt the metabolism of statins resulting in an increase in their plasma concentrations. (69, 70)

One of the most well-known interactions is between grapefruit juice and statins. Grapefruit juice is a CYP3A4 inhibitor as well as a P-gp inhibitor (71) resulting in increased plasma concentrations of statins, specifically a 240% increase in simvastatin concentration when taken concurrently with grapefruit juice, 90% when taken 12 hours later, and an 80% increase in atorvastatin when taken at any time. (72) The two main components that play a role in CYP3A4 inhibition are bergamottin in fresh grapefruit and 6, 7- dihydroxybergamottin (DHB) in juice concentrate. (73) Despite the increase in lipid-lowering when taking statins with grapefruit juice, there is also a rapid increase in the prevalence of adverse events, especially myopathy. (74, 75)

Conversely, rifampicin, which is used for the treatment of tuberculosis, is a potent inducer of CYP3A4 in both the small intestine and liver, and may therefore decrease plasma statin concentrations by enhancing their metabolism. (76, 77) Kyrklund *et al*. (2000) (78) conducted a study on 10 healthy male volunteers to investigate the effects of rifampicin on simvastatin and its active metabolite and demonstrated a significant decrease in the area under the curve (AUC) or total exposure to both simvastatin (87%) and simvastatin acid (93%). Rifampicin also greatly reduced (by ~90%) the peak plasma concentration. No effect was observed in the elimination half-life of either

simvastatin or simvastatin acid. (78) Rapid decrease in the plasma concentration of simvastatin and simvastatin acid may lead to reduced effectiveness, as well as a lower risk of statin intolerance. (76, 77)

Table 8: Drug- drug interactions with statins	
Strong CYP3A4 inhibitors: Increase statin-associated adverse events	
1. Macrolide antibiotics	Clarithromycin Erythromycin
2. Azole antifungals	Itraconazole Ketonazole Posaconazole (Noxafil) Voriconazole
3. Protease inhibitors	Ritonavir Telaprevir Boceprevir
4. Fibric acid	Gemfibrozil
5. Immunosuppressants	Cyclosporine
6. Androgens	Danazol
7. Anti-anginals	Ranolazine
8. Pyridinecarboxylic acids	Niacin (> 1 g/day)
Moderate CYP3A4 inhibitors: Increase in statin-associated adverse events	
1. Antiarrhythmic agents	Amiodarone
2. Calcium channel blocker	Amlodipine Verapamil Diltiazem
3. Pyridinecarboxylic acids	Nicotinic acid
4. Phenylpiperazines	Nefazodone
5. Aryl-phenylketones	Dronedarone
6. Ketolide antibiotics	Telithromycin
7. Fluorobenzenes	Fluconazole
8. Protease Inhibitor	Fosamprenavir Nelfinavir

Minor CYP3A4 inhibitors: Evidence of increased risk of rhabdomyolysis	
1. Macrolide antibiotic	Azithromycin
2. Semi-synthetic macrolide antibiotic	Roxithromycin

CYP3A4 inducers: Decrease in statin efficacy	
1. Macrolactams	Rifampicin
2. Herbal medicines	St John's Wort
3. Protease inhibitor	Fosamprenavir

1.4.2 Pharmacogenomics

Differential drug responses arise from a variety of factors, such as age, sex, comorbid disease, polypharmacy and genetic factors. It has been established that variations in genetics account for ~90% of the differential responses to drug therapy and may result in a > 10-fold difference in drug metabolism and clearance between individuals. (79-81) To date, more than 30 genetic variations that may be associated with differential response to statin therapy have been identified. (82) These variations are divided into different groups, namely variations that affect the pharmacokinetics of a drug and variations that affect their pharmacodynamics. Candidate genes implicated in the import, export and hepatic metabolism of simvastatin and atorvastatin are shown in Table 9. (82)

Table 9: Candidate genes implicated in the import, export and hepatic metabolism of simvastatin and atorvastatin

Statin	Importer	Exporter	CYP enzyme
Simvastatin	SLCO1B1	$ABCG_2$ $ABCB_1$	CYP3A4/5 CYP2C8
Atorvastatin	SLCO1B1	$ABCG_2$	CYP3A4 CYP7A1

$ABCG_2$ ATP-binding cassette super-family G member 2, **$ABCB_1$** ATP-binding cassette super-family B member 1, CYP Cytochrome P450 enzyme, **SLCO1B1** Solute carrier organic anion transporter 1B1

1.4.3 Solute Carrier Organic Anion Transporter Family Member 1B1 (SLCO1B1)

Genome-wide association studies (GWAS) have identified numerous genetic variants associated with differential responses to statins (15, 16, 83, 84) especially in genes involved in LDL homeostasis and metabolism. (15) The most commonly associated genes include those encoding for *SLCO1B1* (*rs4149056*, *rs2306283* and *rs4363657*). (84)

Solute Carrier Organic Anion Transporter Family Member 1B1 *(SLCO1B1)*, located on the short arm of chromosome 12 (84, 85), encodes a 691 amino acid protein with 12 transmembrane helices. (86) *SLCO1B1* is mainly associated with the OATP1B1 influx membrane transporter for many different substrates, which range from drugs, e.g. atorvastatin, simvastatin and methotrexate, to endogenous hormones, e.g. thyroid hormones. (87) (Table 10) Organic Anion transporter polypeptide 1B1 is chiefly expressed on the sinusoidal (basolateral) surface of hepatocytes and distributed evenly throughout the lobules. (88, 89) Gleaser *et al.* (2007) also detected *SCLO1B1* mRNA in the small intestine. (90)

As described previously, when a statin tablet is administered orally, it dissolves in the stomach, is absorbed across the intestinal wall, travels to the liver via the portal circulation where it is metabolized and is either excreted via bile or transported to other tissues via the systemic circulation. (91) During this process statins traverse various biological membranes, either by passive diffusion or by facilitated transport. Membrane transporters play a significant role here. (87) OATP1B1 is specifically known for its role in the hepatic uptake and clearance of statins, referred to as hepatobiliary excretion. Statins function as both a substrate and an inhibitor of this transporter, as shown in Figure 7. (29, 92)

In some cases, patients may present with rotor syndrome, which is the complete or partial absence of OATP1B1. This may result in the inability to transport drugs and endogenous compounds across the hepatic membrane resulting in increased drug toxicity. (93)

Table 10: Substrates and inhibitors of OATP1B1, adapted from Solvo Biotechnology (94)				
Location	**Endogenous substrates**	***In vitro* substrates used experimentally**	**Substrate drugs**	**Inhibitors**
liver: hepatocyte basolateral membrane	bile acids	bromosulfophthalein	statins	rifampicin
	bilirubin	estrone-3-sulfate,	repaglinide	fusidic acid
	steroid hormones	estradiol-17β-glucuronide	olmesartan	clarithromycin
	thyroid hormones	dehydroepiandrosterone-3-sulphate	enalapril	erythromycin
	sulphates	fluvastatin	temocaprilat	roxithromycin
	glucuronide conjugates and peptides		valsartan	telithromycin
	prostaglandin E2		phalloidin	indinavir
	thyroxine (T4) and T3		docetaxel	saquinavir
				ritonavir
				cyclosporine
				gemfibrozil

Genetic polymorphisms in the gene encoding for this transporter may lead to a conformational change in the transporter, which may result in increased or decreased uptake of drugs, which might be an important determinant of drug therapy outcomes. (95, 96) Where genetic variations cause conformational changes in the OATP1B1 transporter, statins can no longer be taken up by the liver effectively, resulting in decreased hepatic concentrations (diminished overall efficacy) and corresponding increased concentrations in the circulation, which potentially increase the likelihood of toxic effects, i.e. muscle related adverse events, as indicated in Figure 8. (87, 97)

Pharmacogenetic studies show that single nucleotide variations (SNVs), which are single base-pair mutations at specific sites in the human deoxyribonucleic acid (DNA) sequence, play an important role in the response and outcome of certain therapies. (98, 99) Of the numerous SNVs identified, *SLCO1B1 rs4149056*, *rs2306283* and *rs4363657* are the strongest contenders for genetic predisposition to statin-intolerance. (100-104)

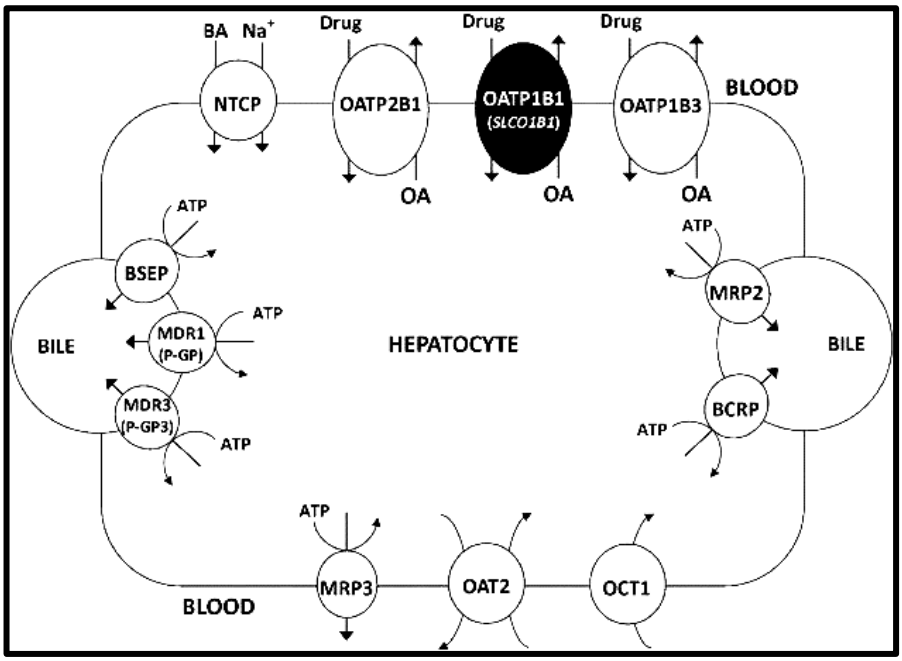

Figure 7: Schematic of the important influx and efflux transporters present on the human hepatocyte (96) Permission for the re-use of image was obtained from Springer Nature. License number: 4938960522881

OATP Organic anion transporter protein, **BCRP** Breast cancer resistant protein, **MRP** Multi-drug resistant associated protein 2, **MDR** Multi-drug resistant/ **P-gp** P-glycoprotein/ **ABC1** ATP-binding cassette, **CYP** Cytochrome P450 enzyme, e.g. CYP3A4, CYP2C9 **BSEP** Bile salt export pump, **OCT** Organic cation transporter, **NTCP** $Na^{=}$ /taurocholate cotransporter, **OAT** Organic anion transporter

The most common and well characterized variants of SCLO1B1 are *rs2306283* and *rs4149056*. These two variants appear to be in partial linkage disequilibrium, meaning these variants are more likely to be associated within a population than variants that are unlinked (105), and most commonly occur in the four different haplotypes as presented in Table 11.

The SEARCH collaborative group (104) conducted a GWAS using archived DNA from a randomized control trial of more than 12 000 participants in order to identify SNVs in a group of 85 participants that could be linked to simvastatin intolerance. A significant association between *rs4363657* and statin intolerance was identified. This non-coding SNV appeared to be in nearly complete linkage disequilibrium with two other well-

known SNVs, located in the *SLCO1B1* gene, namely *rs4149056* and *rs2306283*. Of these SNVs, only *rs4149056* located in the exon region appeared to be nonsynonymous, meaning it alters the encoded protein. Oshiro *et al*. (2011), however found that both *rs4149056* and *rs2306283* are nonsynonymous SNVs. (86) Furthermore, the risk associated with statin intolerance was found to be significantly higher in *rs4149056* CC homozygotes than in T-allele carriers, (104) where T is the wild type allele. (85, 103)

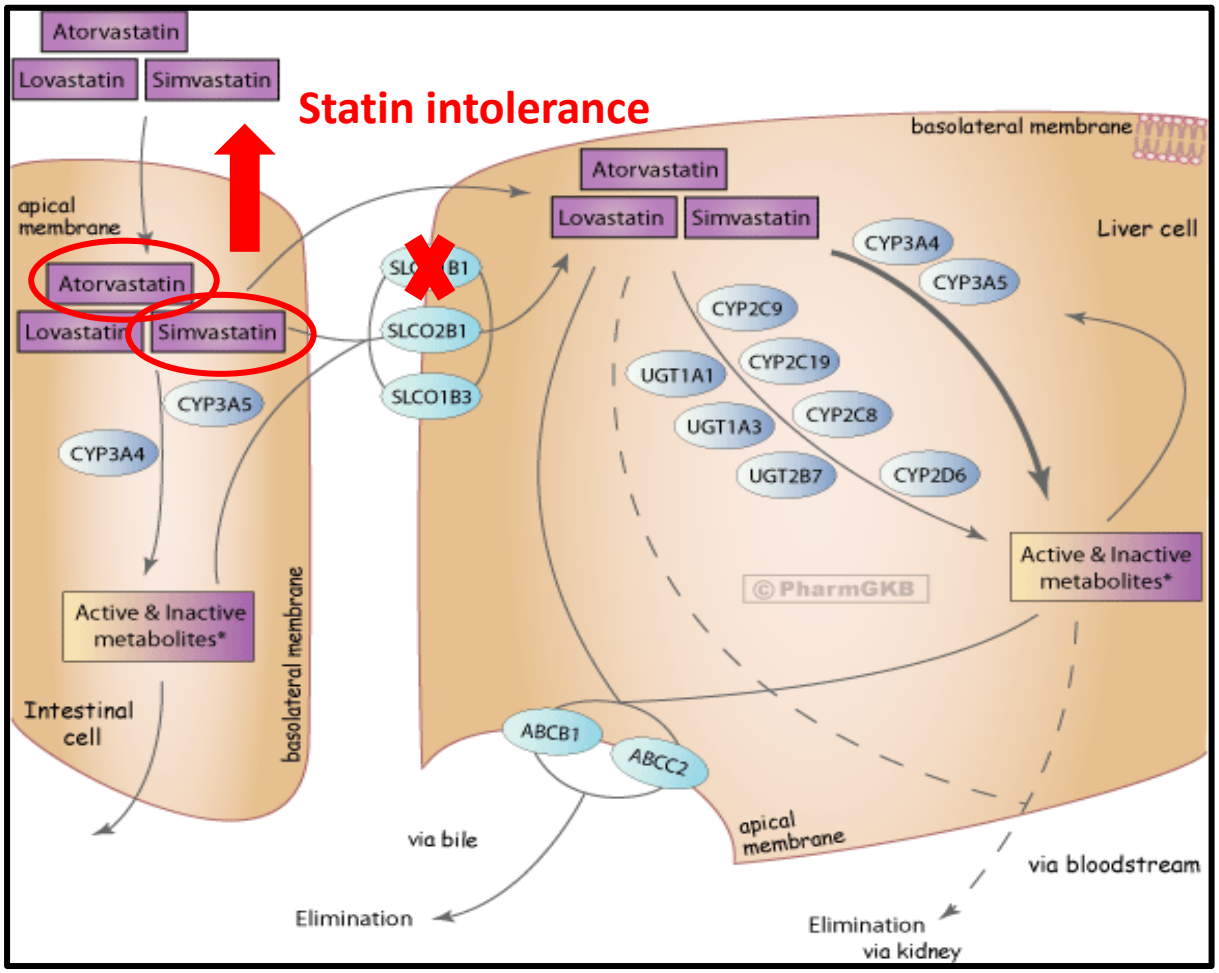

Figure 8: Schematic of altered hepatic uptake of simvastatin and atorvastatin due to the presence of SLCO1B1 variations on OATP1B1 transporter. Image obtained and adapted from PharmaGKB

OATP Organic anion transporter protein, **CYP** Cytochrome P450 enzyme, e.g. CYP3A4, CYP2C9, **ABC1** ATP-binding cassette, **SLCO** Solute carrier organic anion transporter, **UGT** Uridine 5'-diphospho-glucuronosyltransferase

Table 11: Important haplotypes associated with rs2306238 and *rs4149056* (86)
Adapted from Oshiro *et al*. (2011)

Haplotype	Variant	Protein change
SLCO1B1*1a	Contains neither variant	-
SLCO1B1*1b	*rs2306283*	Asn130Asp
SLCO1B1*5	*rs4149056*	Val174Ala
SLCO1B1*15	Both	Val174Ala & Asn130Asp

Val (Valine), **Asn** (Asparagine), **Asp** (Aspartic acid), **Ala** (Alanine)

The *rs4149056* variant is associated with increased circulating concentrations of statins, notably simvastatin, including both the lactone prodrug and acid forms. (86) For instance a study revealed a 221% increase in the AUC and a 200% increase in the Cmax in patients with the homozygous (CC) genotype compared to those with the heterozygous wild type (TC) and homozygous wild type (TT) genotypes. (106) Another single-dose study also showed significant increases in systemic drug exposure in patients with CC genotype. This data is presented in Table 12. (57)

Table 12: Increase in AUC of patients who present with CC genotype of *rs4149056* variant on SLCO1B1 gene
Data reported were obtained from a systematic review conducted by Wilke *et al*. (2012)(57)

Statin	Increase in AUC
Simvastatin acid	221%
Pitavastatin	162% – 191%
Atorvastatin	144%
Pravastatin	57% - 130%
Rosuvastatin	62% – 117%

The *rs2306283* variant on the *SLCO1B1* gene is also suspected of being associated with decreased activity of the *SLCO1B1* transporter, resulting in decreased clearance of statins from the circulation. (107) However, the different haplotypes, i.e. particular combinations of alleles located on one of two homologous chromosomes at a nearby SNV, (108) associated with this SNV have yielded different results. (109) In the study on the effect of *SLCO1B1 rs2306283**15 on the pharmacokinetic profiles of pravastatin and pitavastatin, a strong association between the altered transport of these statins and the specified haplotype was identified. (110) However, two separate studies to

determine the association of this variant with statin intolerance indicated no significant association between *rs2306283* and atorvastatin and rosuvastatin induced myopathy respectively. (111, 112) The data on the effect, as well as its combined effect with other statins is still inconclusive and limited. (103)

In an attempt to resolve some of the questions regarding *SLCO1B1* SNV and their association with statin intolerance, Nagy *et al*. (2015), (84) conducted a study on population groups in Roma and Hungary exploring differences in the prevalence of *rs4149056*, *rs2306283* and *rs4363657*. The researchers included 470 Roma and 442 Hungarian participants in the study. Genotypic prevalence is shown in Table 13. In both the Roma and Hungarian populations homozygous variant genotype for *rs2306283* appeared to be more prevalent.

Previous research has established the prevalence and respective associations of *SLCO1B1* (s4149056, *rs2306283* and *rs4363657*) with statin intolerance in various populations, highlighting ethnic differences in prevalence as strongly noted in Table 13. (84, 109, 113, 114, 116, 117, 119-122) Due to the paucity of research on these SNVs, specifically the lack of data on populations other than the frequently researched European and Asian population groups, further studies, specifically on South African populations, need to be conducted.

Table 13: Genotype frequencies as determined from other studies (84, 109, 113-122)

rs2306283				
	Population	**Homozygous wild type**	**Heterozygous**	**Homozygous variant**
*Roma	470	115	198	157
*Hungarian	442	201	162	79
Finnish	468	137	230	101
Indian (North)	270	86	126	58
Indian (Singapore)	100	17	52	31
Chinese	299	22	108	169
Chinese (Singapore)	100	5	31	64
Chinese (Han)	111	10	39	62
Germans	143	-	-	-
Malays (Singapore)	100	2	22	76
Brazilian	143	80	51	12

rs4149056				
	Population	**Homozygous wild type**	**Heterozygous**	**Homozygous variant**
*Roma	470	315	148	7
*Hungarian	442	288	141	13
Estonia	540	316	188	36
Finnish	468	299	149	20
Indian (Singapore)	100	87	13	0
Chileans	100	73	24	3
Chinese (Singapore)	100	75	24	1
Germans	143	-	-	-
Chinese (Han)	111	82	27	2
Malays (Singapore)	100	73	20	1
African	97	86	11	0
Brazilian	143	106	34	3
European (Lithuania)	206	108	98	0
European (Lithuania)	290	195	95	11

rs4363657				
	Population	**Homozygous wild type**	**Heterozygous**	**Homozygous variant**
*Roma	470	308	150	12
*Hungarian	442	285	141	16
European	113	77	35	1
Italian	102	60	38	4
Indian	101	88	13	0
Japanese (Tokyo)	113	44	49	20
Chinese (Han)	135	44	60	31
Chinese (Colorado)	108	34	44	30
African (USA)	57	36	18	3
Kenya (Luhya)	109	78	29	2
Kenya (Masaai)	156	106	43	7
Nigeria (Yoruban)	147	109	36	2
Mexican (La)	57	46	10	1

In South Africa where health care can be described as a disproportionate blend between first world (for a small minority) and developing (for the majority of the population) countries, health care service is likely to change dramatically with the implementation of National Health Insurance. South Africa lags behind the rapid progress in the personalized and precision medicine fields. Large-scale databases,

technological advances to characterize patients and tools to analyze this data are required to improve diagnosis, therapeutic responses and ultimately health outcomes in this resource-limited setting.

The aim of this study was therefore to determine the background prevalence of *SLCO1B1* SNVs in a randomly selected sample of the general population in Gauteng, South Africa, and to investigate if there are associations between *SLCO1B1* SNVs and statin intolerance in patients diagnosed with hypercholesterolemia.

The objectives of this study were to:

1. Determine the prevalence of *SLCO1B1 rs4149056*, *rs2306283* and *rs4363657* SNVs in (i) a general population (control), and in (ii) patients with hypercholesterolemia on a 12-week stable simvastatin or atorvastatin dose, using Polymerase Chain Reaction - Restriction Fragment Length Polymorphism.
2. Determine the statin-intolerance severity scores of hypercholesterolaemic patients using the American College of Cardiology's (ACC) Statin Intolerance Application
3. Determine CK levels of hypercholesterolaemic patients from a collected serum sample, using Enzyme-linked immunosorbent assay (ELISA).
4. Compare possible associations of SNVs and statin intolerance (elevated CK levels) between simvastatin and atorvastatin hypercholesterolaemic subgroups using statistical methods.
5. Determine if there are associations between symptoms and signs of statin intolerance and individual SNVs using statistical methods

Chapter 2: Materials and methods

This clinical study aimed to determine the background prevalence of *SLCO1B1* SNVs in a randomly selected sample of the general population in Gauteng, South Africa, and to investigate if there are associations between *SLCO1B1* SNVs and statin intolerance in patients diagnosed with hypercholesterolemia.

The study design, materials, methods, statistical analysis, and ethics are described in detail in this chapter, and are summarized in the schematic, presented in Figure 9.

2.1 Ethics

The study was granted ethics approval by the Faculty of Health Sciences Research Ethics Committee on 26 April 2019. (Ethics Reference No.: 154/2019, appendix 4) The clinical study was conducted in accordance with the International Conference on Harmonization Good Clinical Practice (ICH GCP) guidelines. Written informed consent to participate in the study was obtained from all participants before any sample or data was collected. Confidentiality and anonymity were maintained by using numerical codes as patient identifiers. Blood samples for analysis were collected by a physician or nurse. By determining the presence and the prevalence of *SLCO1B1 rs4149056*, *rs2306283* and *rs4363657* SNVs, a better understanding of hypercholesterolemia therapy in a diverse South African population was gained.

2.2 Study design

This explorative, retrospective, experimental study, with a quantitative questionnaire, examined the background prevalence of *SLCO1B1 rs4149056*, *rs2306283* and *rs4363657* SNVs in Gauteng, South Africa, and the possible associations between these SNVs and statin intolerance in patients with hypercholesterolemia.

2.3 Setting

To determine the background prevalence of SNVs, 181 control participants who had never been diagnosed with hypercholesterolemia, were recruited from the general population in Gauteng, e.g. from shopping centers, the University of Pretoria's clinical research unit (CRU), private practice, the University of Pretoria, and from gyms and public spaces. To determine the prevalence of SNVs in hypercholesterolaemic

patients specifically, 100 hypercholesterolaemic patients were recruited from the University of Pretoria's Clinical Research Unit (CRU), and from other medical centers in Gauteng. These patients were generally recruited from public health institutions which are government funded. This means that this cohort was predominantly of a low-socio-economic population dependent on public health care.

2.4 Patient selection

2.4.1 Sample size and selection

One hundred statin-treated hypercholesterolaemic patients at the CRU and other identified medical centers/ hospitals across the Pretoria and Johannesburg area, were recruited during their respective follow up visits. A total of 181 participants served as the general population controls. Controls were any person, from the general public, who had not been diagnosed with hypercholesterolemia previously. The number of control and test samples were determined using the power calculation to ensure minimal statistical power in consultation with a statistician.

2.4.2 Inclusion Criteria for participants with hypercholesterolemia on statin therapy

A statin-treated patient was eligible for participation in this study if all the following inclusion criteria were met:

1. Patients > 18 years
   Provided written informed consent for study participation prior to the start of any study related procedures.
2. Diagnosed with hypercholesterolemia.
3. On continuous and stable 12-week atorvastatin-/ simvastatin dose.

2.4.3 Exclusion Criteria for participants with hypercholesterolemia on statin therapy

A statin-treated patient was not eligible for participation if any of the following exclusion criteria were met:

1. Unwilling to give consent.
2. Patients < 18 years

3. Any disruption of atorvastatin or simvastatin therapy within the preceding 12-week period.

2.4.4 Inclusion Criteria for control participants

A participant was eligible for participation if all the following inclusion criteria were met:

1. Provided written informed consent for study participation prior to the start of any study related procedures.
2. Participants > 18 years

2.4.5 Exclusion Criteria for control participants

A participant was excluded from study participation if any of the following exclusion criteria were met:

1. Unwilling to give consent
2. Participants < 18 years
3. Diagnosed with hypercholesterolemia.
4. Receiving any lipid-lowering therapy

These factors were added to the exclusion criteria as they will be confounding factors and will therefor skew the data.

2.5 Measurements and laboratory work

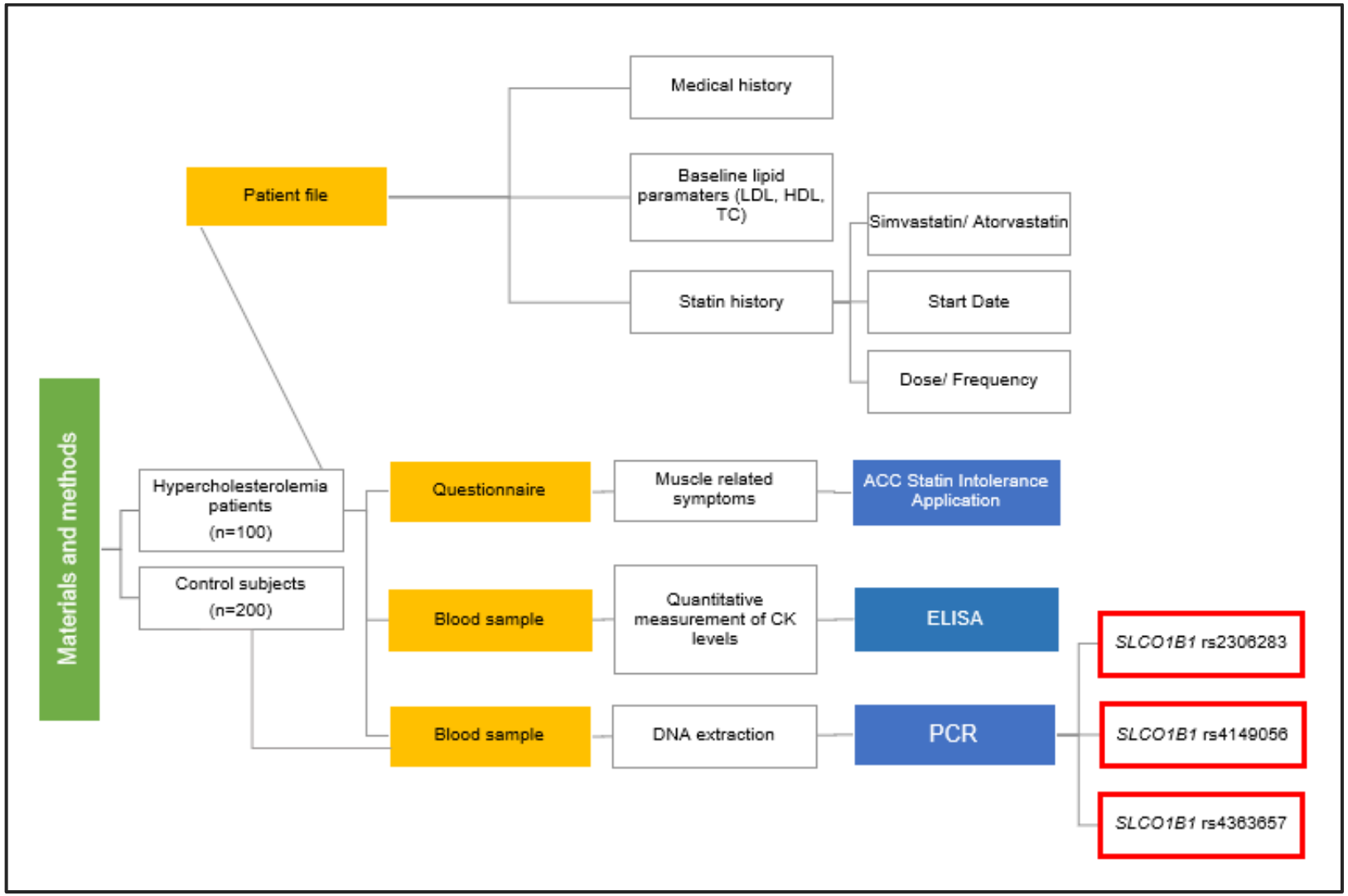

Figure 9: Schematic representation of the experimental design of the study

2.5.1 Questionnaire

A validated questionnaire, completed by each participant, was completed to evaluate statin intolerance of the 100 hypercholesterolaemic patients on their current statin therapy using the American College of Cardiology's (ACC) Statin Intolerance Application. Statin intolerance risk was calculated using the calculator provided by the ACC Statin Intolerance app. (123) (See Appendix 1)

2.5.2 Blood collection

Following informed consent, a 5 ml whole blood (WB) sample was collected in a citrate tube by venipuncture from all study participants in order to evaluate their *SLCO1B1 rs4149056*, *-rs2306283* and *-rs4363657* SNV status. A total of 1 ml of WB was transferred into a collection tube and stored at -80^{o}C until DNA extraction.

2.5.3 DNA extraction

Genomic DNA was isolated from 200 µl of WB using the Quick-DNA™ Miniprep Plus Kit (Zymo Research).

At the time of extraction, frozen WB was thawed on ice. Thereafter, an aliquot of 800 µl cell lysis buffer was added to 200 µl WB in 1.5 ml microcentrifuge tubes. The tubes were vortexed for 10-15 seconds (s) and incubated at room temperature (RT) for 10 minutes (min). Following incubation, the sample was transferred into a Zymo-Spin™ IIC-XL column in a collection tube and centrifuged at 12 000 x *g* for 1 min. The collection tube with the flow through was discarded and the column was placed into a new collection tube. A total volume of 200 µl of DNA pre-wash buffer was added to the spin column and centrifuged at 12 000 x *g* for 1 min. The eluate collection tube was emptied, and 500 µl g-DNA wash buffer was added to the spin column and centrifuged at 12 000 x *g* for 1 min. The collection tube with the flow through was discarded. The spin columns were transferred into a clean 1.5 ml microcentrifuge tube, 50 µl DNA elution buffer was added directly on to the matrix and the sample was incubated for 15 min at RT. Following incubation, the tubes were centrifuged at 12 000 x *g* for 30 s to elute the DNA. The eluted DNA was stored at -20ºC and quantified using spectrophotometry (Nanodrop 2000c, Thermo-Fischer, South Africa), standardized to 10 ng/µl and used for polymerase chain reaction (PCR). The ratio of spectrophotometric absorbance at 260 nm to that of 280 nm (A260/A280) was used

to assess the purity and quality of the extracted DNA, where a value of ~1.8 was expected.

2.5.4 Polymerase Chain Reaction optimization and cycle conditions

To avoid non-specific binding and primer dimers, primer concentration of the 3 specified SNVs were optimized using Go Taq® Hot start green master mix: Go Taq® Hot start green master mix contains Go Taq® Hot start polymerase, dTNPs, $MgCl_2$ and reaction buffers. All components were kept constant while the primer concentrations were varied between 400 nM, 500 nM, and 600 nM. The PCR products were electrophoresed on a 1.8% GR Green stained agarose gel at 120 V for 30 min and gels were viewed using Image Lab software (BioRad, version 5.21) on the Gel Doc EZ Imager (BioRad, South Africa). A gel documentation system with a UV transilluminator was used to detect the bands in the agarose gel following electrophoresis. The concentration selection was based on the PCR band that had the appropriate weight PCR product and no primer dimers.

2.5.5 Polymerase Chain Reaction

SLCO1B1 rs2306283, *rs4149056* and *rs4363657* were amplified in a 25 µl reaction using the primers described in Table 14. (84, 124)

The PCR conditions for *SLCO1B1 rs2306283* were pre-denaturated for 2 min at 95°C, followed by a three-step amplification, denaturation at 95°C for 45 s, annealing at 55.5°C for 45 s and extension at 72°C for 45 s. This was followed by a final extension at 72°C for 300 s and cooling at 37°C for 300 s. For *SLCO1B1 rs4149056,* were pre-denaturated for 2 min at 95°C, followed by a three-step amplification, denaturation at 95°C for 45 s, annealing at 52°C for 45 s and extension at 72°C for 45 s. This was followed by a final extension at 72°C for 300 s and cooling at 37°C for 300 s. Predenaturation for *SLCO1B1 rs4363657* was for 2 min at 96°C, followed by a three-step amplification, denaturation at 95°C for 45 s, annealing at 58°C for 45 s and extension at 72°C for 45 s. This was followed by a final extension at 72°C for 300 s and cooling at 37°C for 300 s.

Table 14: Primer sequences and restriction enzymes			
Reference SNV cluster ID	**Primers**	**Restriction enzymes**	**Primer concentration**
SLCO1B1 rs2306283	F: 5'-CTGTGTTGTTAATGGGCGAA-3' R: 5'-GGGGAAGATAATGGTGCAAA-3'	TaqI	400 nM
SLCO1B1 rs4149056	F: 5'- TTGTCAAAGTTTGCAAAGTG -3' R: 5'- GAAGCATATTACCCATGAGC -3'	Hin6I	400 nM
SLCO1B1 rs4363657	F: 5'-CAGTTTGCTAGTGTTTTGTTGAGG-3' R: 5'-ACCATCCAAGACGAACAAAGAG -3'	KpnI	400 nM

2.5.6 Polymerase Chain Reaction - Restriction Fragment Length Polymorphism

The restriction of the SNVs described in Table 14 were conducted in a 25 µl reaction (3 µl PCR product, 5 µl 1X New England (NE) buffer and 1 µl RE (1 000U)) (New England BioLabs) using the restriction enzymes specified in Table 14. The restriction fragments were then analyzed using gel electrophoresis on a 3% agarose gel for the *SLCO1B1* SNVs for 30 min and then visualized with a ultra violet (UV) transilluminator. Expected PCR and restriction products are shown in Table 15.

Table 15: Expected PCR and restriction products (84, 124)			
SNV	**Expected length of PCR product**	**Expected length of Restriction productions**	**Interpretation of cleavage**
SLCO1B1 rs2306283	406 base pairs (bp)	**Homozygous wild type (AA):** 159 bp and 247 bp **Heterozygous (AG):** three bands of 247 bp, 136 bp and 23 bp. **Homozygous variant (GG):**	**No restrictions:** Homozygous wild type **Both initial PCR and restriction products detected:** Heterozygous **Complete digestion of**

		three bands of 247 bp, 136 bp and 23 bp	**PCR products:** Homozygous variant
SLCO1B1 rs4149056	209 bp	**Homozygous wild type (TT):** 209 bp **Heterozygous (TC):** three bands of 209 bp, 21 bp and 188 bp. **Homozygous variant (CC):** two bands of 188 bp and 21 bp	**No restriction:** Homozygous wild type **Both initial PCR and restriction products detected:** Heterozygous **Complete digestion of PCR products:** Homozygous variant
SLCO1B1 rs4363657	Two fragments of 133 bp and 236 bp	**Homozygous wild type (TT):** two fragments of 133 bp and 236 bp **Heterozygous (CT):** four fragments of 84 bp, 133 bp, 152 bp and 236 bp. **Homozygous variant (CC):** three fragments of 133 bp, 84 bp, 152 bp.	**No restrictions:** Homozygous wild type **Both initial PCR and restriction products detected:** Heterozygous **Complete digestion of PCR products:** Homozygous variant

2.5.7 Quantitative measurement of plasma Creatine Kinase

Creatine kinase levels were estimated using the CKM Human SimpleStep ELISA® Kit: Blood samples were collected in 5 ml citrate tubes and centrifuged at 2000 x *g* for 10 min. Plasma samples were transferred to collection tubes and stored at -80°C. Plasma samples were thawed at RT and diluted with Sample Diluent in a 1:50 ratio. Standards were also reconstituted and prepared in sample diluent. A total of 50 µl of each sample or standard with known concentration was added in duplicate to the appropriate wells in a 96 well plate, after which 50 µl of the antibody cocktail was added to each well. The plate was sealed and incubated for 1 hour at RT on a plate shaker set to 400 rpm. After incubation, each well was washed with 3 x 350 µl 1 x Wash Buffer PT by aspirating from each well and then dispensing 350 µl 1 x Wash Buffer PT into each well. Complete removal of liquid at each step was essential. After the last wash, the plate was inverted and blotted with clean paper to ensure removal of all excess liquid. A total of 100 µl of TMB substrate was added to each well and the plate was incubated for 5 min on a plate shaker set at 400 rpm. After incubation, 100 µl of Stop Solution was added to each well and the plate was placed back onto the plate shaker for 1 min to ensure thorough mixing. The optical density was measured using a spectrophotometer (Biotek Synergy HT, Software GEN 5.1) at an absorbance 450 nm. A standard curve was constructed, and concentration of the unknown samples were calculated using the equation derived from the standard curve (y=mx+c).

2.6 Statistical analysis

A Mann Whitney U-test was conducted to determine differences between demographic parameters. The background prevalence of *SLCO1B1 rs4149056*, *rs2306283* and *rs4363657* SNVs were determined by previous study reports (84, 109, 113, 114, 116, 117, 119-122) as well as from a general population in the Gauteng region. SNVs in a hypercholesterolaemic population were reported using appropriate descriptive and inferential statistics and the Hardy-Weinberg equilibrium (HWE) test to determine statistical significance. The Fishers exact test was used to determine the odds ratio (OR) and relative risk (RR) for each SNV. Analysis for CK levels included column statistics using the recommended D'Agostino and Pearson test to determine if the data followed a normal distribution. Comparisons between groups were achieved using the non-parametric tests (Mann Whitney U-test and one-way ANOVA (Kruskal-

Wallis test with Dunn’s post-test for multiple comparisons between groups). The level of significance was set at $p<0.05$. The analysis was carried out on GraphPad Prism v6 (San Diego, California).

Chapter 3: Results

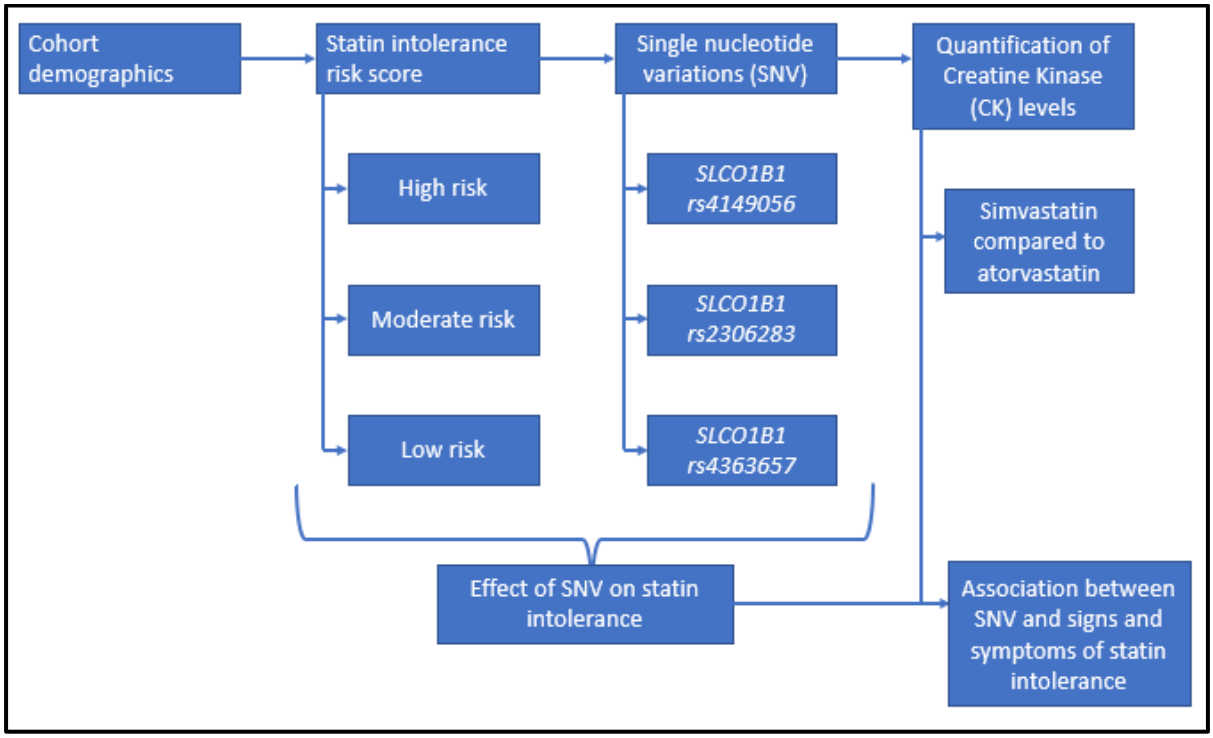

Figure 10: Flow diagram to illustrate the presentation of results

3.1 Cohort demographics

The cohort demographics for this study included 281 Caucasian and Black participants between the ages of 19 and 75. Of these, 100 were included in the hypercholesterolaemic group and 181 were included in the control group. The male to female ratio was kept the same between the hypercholesterolemic and control group. The full cohort demographics are shown in Table 16. Most (70%) of the hypercholesterolaemic patients were treated with the first line statin, simvastatin.

Table 16: Participant (cohort) demographics		
	Hypercholesterolemic group (*n*= 100)	**Control group (*n*= 180)**
Age median (range) in years	51 (19 – 75)	49 (20 – 75)
Sex		
Male (%)	49 (49%)	89 (49.2%)
Female (%)	51 (51%)	92 (50.8%)
Race		
Black (*n*)	86	142
White (*n*)	14	39
Co-morbidities		
Rheumatoid arthritis (*n*)	2	0
Psoriatic arthritis (*n*)	4	0
Osteoarthritis (*n*)	9	0
Type 2 Diabetes Mellitus (*n*)	12	0
Hypothyroidism (*n*)	2	0
Low body mass index (*n*)	2	0
Treatment group		
Atorvastatin (*n*)	29	0
Simvastatin (*n*)	71	0

3.2 Statin intolerance risk score

All participants in the hypercholesterolaemic group (n=100) completed a detailed questionnaire (Appendix 1) adapted from the American College of Cardiology's (ACC) Statin Intolerance Application. The questionnaire collected data on muscle symptoms, their severity, frequency, patient characteristics and medical history that may increase a patient's predisposition for statin intolerance. Table 17 illustrates the distribution of patients on different statin treatments as high, moderate, or low risk. Of the 100 patients in the hypercholesterolaemic group, four presented with risk factors that may worsen or contribute to statin intolerance, i.e. low body mass index (BMI) and hypothyroidism. Further assessment of these particular four patients using the statin intolerance questionnaire, demonstrated moderate risk for statin intolerance for all.

Furthermore, two of the four were carriers of at least one of the assessed SNVs.

Table 17: Statin intolerance risk score					
Statin intolerance risk score	**Treatment groups**				
	Atorvastatin 10 mg	Atorvastatin 20 mg	Simvastatin 10 mg	Simvastatin 20 mg	Simvastatin 40 mg
High risk (*n*)	-	2 (10%)	7 (26%)	4 (11%)	2 (29%)
Moderate risk (*n*)	4 (40%)	12 (60%)	13 (48%)	16 (44%)	4 (57%)
Low risk (*n*)	6 (60%)	6 (30%)	7 (26%)	16 (44%)	1 (14%)
Total	10 (10%)	20 (20%)	27 (27%)	36 (36%)	7 (7%)

3.3. Single nucleotide variations

Hardy-Weinberg equilibrium (HWE) is a very common analysis used in population genetics. The theory states that when allelic frequencies conform with HWE, the allelic frequency is constant from generation to generation and the distribution can be determined. If the allelic frequency does not conform with the HWE, researchers can make suggestions that evolutionary influences might be playing a role.

The genotype distribution in the statin-treated hypercholesterolaemic (test) group conformed with the HWE ($p>0.05$). However, the *SCLO1B1* rs4149056 and rs2306283 genotype distribution in the control group did not conform with the HWE ($p<0.05$).

The prevalence of the *rs4149056* variant was 16% for the control group and 20% for the test group. Although the prevalence was 4% higher in the test group, it did not reach statistical significance. (OR= 1.324; 95% confidence interval (CI)=0.8430 to 2.078; $p=0.2405$).

The *rs2306283* variant was significantly more prevalent in the control group (31.5%) compared to the test group (10.5%), (OR= 0,2552 95% CI=0.1542 to 0.4223, $p< 0.0001$. The prevalence of the *rs4363657* variant was similar in both the test and control group. (OR=1.345, 95% CI=0.8492 to 2.129, $p=0.2380$).

Table 18 illustrates the frequency and distribution of the *SLCO1b1* rs4149056, rs2306283 and rs4363657 SNVs.

Table 18: Genotype and Allele frequencies						
Population	***n***	**Homozygous wild type *n (%)***	**Heterozygous *n (%)***	**Homozygous variant *n (%)***	**Presence of variant *n (%)***	**P value[1]**
rs4149056	**281**	**TT**	**TC**	**CC**	**C allele**	
Control	181	138 *(76.7)*	30 *(16.6)*	13 *(7.3)*	56 *(15.5)*	*p*= 0.2405
Test	100	62 *(62)*	37 *(37)*	1 *(1)*	39 *(19.5)*	
rs2306283	**281**	**AA**	**AG**	**GG**	**G allele**	
Control	181	95 *(52.5)*	58 *(32)*	28 *(15.5)*	114 (*31.5)*	*p*< 0.0001
Test	100	79 *(79)*	21 *(21)*	0 *(0)*	21 *(10.5)*	
rs4363657	**281**	**TT**	**TC**	**CC**	**C allele**	
Control	181	140 *(77.3)*	40 *(22.1)*	1 *(0.6)*	42 *(11)*	*p*= 0.2380
Test	100	60 *(60)*	38 *(38)*	2 *(2)*	42 *(21)*	

[1] *P*-values were derived from Fisher's exact test

Taken together, of the atorvastatin-treated patients included in the study, 2 *(7%)* had a high risk of developing statin intolerance, 16 *(53%)* had a moderate risk of statin intolerance and 12 *(40%)* a low risk of statin intolerance. Of the simvastatin-treated patients, 13 *(19%)* presented with high risk to statin intolerance, 33 *(47%)* with moderate risk to statin intolerance and 24 *(34%)* with low risk to statin intolerance (Table 17). The wild type genotype was more prevalent than the variant for all SNVs. The variant allele for rs2306283 was more prevalent in the control group than in the test group, 31.5% and 10.5% respectively.

3.4 The effect of the presence the *SLCO1B1* single nucleotide variations on statin intolerance

Following the determination of the frequency and prevalence of each variant and the statin intolerance severity risk score of each included patient, an analysis was performed to ascertain if there was an association between the presence of either of the single nucleotide variants and the risk outcome of the statin intolerance questionnaire in order to assess if a genotype had an effect on muscle fatigue and

muscle weakness. In addition to the analysis between controls and hypercholesterolemic subjects, frequencies were compared within the test group based on calculated risk.

The genotype frequency for each SNV was compared within two groups, namely low risk statin intolerance and moderate to high risk statin intolerance. This analysis showed no significant association for any of the three SNVs and either low risk or moderate to high risk statin intolerance, *rs4149056* (Figure 11, OR=0.7857, 95% CI=0.2115 to 2.919, RR= 0.8800, 95% CI= 0.4433 to 1.747, *p*=0.7496) and *rs2306283* (Figure 12, OR=0.4911, 95% CI=0.1234 to 1.954, RR=0.9659, 95% CI 0.4888 to 1.909, *p*=0.4877) and *rs4363657* (Figure 13, OR= 0.9375, 95% CI= 0.2634 to 3.3337, RR=0.6984, 95% CI=0.3609 to 1.352, *p*=1.0000).

There was no difference in the frequency of either variant between moderate to high risk to statin intolerance and patients with a low risk of statin intolerance. For rs4149056 (Figure 11), low risk wild type frequency was 28.3% compared to 35.9% in the moderate to high risk and the variant (C-allele) occurred 17.9% times in both groups. For rs2306283 (Figure 12) the variant allele was present in 17.9% of the low risk participants and in 12.8% or moderate to high risk participants, while the wild type genotype was present in 28.3% and 41% of the low risk and moderate to high risk patients, respectively. For rs4363657 (Figure 13) the wild type genotype was seen in 25.6% of the low risk participants and 30.8% of the moderate to high risk participants while the variant allele was only present in 20.5% of the low risk and in 23.1% of the moderate to high risk participants.

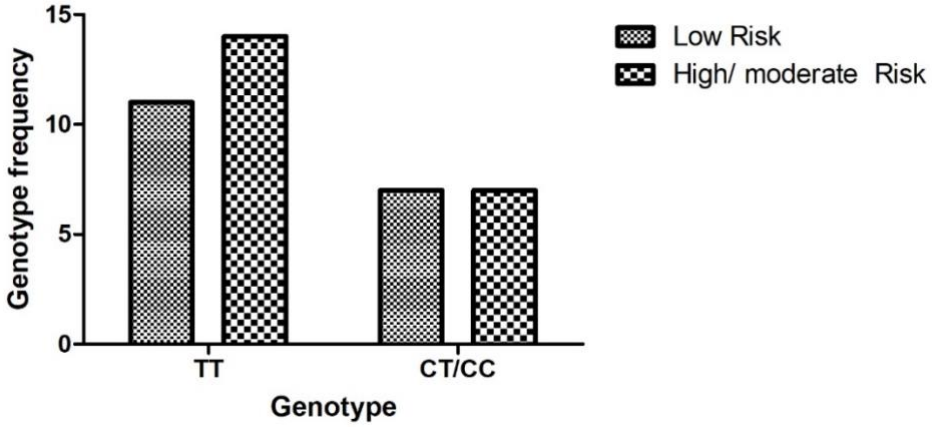

Figure 11: Distribution of *SLCO1B1 rs4149056* genotypes among patients with low and moderate/high risk of statin intolerance

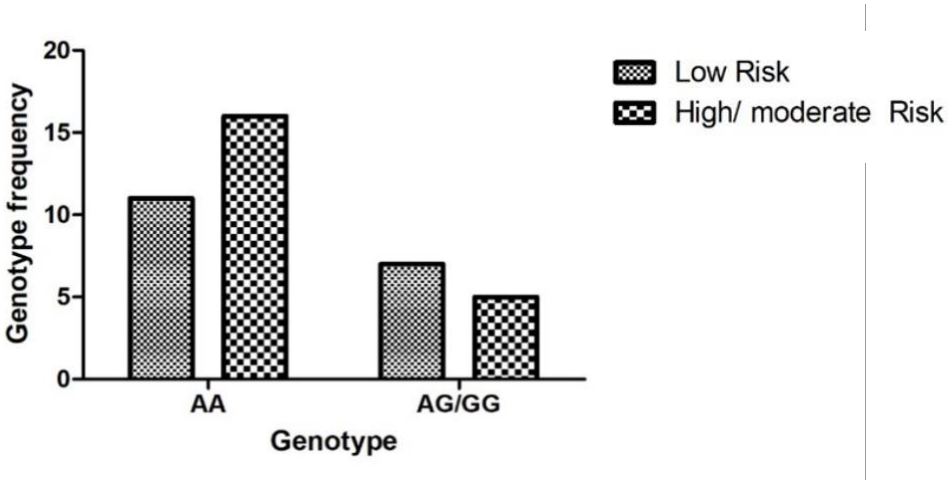

Figure 12: Distribution of *SLCO1B1 rs2306283* genotypes among patients with low to moderate/high risk of statin intolerance statin intolerance

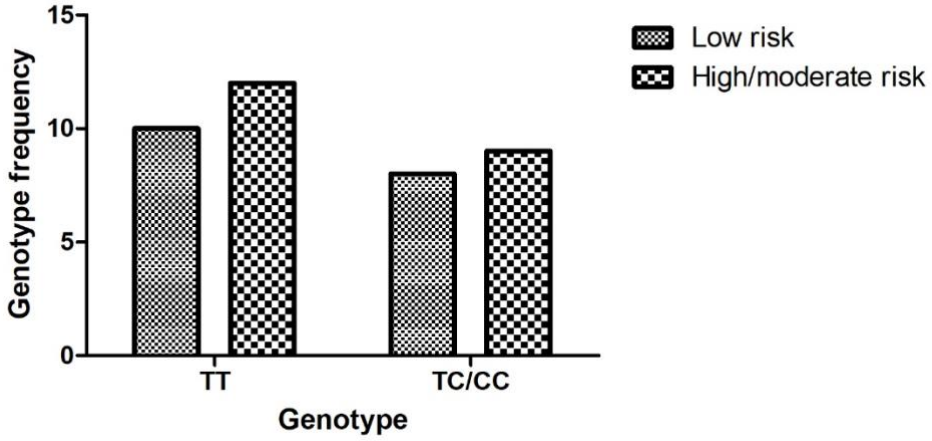

Figure 13: Distribution of *SLCO1B1 rs4363657* genotypes among patients with low to moderate/high risk of statin intolerance

3.5 Quantification of creatine kinase

Raised circulating plasma CK is often used as an indirect biomarker of skeletal muscle damage or breakdown. It is thus a sign commonly associated with statin intolerance and is therefore used as a surrogate endpoint To determine the serum levels of CK in patients on statin therapy, 40 patients were selected, based on predefined selection criteria (none with co-morbidities as listed in Table 16) as shown below in groups 1 to 6:

1. 3 test samples - moderate/high risk with 3 SNVs present
2. 8 test samples - moderate/high risk with SNV *rs4149056* and SNV *rs4363657*
3. 5 test samples - moderate/high risk with SNV *rs2306283* and SNV *rs4363657*
4. 2 test samples - moderate/high risk with SNV *rs4149056* and SNV *rs2306283*
5. 7 test samples - low risk with 1 SNV only
6. 15 test samples - low risk wild type

Upon completion of the ELISA, CK values were calculated as described in Chapter 2, section 2.5.7. Numerical values of CK were recorded in pg/ml units. A total of 29 patients were on atorvastatin and the remaining 71 were on simvastatin. The ratio of the number of patients treated with simvastatin to atorvastatin ratio was kept consistent when the samples were selected for CK analysis: 15 were on atorvastatin and 25 on simvastatin.

The average CK level in the atorvastatin group was 16 517 pg/ml compared to 26 744 pg/ml in the simvastatin group (Figure 14). Statistical analysis showed that the CK levels of patients in the simvastatin treatment group were significantly higher compared to the CK levels of patients in the atorvastatin treatment group (p=0.0418).

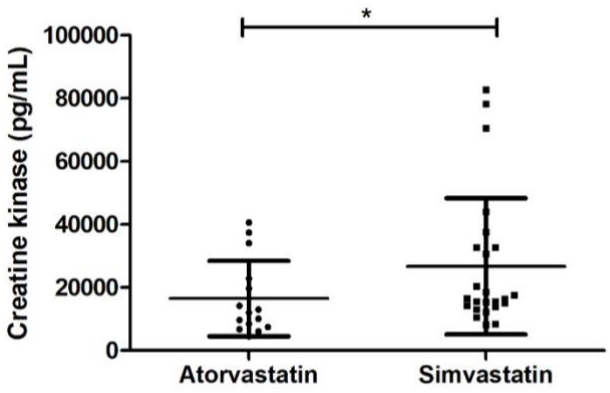

Figure 14: Serum CK levels of patients within the simvastatin and atorvastatin treatment groups

*p= 0.0418, Indicates statistical significance with p<0.05.

3.6 Determining if there are associations between symptoms and signs of statin intolerance and individual SNVs.

After establishing an association within treatment groups, an analysis was done to determine if there was a significant difference between the presence of either SNVs, the statin intolerance severity risk score and CK elevation. This was done by comparing the CK levels of the different subgroups (identified in the selection criteria, i.e. moderate/high risk with SNVs present; moderate/high risk with SNV rs4149056 and SNV rs4363657; moderate/high risk with SNV rs2306283 and SNV rs4363657; moderate/high risk with SNV rs4149056 and SNV rs2306283; low risk with 1 SNV only; low risk wild type) set out by comparing the difference between the CK values of patients who presented with low risk to statin intolerance and no SNV (wild type) and patients with moderate to high risk of SNV with at least 1 SNV. The aim of this analysis was to determine if there are associations between symptoms and signs of statin intolerance and individual SNVs.

Figure 15 illustrates the estimated CK levels of each patient within every subgroup. The range in CK levels for each subgroup was; Group 1: 4567 pg/ml – 15650 pg/ml, Group 2: 6150 pg/ml – 37483 pg/ml, Group 3: 12317 pg/ml – 32733 pg/ml, Group 4: 15567 pg/ml – 34150 pg/ml, Group 5: 10650 pg/ml – 44067 pg/ml, Group 6: 7483 pg/ml – 40650 pg/ml. Analysis showed no significant difference between CK levels of each subgroup (p=0.2048). The column statistics are presented in Table 19.

Table 19: Column statistics of 1-way ANOVA of difference between CK levels of each subgroup						
Subgroup	**1**	**2**	**3**	**4**	**5**	**6**
Minimum	4567	6150	12317	15567	10650	7483
25% Percentile	4567	6817	12692	15567	14213	9942
Median	8567	15067	13983	24859	31733	14233
75% Percentile	15650	17567	24567	34150	39254	20108
Maximum	15650	37483	32733	34150	44067	40650

[1] Moderate/high risk with 3 SNVs present; [2] moderate/high risk with SNV rs4149056 and SNV rs4363657; [3] moderate/high risk with SNV rs2306283 and SNV rs4363657; [4] moderate/high risk with SNV rs4149056 and SNV rs2306283; [5] low risk with 1 SNV only; [6] low risk wild type.

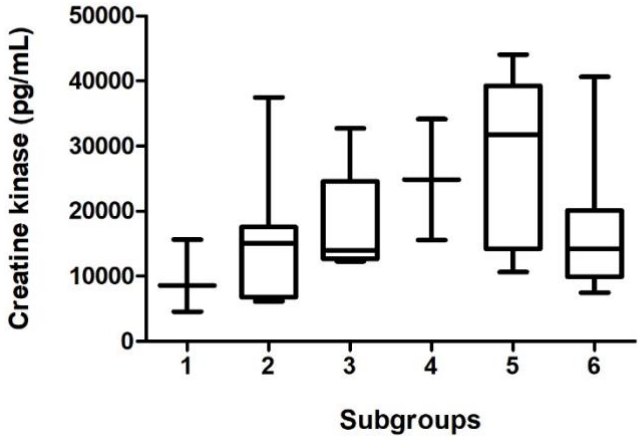

Figure 15: Serum CK levels of patients in subgroups. Box whisker plot representing median, interquartile range and standard deviation.

[1] Moderate/high risk with 3 SNVs present; [2] moderate/high risk with SNV rs4149056 and SNV rs4363657; [3] moderate/high risk with SNV rs2306283 and SNV rs4363657; [4] moderate/high risk with SNV rs4149056 and SNV rs2306283; [5] low risk with 1 SNV only; [6] low risk wild type.

Figure 16 shows no significant difference between the CK levels of patients with wildtype genotypes for all SNVs and those who presented with low risk statin intolerance compared to patients who presented with moderate to high risk statin intolerance and the presence of at least 1 SNV (p=0.9885). The Column statistics are presented in Table 20. CK levels of the low risk group ranged from 7483 pg/ml – 78230 pg/ml compared to the moderate to high risk group which ranged from 4567 pg/ml – 82730 pg/ml.

Table 20: Column statistics of t-test of difference between CK levels of low risk wild type patients and high/moderate risk with at least 1 SNV		
Subgroup	**Low risk wild type**	**Moderate/high risk with SNV**
Minimum	7483	4567
25% Percentile	10150	11070
Median	14230	15610
75% Percentile	22900	32730
Maximum	78230	82730

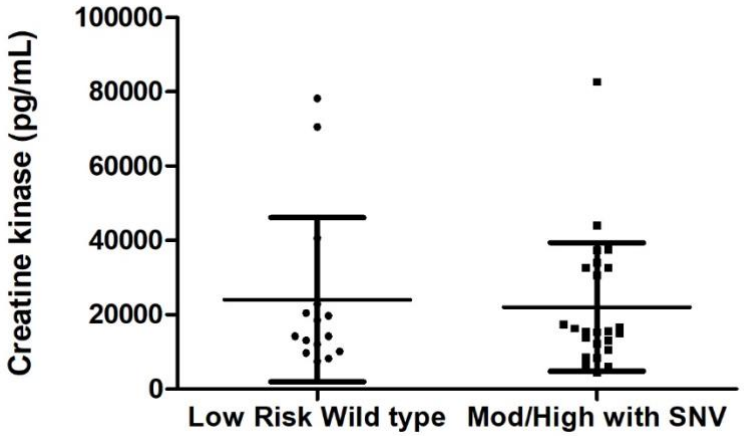

Figure 16: CK levels of patients within the Low risk Wild type and moderate/high risk with one SNV subgroups

Although no significant association between the presence of at least one variant and elevated CK levels was made, there was a slightly higher elevation in the maximum CK level of the moderate/high risk group, i.e. 82 730 pg/ml compared to the low risk group, 78 230 pg/ml.

In conclusion, there was no significant association between the presence of one or more of the SNVs and signs and symptoms of statin intolerance reported by the patients. Patients on simvastatin, however, presented with significantly elevated levels of serum CK than atorvastatin-treated patients.

Chapter 4: Discussion

Hypercholesterolaemia is a worldwide problem that poses an ever-growing risk for adverse cardiovascular disease outcomes. Atherosclerosis may lead to coronary artery disease, myocardial infarction, stroke and death. An estimated 1 in 75 South Africans are hypercholesterolaemic. (125) Statins are HMG-CoA reductase inhibitors that rapidly decrease LDL levels. Statins, including simvastatin and atorvastatin, are currently the gold standard lipid-lowering therapy for hypercholesterolemia. (3, 126) However, they may be associated with adverse effects, including statin intolerance.

The considerable (~20%) risk of statin intolerance, ranging from mild asymptomatic myopathy to life-threatening rhabdomyolysis, (48-52) has prompted an urgent need to investigate potentially relevant pharmacokinetic factors, including population differences in statin absorption, distribution, metabolism and excretion that may be influenced by genetic variation. Both simvastatin and atorvastatin are administered orally and are absorbed by the small and large intestines. (30, 32, 33) After initial absorption, simvastatin and atorvastatin are partially metabolized by gastro-intestinal CYP3A4 isoenzymes and then transported to the liver for further metabolism and distribution. (30)

The *SLCO1B1* gene encodes for the basolateral membrane transporter OATP1 (92) which aids in hepatic uptake of statins. Single nucleotide variations in the *SLCO1B1* gene lead to conformational changes in this transport mechanism, which may decrease hepatic uptake of statins (127), and thereby decrease their metabolism, leading to potentially toxic concentrations of statins in their native forms in circulation. (101) Single nucleotide variations may profoundly affect this transporter capability and influence how patients respond to statins. In fact, emerging research shows an association between statin intolerance and genetic variation. (100-104) Single nucleotide variations have been implicated in statin toxicity, in particular statin-intolerance, which is defined as statin-induced muscle related adverse events. (84, 102) Signs and symptoms associated with statin-intolerance include, muscle pain and weakness, muscle cramps, myositis and elevated CK levels. (48-52)

Genetic variations such as SNVs influence drug metabolism – which is an important rate-limiting step in drug pharmacokinetics. There are ~10 million SNVs present in the human genome. (128) These single base-pair changes occur at specific sites in the human DNA sequence. (98, 99) The most common form of genetic variations are SNV substitutions. These genetic variations result in a nucleotide / base pair substitution that may lead to a change in the amino acid codon and this ultimately changes the protein structure. These SNVs are known as nonsynonymous SNVs. A nonsynonymous SNV may result in a conformational change in the *SLCO1B1* transporter. (128-130) This change may hinder drug clearance which ultimately increases drug concentrations and toxicity. (84, 102)

Single nucleotide variations have been identified in *SLCO1B1 rs4149056*, *rs2306283* and *rs4363657* and are believed to influence statin intolerance. (84) A global analysis of the genetic variation in *SLCO1B1* stated that the *rs4149056* has an allele frequency of ~15 – 20% in Caucasian populations and a ~1 – 2% in black populations. The *rs2306283* variant has a frequency of ~40% in Caucasian populations with little to no data on black populations. The *rs4363657* variant illustrated a high allelic frequency in Caucasian, East African and African American populations of ~30%, (84, 109, 113-122, 131-133) There is a paucity of research on the prevalence of these SNVs in the diverse South African setting.

The aim of this study was therefore to determine the background prevalence of *SLCO1B1* SNVs in a randomly selected sample of the general population in Gauteng, South Africa, and to investigate if there are associations between *SLCO1B1* SNVs and statin intolerance in patients diagnosed with hypercholesterolaemia.

The first objective of this study was to determine the prevalence of *SLCO1B1* SNVs (*rs4149056*, *rs2306283* and *rs4363657)* in a general population as well as in patients with hypercholesterolemia on either simvastatin or atorvastatin therapy.

It was demonstrated for the first time that SNVs for *SLCO1B1* are present in South African populations. Of note is that only Black and Caucasian patients were included in the study in an attempt to provide a close representation of the population ethnic

ratio in the region. The prevalence of the variant allele for the total cohort was 15.1% for *rs4363657*, 24% for *rs2306283* and 17.4% for *rs4149056*. The prevalence of each SNV varied between ethnicities and no difference was found for *rs4363657* and rs2306283. Interestingly, the frequency of the *rs4149056* variant was significantly higher amongst the black participants with hypercholesterolaemia (53.3%) compared to Caucasians (15.3%), *p*=0.0206, OR: 3.467.

Other studies have also shown diverse results in Asian, European, North and South American and East African populations. Agnes Nagy (2015) explored the differences in frequencies of *SLCO1B1* variants between Roma (*n*= 470) and Hungarian (*n*=442) populations and found the *rs2306283* variant to be the most prevalent in both ethic groups (presence of G allele/variant 54.5% and 36.2%). The *rs4149056* variant was evident in 17.2% of the Roma participants and 18.9% of the Hungarian participants, while the *rs4363657* variant appeared to be the least frequent with the variant in only 19.8% and 19.5% of the Roma and Hungarian participants, respectively. (84) Mwinyi *et al*. (2008) (134) found that the prevalence of the *SLCO1B1* gene variations were low in a selected African cohort from Uganda compared to other populations. Eighteen *SLCO1B1* variants were explored in this study. However only 6 were present in the African cohort. Prevalence of the *rs4149056* variant was only 3.9% but the prevalence of the *rs2306283* variant was significantly higher at 77.8% (95% CI= 71.9 -83.0). (134) In the cohort from the current study, prevalence of the *rs4149056* variant (16.9%) was similar to the prevalence of the *rs2306283* variant (24%).

The SNVs most commonly and widely associated with statin intolerance are *rs2306283* and *rs4149056*. These SNVs are in linkage disequilibrium and are most commonly identified together in the same population. (105) In this study the *rs4149056* variant was more prevalent in the hypercholesterolaemic (test) group compared to the control group. The *rs2306283* variant was most commonly identified in the control group. In this study the presence of both *rs4149056* and *rs4363657* were more common amongst subjects in the control and test groups. This result is substantiated by the GWAS conducted by the 'SEARCH collaborative' in 2008 which concluded that *rs4149056* and *rs4363657* are in complete linkage disequilibrium. (135)

The second objective of this study was to determine the presence and severity of statin-intolerance in hypercholesterolaemic patients using the American College of Cardiology's (ACC) Statin Intolerance Application. In 2013 the American College of Cardiology and the American Heart Association (ACC/AHA) developed a guideline on the treatment of blood cholesterol to reduce atherosclerosis in order to assist physicians in optimizing patient health care and reduce the cardiovascular risks associated with elevated LDL levels. (136) In conjunction with this guideline, researchers developed an application through patient testing, and optimized by physicians and nurses, to provide a first line assessment for patients who present with statin intolerance. In this application, muscle symptoms are graded according to a scale of 0–10, where 0-2 is considered mild, 3-5 moderate and 6–10 severe. (53, 123, 136) The questionnaire used in this study (Appendix 1), was adapted from this application.

Majority (64%) of the hypercholesterolaemic patients included in this study reported muscle-related adverse effects. Most (49%) reported their muscle pain as mild to moderate. Moderate severity indicates that muscle related symptoms only slightly reduce everyday activities such as experiencing difficulty in working, sleeping, performing household chores and climbing stairs. Reducing the statin dose may be a feasible treatment option while monitoring these patients' CK levels. (7) A total of 17% reported severe pain. Other studies have reported an overall prevalence of ~30% of severe myalgia, myopathy and in some cases rhabdomyolysis. In these reports, the therapy used included various statins, with myopathy observed in subjects on simvastatin and atorvastatin. (26, 41, 42, 44-46)

The risk of developing statin intolerance was found to be low (in 36%), moderate (in 49%), or high (in 15%) in the statin-treated hypercholesterolaemic patients and was based on what was reported in the questionnaires. The prevalence in the latter two categories (64%) correlated well with the actual development of muscle-related adverse effects (65%).

Statin intolerance may vary in definition and severity. Currently there is no accepted definition of statin intolerance, but various definitions have been constructed by drug

regulatory authorities, making the results from investigational studies difficult to compare. The definition used in this study, was that statin intolerance is a clinical syndrome, which manifests as the inability to tolerate at least two statins, (one of which is at its lowest daily dose, 5 mg), due to symptoms and signs related to statin treatment, e.g. increase in laboratory markers and / or myopathy, which is agreed by the regulatory bodies (NAL, ILEP and CCWG). (9-12, 137)

The third objective of this study was to determine the CK levels of simvastatin and atorvastatin-treated hypercholesterolaemic patients. Creatine kinase is expressed in high levels in the heart and skeletal muscle tissues. (138) Elevated plasma CK is therefore one of the most commonly used biomarkers of statin-induced myopathy, indicative of myositis, myopathy and in severe cases, rhabdomyolysis. (138, 139) Levels of CK may be graded into three different classes: incipient myopathy, myopathy, and rhabdomyolysis. (56, 57)

A total of 10% of patients had elevated plasma CK, compared with 64% of patients who reported muscle symptoms. Ballantyne *et al*. (2003) (43) described various symptoms reported by patients on simvastatin and atorvastatin. These ranged from low grade muscle cramps and body aches to severe muscle weakness. However, these symptoms were not accompanied by a significant plasma CK elevation. (43) Although statin intolerance is not always accompanied by extreme elevation in the CK level (> 10 to 50 x ULN), a slight increase can result from myopathy and muscle breakdown. (12). This highlights a consistent lack of correlation between CK levels and signs and symptoms of statin intolerance.

The highest CK levels reported in this study were 70 567 pg/ml, 78 233 pg/ml, 82 733 pg/ml. Of these the patient who presented with the highest CK level (82 733 pg/ml), carried the variant for both the *rs4149056* and *rs4363657* SNVs, but reported little to no muscle related symptoms. Furthermore, CK analyses indicated a slight (27.7%), elevation in the median CK level of the low risk hypercholesterolaemic participants who presented with only 1 of the 3 SNVs. These differences, however, were not statistically significant.

The fourth objective was to determine and compare possible associations of SNVs and elevated CK levels between simvastatin and atorvastatin subgroups. Although all statins are substrates of the OATP transporter, the effect the *SLCO1B1* SNVs has on the pharmacokinetics differs between statins. This study compared the effect of simvastatin and atorvastatin on CK levels and statin intolerance in order to determine which statin is safer in patients who carry any or all of the three SNVs.

Majority of participants were on 20 mg atorvastatin (20%), 10mg simvastatin (27%) or 20 mg simvastatin (36%). While this study did not specifically assess the pharmacokinetic influence of individual SNVs, CK analysis did show that the CK level of hypercholesterolaemic patients on simvastatin was significantly higher compared to atorvastatin. Simvastatin yielded a mean CK level of 26 744 pg/ml almost double the mean CK level 16 517 pg/ml for atorvastatin. This finding reiterates that Atorvastatin might be a safer and more tolerable option for hypercholesterolaemic patients.

Other studies also suggest that atorvastatin might be safer and more tolerable in hypercholesterolaemic patients. (104, 140) Carr *et al*. (2013) included 76 statin-induced myopathy cases reported between June and November of 2011, of which 59 were receiving simvastatin, 11 atorvastatin and 6 other statins. Their findings indicated that having just one allele of the *rs4149056* variant resulted in severe myopathy and an elevation in the CK level of at least four times the UNL in simvastatin treated patients. Although atorvastatin was their second most implicated drug there was no significant association between the *SLCO1B1* variant and significant elevations of CK or severe myopathy. (140) These results (104) were comparable with those found in the GWAS conducted by Link *et al*. (2008) and the study by Brunham *et al*. (2011) which aimed to investigate the statin-specificity of the association between *SLCO1B1* variations and severe statin-induced myopathy. (8, 104, 141) In both these studies they found that although the *SLCO1B1* variations might not specifically be associated with statin-induced myopathy, there was a significant association (Brunham at al.(2011)(8) OR= 3.2, 95% CI 0.83–11.96, χ^2 p= 0.042, Fisher's exact p= 0.064 and Link *et al*. (2008)(104) OR= 4.3 (95% CI, 2.5 to 7.2)) between the patients receiving simvastatin and myopathy. (8, 104) This finding parallels the findings from the current study.

A study by Pasanen *et al*. (2006) (106) that aimed to assess the effect that transporter variations have on statins, reported that the *SLCO1B1* variations had the greatest effect on simvastatin uptake. The researchers specifically investigated *rs4149056* and demonstrated that the presence of the variation markedly affected the pharmacokinetic profile of the drug. The Cmax was 221% higher in participants with the variation compared to those without. (106) A GWAS study conducted in 2008 identified SNVs in a group of 85 participants that could have an association with statin intolerance. A significant association was drawn between *rs4363657* and *rs4149056* and statin induced myopathy. After genotyping both SNVs, the risk associated with simvastatin intolerance was significantly higher in patients who presented with the homozygote (CC) genotype compared to the T allele carriers. (85, 103, 104)

The final study objective was to determine if there are associations between symptoms and signs of statin intolerance and individual SNVs.

Previous research has established an association between *SLCO1B1* (s4149056, *rs2306283* and *rs4363657*) and statin intolerance in various populations, highlighting ethnic differences in the prevalence of these variations. (84, 109, 113, 114, 116, 117, 119-122) For instance, Lin *et al*. (2011) (107) identified the *rs2306283* and *rs4149056* variants on the *SLCO1B1* gene as two of the *SLCO1B1* variants most commonly associated with decreased activity of the OATP1 transporter, resulting in a decreased clearance of statins from the circulatory system and eventual statin intolerance. (107)

Surprisingly, despite the published evidence in other populations, statistical analyses indicated no significant association between the signs and symptoms associated with statin intolerance and the presence of an SNV in this cohort.

Comorbidities in 31% of the study population included; hypothyroidism, T2DM, rheumatoid arthritis, osteoarthritis, psoriatic arthritis, and small body frame (low BMI). These are risk factors that could initiate or worsen symptoms associated with statin induced myopathy, potentially confounding the data. (82, 142) Despite this, only a minority (13%) of patients with comorbidities were considered to have a high risk of

developing statin intolerance. However, this may have been due to the statin dose (mostly simvastatin 10 – 40 mg), which is currently considered the most significant risk factor for developing statin intolerance. (26)

Chapter 5: Conclusion

Two hundred and eighty one participants were included in the study of which 100 were hypercholesterolaemic and 181 were healthy volunteers. Prevalence of the variant allele of all three SNVs differed to previous studies.

In this study, no association could be drawn between the presence of SNV, signs and symptoms of statin intolerance and elevated CK levels. This study showed conclusively that simvastatin treated patients had a higher risk of statin induced myopathy compared to the atorvastatin treated patients which is in line with previous literature (8, 104, 140, 141)

The results of this study provide a better understanding and is the first to explore the prevalence of these SNVs and their association with statin intolerance in a South African cohort. These findings will allow a more personalized approach to statin therapy, especially relevant within the diverse South African population. This prompts a need to investigate a larger population, especially to determine if the presence of the SNVs affects CK levels; and to determine whether there is a differential effect in patients who present with the homozygous variant compared to heterozygous or homozygous wild type.

Limitations

Lack of significance seen with CK analysis can be ascribed to the small sample size following group selections for analysis due to financial constraints.

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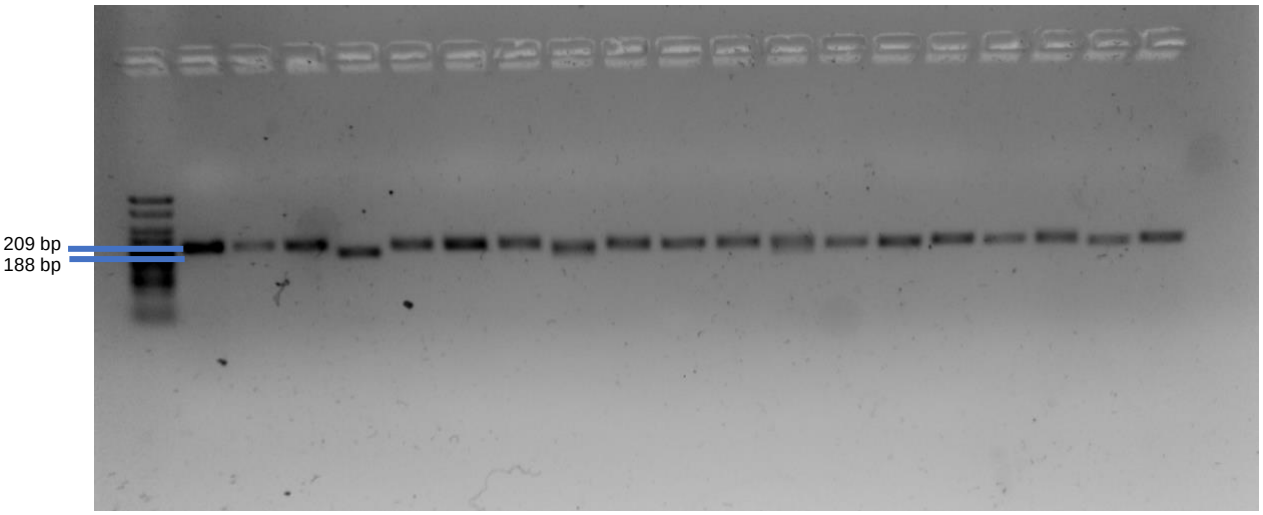
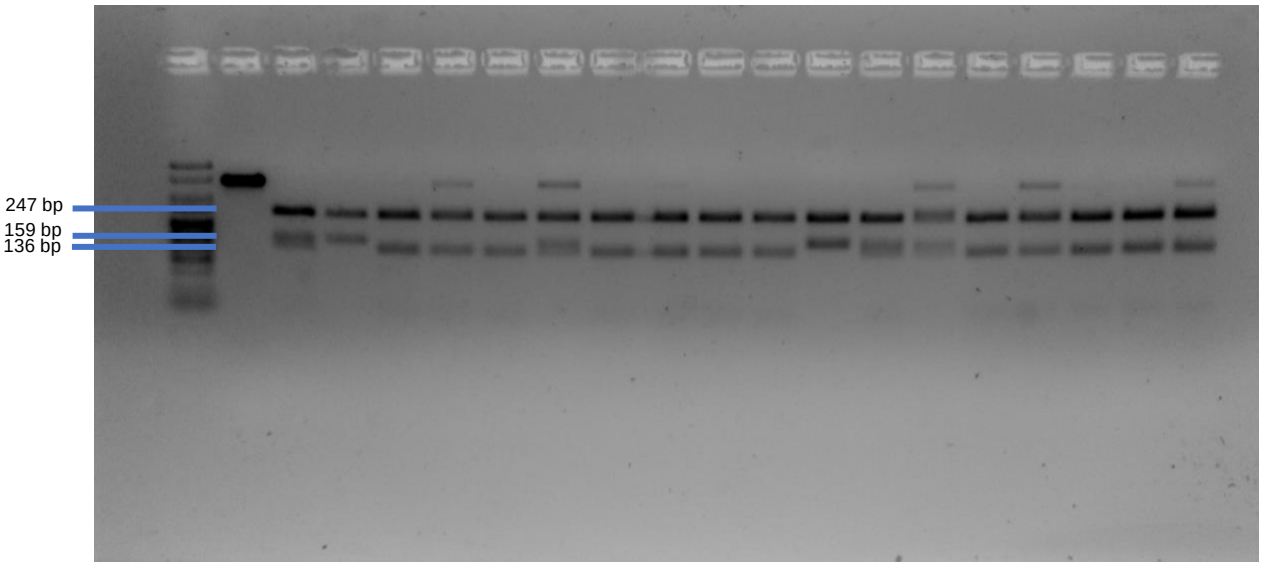
Appendix 1

Gel image examples

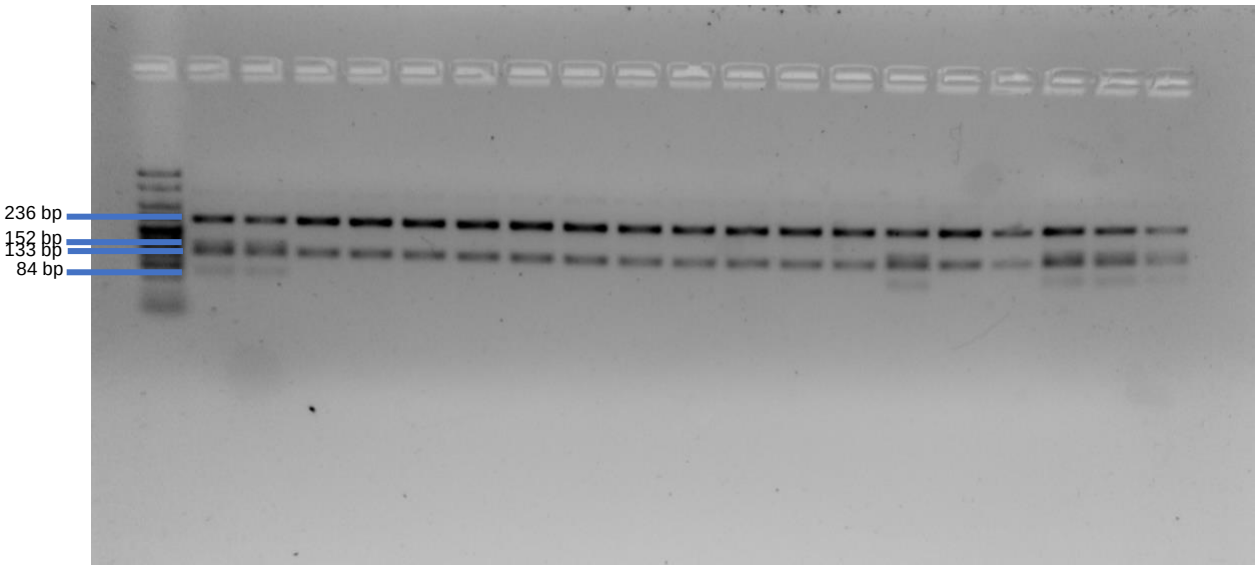
a. rs4149056

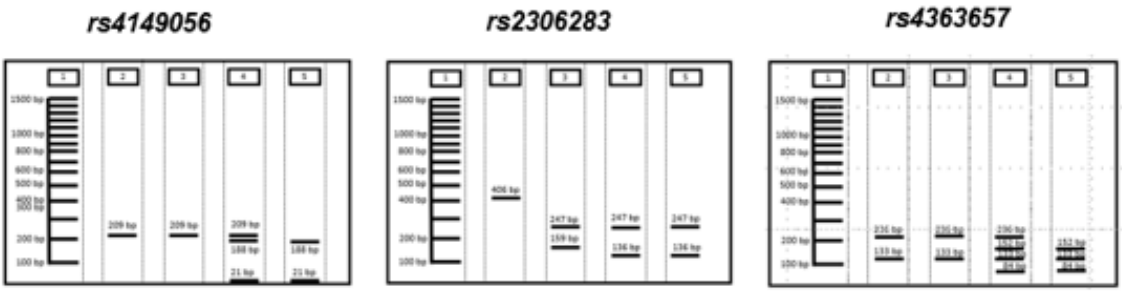


b. rs2306283



c. rs4363657





Schematic of PCR and restriction products. 1. DNA Ladder, 2. PCR product, 3. Homozygous wild type, 4. Heterozygous, 5. Homozygous variant

Appendix 2

Questionnaire: American College of Cardiology's (ACC) Statin Intolerance Application

Demographics

Sex:

- Male
- Female

Age: _____ years

Race:

_____

Rhabdomyolysis Assessment

1. Is your patient's CK above 5x the upper normal limit (UNL)?
   - Yes
   - No
   - I don't know

Muscle symptoms

2. Select the group that best describes the symptoms
   - Muscle ache, weakness, soreness, stiffness, cramping, tenderness, or general fatigue.
   - Tingling, twitching, shooting pain, nocturnal cramps, or joint pain
3. Select symptom area
   - Bilateral (Muscle symptoms are generalized, e.g. neck and shoulder pain or lower back)
   - Unilateral (Muscle symptoms are isolated, e.g. only on knee of shoulder)
4. Severity of symptoms
   a. How severe is the pain?
      (0 = no pain to 10 = worse pain)
      - 0 – 2 mild
      - 3 – 5 moderate
      - 6 – 10 severe
   b. How many of the last seven days has the patient had the symptoms?
      - 1 – 2 mild
      - 3 – 4 moderate
      - 5 – 7 severe

c. How much have the muscle symptoms impacted everyday activities?
    - Only limits exercise
    - Slightly reduces everyday activity (trouble working, sleeping, performing household chores, climbing stairs etc.)
    - Greatly restricts everyday activities (cannot work, sleep, perform household chores or climb stairs)

5. <u>When did the muscle symptoms start?</u>

   _____ / _____ / _____

6. <u>Factors that increase risk of statin intolerance</u>

   Patient Characteristics

- Low BMI
- Excessive grapefruit juice consumption
- Heavy exercise/ physical exertion
- Personal or immediate family history of statin intolerance
- Frailty
- High alcohol consumption
- Drug abuse
- Dehydration or decrease daily fluid intake

Medical History

- Unexplained ALT elevations >3 times ULN
- Renal insufficiency
- Multiple or serious comorbidities
- Hepatic dysfunction

7. Non-statin cause for muscle symptoms

Medical history

- ☐ Multiple or serous comorbidities
- ☐ Heavy exercise or physical exertion
- ☐ Seizures
- ☐ Vitamin D deficiency
- ☐ Multiple-organ disease
- ☐ Elevate erythrocyte sedimentation rate (ESR)
- ☐ Previous muscle disorder history
- ☐ Trauma
- ☐ Electrolyte abnormalities
- ☐ Hypothyroidism
- ☐ Post-op state, especially surgery with high metabolic demands

Medical conditions

Primary muscle diseases

- ☐ Muscular dystrophy
- ☐ Polymyositis
- ☐ Steroid myopathy
- ☐ Polymyalgia rheumatica
- ☐ Rhabdomyolysis

Rheumatological disorders

- ☐ Arthritis
- ☐ Fibromyalgia
- ☐ Systemic lupus
- ☐ Tendonitis or joint disorder

Additional disorders

- ☐ Diabetes

- ☐ Adrenal insufficiency/ Cushing Syndrome
- ☐ Addison's disease
- ☐ Anemia
- ☐ Hypoparathyroidism
- ☐ Viral illness
- ☐ Anemia
- ☐ Peripheral arterial disease

8. <u>Current statin and drug interactions</u>

   Current statin: _____

   Dose: _____

   Frequency: _____

   Time of day:

   - ☐ Morning
   - ☐ Afternoon/ Evening
   - ☐ Bedtime

   Start date: _____ / _____ / _____

   Has the patient had muscle pain while taking a previous statin?

   - ☐ Yes
   - ☐ No

   Contraindicated medication

   _____

   _____

   _____

Appendix 3

Informed Consent Documents

a. Control group

PARTICIPANT'S INFORMATION & INFORMED CONSENT DOCUMENT

STUDY TITLE:

Prevalence of *SLCO1B1* single nucleotide variations, and their association with statin intolerance in hypercholesterolaemic patients in Gauteng, South Africa

Protocol no: …… Researcher: René de Beer

Dear Mr. / Mrs. .....

1) INTRODUCTION

You are invited to volunteer for a research study involving the medication that is commonly prescribed for the treatment of high cholesterol called statins. I am doing research for a master's degree at the University of Pretoria. The information in this document is to help you to decide if you would like to participate or not. Before you agree to take part in this study you should fully understand what is involved. If you have any questions, which are not fully explained in this document, do not hesitate to ask the researcher. You should not agree to take part unless you are completely happy about all the procedures involved.

2) THE NATURE AND PURPOSE OF THIS STUDY

Statins are normally taken up by the liver where it is broken down so it can be excreted as waste. However, in some cases this up take does not happen as it should which mean the statins can no longer be broken down as it is supposed to. This causes some patients to have more severe adverse / side effects than others do.

This generally happens due to a type of change to the gene responsible for the uptake of statins by the liver. In this study we will be testing whether these genes are present in a selected population in Gauteng.

3) EXPLANATION OF PROCEDURES AND WHAT WILL BE EXPECTED FROM PARTICIPANTS.

Once you have signed the informed consent document, a 5ml (about a teaspoon full) tube of blood

will be collected to determine whether you do have any of the SNVs.

To determine whether you are suitable for the specific study you will have to be able to answer YES to all the following:

1. Are you older than 18 years?

To determine whether you are suitable for the specific study you will have to be able to answer NO to all the following:

1. Are you younger than 18 years?
2. Have you been diagnosed with abnormally high levels of cholesterol?
3. Are you currently taking any cholesterol medication?

4) POSSIBLE RISKS AND DISCOMFORTS INVOLVED

There are no medical risks associated with the study. The only possible risk and discomfort involved is drawing of the blood which can result in pain, bruising and bleeding from the site where the needle is inserted, but usually this does not last long.

5) POSSIBLE BENEFITS OF THIS STUDY

There will be no direct healing benefit for you from this specific research project. However, by determining the presence and the prevalence of these SNVs, a better understanding of hypercholesterolemia therapy in a diverse South African population will be gained.

6) COMPENSATION

You will not be paid to take part in the study. There are no costs involved for you to be part of the study.

7) YOUR RIGHTS AS A RESEARCH PARTICIPANT

Your participation in this study is entirely voluntary and you can refuse to participate or stop at any time without stating any reason. Your withdrawal will not affect your access to other medical care.

8) ETHICS APPROVAL

This Protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, telephone numbers 012 356 3084 / 012 356 3085 and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2013), which deals with the recommendations guiding doctors in biomedical

research involving human/participants. A copy of the Declaration may be obtained from the researcher should you wish to review it.

9) CONFIDENTIALITY

All information obtained during this study will be regarded as confidential. Each participant that is taking part will be provided with a number e.g. 001. This will ensure confidentiality of information collected. Only the researcher, René de Beer, will be able to identify you as participant. Results will be published or presented in such a fashion that patients remain unidentifiable. The hard copies of the anonymous date and the sample we collected will be kept in a locked facility at the Department of Pharmacology, the University of Pretoria.

10) CONSENT TO PARTICIPATE IN THIS STUDY

	Initials
I have also received, read and understood the above written information about the study.	
I have had adequate time to ask questions and I have no objections to participate in this study.	
I am aware that the information obtained in the study, including personal details, will be anonymously processed and presented in the reporting of results.	
I understand that I will not be penalized in any way should I wish to discontinue with the study and that withdrawal will not affect my further treatments.	
I am participating willingly.	

_____

Participant's name (Please print)

_____ _____

Participant's signature Date

_____

Researcher's name (Please print)

_____ _____

Researcher's signature Date

b. Hypercholesterolaemic group

PARTICIPANT'S INFORMATION & INFORMED CONSENT DOCUMENT

STUDY TITLE:

Prevalence of *SLCO1B1* single nucleotide variations, and their association with statin intolerance in hypercholesterolaemic patients in Gauteng, South Africa

Protocol no: ……. Researcher: René de Beer

Dear Mr. / Mrs. .....

1) INTRODUCTION

You are invited to volunteer for a research study involving the medication called statins, that you are currently using as treatment for your high cholesterol. I am doing research for a master's degree purpose at the University of Pretoria. The information in this document is to help you to decide if you would like to participate or not. Before you agree to take part in this study you should fully understand what is involved. If you have any questions, which are not fully explained in this document, do not hesitate to ask the researcher. You should not agree to take part unless you completely understand all the procedures involved.

2) THE NATURE AND PURPOSE OF THIS STUDY

Statins are normally taken up by the liver where it is broken down so it can be excreted as waste. However, in some cases this up take does not happen as it should which mean the statins can no longer be broken down as it is supposed to. This causes some patients to have more severe adverse / side effects than others do.

This generally happens due to a type of change to the gene responsible for the uptake of statins by the liver. In this study we will be testing whether these genes are present in people in Gauteng.

3) EXPLANATION OF PROCEDURES AND WHAT WILL BE EXPEXTED FROM PARTICIPANTS.

Once you have signed the informed consent document, we will start by asking you a series of questions based on your statin treatment, which we obtained from the American College of Cardiology's (ACC) Statin Intolerance Application. We will then collect the data we require from your patient file. We will collect data on your high cholesterol history, such as when you were first

diagnosed, which statin you are taking and what dose, which other disease you have that might affect your high cholesterol, which other medications you are currently taking, and have taken in the past.

A 5ml (about a teaspoon full) tube of blood will be collected to determine whether you do have any of the genes and to determine your Creatine Kinase (CK) levels to see if there is an abnormal increase in your CK levels. CK is an enzyme commonly found in the heart, brain and muscles. It is secreted into the blood when muscle damage / muscle breakdown happens, which may be caused by statin therapy.

To determine whether you are suitable for the specific study you will have to be able to answer YES to all the following:

2. Are you older than 18 years?
3. Have you been diagnosed with high cholesterol?
4. Have you been on a stable and continuous atorvastatin or simvastatin dose12-weeks prior to this date?

To determine whether you are suitable for the specific study you will have to be able to answer NO to all the following:

4. Are you younger than 18 years?
5. Did you have any disruptions in your atorvastatin or simvastatin therapy within the preceding 12-week period?

4) POSSIBLE RISKS AND DISCOMFORTS INVOLVED

There are no medical risks associated with the study. The only possible risk and discomfort involved is associated with drawing of the blood, which can result in pain, bruising and bleeding from the site where the needle is inserted, but usually this does not last long, and resolves within minutes to hours.

5) POSSIBLE BENEFITS OF THIS STUDY

There will be no direct healing benefit for you from this specific research project. However, by determining the presence and the prevalence of SNVs, a better understanding of cholesterol therapy in a diverse South African population will be gained.

6) COMPENSATION

You will not be paid to take part in the study. There are no costs involved for you to be part of the study.

7) YOUR RIGHTS AS A RESEARCH PARTICIPANT

Your participation in this trial is entirely voluntary and you can refuse to participate or stop at any time without stating any reason. Your withdrawal will not affect your access to other medical care.

8) ETHICS APPROVAL

This Protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, telephone numbers 012 356 3084 / 012 356 3085 and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2013), which deals with the recommendations guiding doctors in biomedical research involving human/participants. A copy of the Declaration may be obtained from the researcher should you wish to review it.

9) CONFIDENTIALITY

All information obtained during this study will be regarded as confidential. Each participant that is taking part will be provided with a number e.g. 001. This will ensure confidentiality of information collected. Only the researcher, René de Beer, will be able to identify you as participant. Results will be published or presented in such a fashion that patients remain unidentifiable. The hard copies of the anonymous data and the samples we collected will be kept in a locked facility at the Department of Pharmacology, the University of Pretoria.

10) CONSENT TO PARTICIPATE IN THIS STUDY

	Initials
I have also received, read and understood the above written information about the study.	
I have had adequate time to ask questions and I have no objections to participate in this study.	
I am aware that the information obtained in the study, including personal details, will be anonymously processed and presented in the reporting of results.	
I understand that I will not be penalized in any way should I wish to discontinue with the study and that withdrawal will not affect my further treatments.	

I am participating willingly.	
I have received a signed copy of this informed consent agreement.	

_____

Participant's name (Please print)

_____ _____

Participant's signature Date

_____

Researcher's name (Please print)

_____ _____

Researcher's signature Date

Appendix 4

Ethics approval (Reference nr: 154/2019)

a. Original approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/28/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

26 April 2019

Approval Certificate
New Application

Ethics Reference No.: 154/2019

Title: Prevalence of SLCO1B1 single nucleotide variations, and their association with statin intolerance in hypercholesterolaemic patients in Gauteng, South Africa

Dear Ms R de Beer

The **New Application** as supported by documents received between 2019-03-25 and 2019-04-24 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2019-04-24.

Please note the following about your ethics approval:

- Ethics Approval is valid for 1 year and needs to be renewed annually by 2020-04-26.
- Please remember to use your protocol number (154/2019) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

Research Ethics Committee
Room 4-60, Level 4, Tuiwelopele Building
University of Pretoria, Private Bag X323
Arcadia 0007, South Africa
Tel: +27 (0)12 356 3084
Email: deepika.behari@up.ac.za
www.up.ac.za

Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

b. Annual renewal

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved od 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001782. OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 09/04/2023.

14 May 2020

Approval Certificate
Annual Renewal

Ethics Reference No.: 154/2019
Title: Prevalence of SLCO1B1 single nucleotide variations, and their association with statin intolerance in hypercholesterolaemic patients in Gauteng, South Africa

Dear Ms R de Beer

The **Annual Renewal** as supported by documents received between 2020-04-16 and 2020-05-13 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2020-05-13.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2021-05-14
- Please remember to use your protocol number (154/2019) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

Research Ethics Committee
Room 4-66, Level 4, Tuanskamer Building
University of Pretoria, Private Bag x323
Gezina 0031, South Africa
Tel: +27 (0)12 356 3084
Email: deepeka.behari@up.ac.za
www.up.ac.za

Fakulteit Gesondheidswetenskappe
Lefapha la Dicaense tša Maphelo

Appendix 5

MSc Committee approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

MSc Committee
School of Medicine
Faculty of Health Sciences

MSc Committee
7 May 2019

Prof P Soma
Department of Anatomy
Faculty of Health Sciences

Dear Prof,

Ms R de Beer, Student no 15048323

Please receive the following comments with reference to the MSc Committee submission of the abovementioned student:

Student name	Ms René de Beer	**Student number**	15048323
Name of study leader	Prof P Soma		
Department	Pharmacology		
Title of MSc	Prevalence of SLCO1B1 single nucleotide variations, and their association with statin intolerance in hypercholesterolaemic patients in Gauteng, South Africa		
Date of first submission	**March 2019**		
April 2019	• Thank you for submitting the revised protocol and new MSc form.		
May 2019	• Thank you for submitting the ethics approval certificate.		
Decision	This protocol has been approved. Ethics approval has been obtained. The internal and external examiners can be nominated and submitted to the MSc Committee six months prior to submission of the dissertation. Please ensure that the CV of the examiners includes: supervision, examination and publication records.		

Yours sincerely

Prof Marleen Kock
Chair: MSc Committee

MSc Committee, School of Medicine
Faculty of Health Sciences
University of Pretoria,
Private Bag X323
Pretoria 0001, South Africa
Tel +27 (0)12 319 2325
Fax +27 (0)12 323 0732

Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tSa Maphelo

Appendix 6

Letter from Biostatistician

Date: 05/03/2019

LETTER OF CLEARANCE FROM THE BIOSTATISTICIAN

This letter is to confirm that the student(s),

with the Name Rene de Beer, Studying at the University of Pretoria, discussed the Project with the title; An investigation of the background prevalence of SLCO1B1 single nucleotide polymorphisms, and their association with statin intolerance in hypercholesterolaemic patients in Gauteng, South Africa with me.

I hereby confirm that I am aware of the project and also undertake to assist with the Statistical analysis of the data generated from the project.

The analytical tool that will be used will be Hardy-Weinberg equilibrium test and exploratory SNP analysis for quantitative traits, haplotype-based logistic modelling and appropriate descriptive methods to indicate prevalence utilizing STATA 13 SE, StataCorp, Texas, USA, to achieve the objective(s) of the study.

Name: Prof Pieter WA Meyer Date: 5 Maart 2019

Signature: Tel: 012 319 2624

Department or Unit: Immunology

Prof PWA Meyer
Head of Department
Department of Immunology
TAD / Faculty of Health Sciences
NHLS / UP