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The prevalence of 3-hydroxy-3-methylglutaryl-Coenzyme A reductase variants in hypercholesterolaemic patients with statin intolerance

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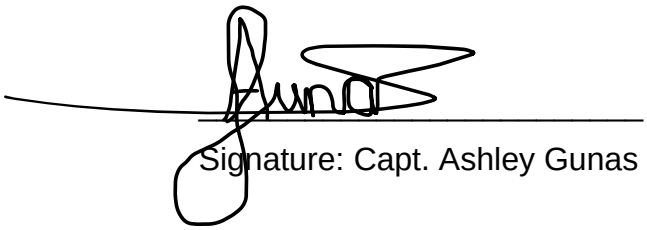

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Abstract

Background: High cholesterol raises the risk of heart disease, the leading cause of death around the world. The World Health Organization (WHO), reports that elevated cholesterol levels contribute to more than 2.6 million deaths, representing 4.5% of all global fatalities.

The advent of statins, known as HMG-CoA reductase inhibitors, has revolutionised the treatment of hypercholesterolaemia. These drugs function by blocking cholesterol production and by boosting the number of low-density lipoprotein (LDL) receptors in the liver, which leads to reduced cholesterol levels in the blood.

In the South African public healthcare system, simvastatin and atorvastatin are the recommended treatments for hypercholesterolaemia. When combined with dietary and lifestyle modifications, these medications have shown great potential to reduce LDL levels. Although statins are typically safe, some individuals may encounter side effects such as muscle-related symptoms. These adverse effects can occur when the drug is not metabolised and cleared efficiently, leading to an accumulation of the drug, and its metabolites, or prolonged exposure. This altered metabolism may be due to variants of proteins involved in drug metabolism pathways, such as 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR).

Therefore, this study explored the prevalence of HMGCR variants (*rs12916* and *rs17244841*) in healthy individuals compared to hypercholesterolaemic patients on statin treatment, as well as possible associations between intolerance and creatine kinase (CK) levels in patients diagnosed with hypercholesterolaemia and who are receiving statin therapy.

Methodology:
The study included 186 participants aged 19 to 75 for the *rs12916* analysis. Among them, 100 were healthy volunteers serving as the control group, while 86 were hypercholesterolaemic patients constituting the test group. The patients were treated with either simvastatin (67%) or atorvastatin (33%).

For the *rs17244841* analysis, 192 participants aged 19 to 75 were studied, comprising 100 healthy volunteers as the control group and 92 hypercholesterolaemic patients as the test group. In this group, 72% received simvastatin and 28% were on atorvastatin.

TaqMan® Polymerase Chain Reaction (PCR) genotyping assays were employed for the identification of single nucleotide variants (SNVs) *rs12916* and *rs17244841*. The CKM Human SingleStep ELISA® Kit was used to assess CK levels, and to evaluate the likelihood of statin intolerance, a quantitative questionnaire was utilised.

Results:
Among the 86 samples analysed from individuals with diagnosed hypercholesterolaemia, 14% exhibited a high risk of statin intolerance, 48% demonstrated moderate risk, and 38% showed low risk based on the *rs12916* variant. Similarly, among the 92 hypercholesterolemia samples analysed, 14% showed high risk, 48% moderate risk, and 38% low risk for statin intolerance associated with the *rs17244841* variant.

The genotype distribution for HMGCR *rs12916* in the test group and for *rs17244841* in both the test and control groups did not align with the Hardy-Weinberg equilibrium (HWE) (p<0.05). However, the genotype distribution for HMGCR *rs12916* in the control group was consistent with the HWE.

The *rs12916* variant was significantly more common in the control group (42%) than in the test group (36%), (Odds ratio (OR)=0.5318; 95% confidence interval (CI)=0.3543 to 0.8081; p=0.0034).

The frequency of *rs17244841* was more common in the control group (61%) than in the test group (50%), (OR)=0.7846; 95% CI=0.5093 to 1.204; p=0.2774).

No significant association was found between the risk levels (low, moderate, and high) and statin intolerance for the genotype frequencies for *rs12916* (OR=0.5318, 95% CI=0.3543 to 0.8081, relative risk (RR)=0.7439, 95% CI=0.6066 to 0.9036, p=0.0034) or for *rs17244841* (OR=0.7846, 95% CI=0.5093 to 1.204, RR=0.888, 95% CI=0.7103 to 1.091, p=0.2774) when based on calculated statin intolerance risk (low risk vs.

moderate to high risk). Relative risk quantifies the likelihood of statin intolerance in individuals with these genetic variants compared to those without them.

Although patients on simvastatin had higher mean CK levels (25041 pg/mL) compared to atorvastatin users (17650 pg/mL), this difference was not statistically significant (p=0.2111).

There was no correlation found between the *rs12916* variant and statin intolerance or CK levels (p=0.3658), a positive correlation was observed between the *rs17244841* variant and both the severity of statin intolerance and elevated CK levels (p=0.2314).

Conclusion: This study is the inaugural report on the occurrence of *rs12916* and *rs17244841* in the South African population. Consistent with earlier studies, this research suggests that patients treated with simvastatin experience statin intolerance than those treated with atorvastatin. From a clinical perspective, these findings contribute to the expanding field of pharmacogenomics and precision medicine. Identifying population-specific genetic risk factors for statin intolerance can facilitate more personalised treatment strategies, enabling healthcare practitioners to tailor statin prescriptions based on an individual's genetic profile. This approach has the potential to improve patient adherence, reduce adverse effects, and ultimately enhance cardiovascular health outcomes.

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Acronym Guide:

ABC	ATP-binding cassette
ACC	American College of Cardiology
AHA	American Heart Association
APOB	Apolipoprotein B
ATP	Adenosine triphosphate
BMI	Body Mass Index
CETP	Cholesterol ester transfer protein
CHD	Coronary heart disease
CI	Confidence interval
CK	Creatine kinase
CRU	Clinical Research Unit
CoQ10	Co-enzyme Q10
CVD	Cardiovascular disease
CWG	Canadian Working Group
CYP450	Cytochrome P450
DDI	Drug-drug Interaction
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ETC	Electron transport chain
Farnesyl-PP	Farnesyl pyrophosphate
Geranylgeranyl – PP	Geranyl – geranyl pyrophosphate
Geranyl – PP	Geranyl pyrophosphate
HDL	High-density lipoprotein
HMGCR	3-hydroxy-3-methylglutaryl-coenzymeA reductase
HWE	Hardy-Weinberg equilibrium
LDL	Low-density lipoprotein
LDL-C	LDL cholesterol
LDLR	LDL-receptor
MDR1	Multidrug resistance-associated protein 2
MGB	Minor groove binder
MIN	Minutes
NFQ	Nonfluorescent quenchers

NLA	National Lipid Association
OATP	Organic anion transporting polypeptide
OD	Optical density
OR	Odds ratio
PCR	Polymerase chain reaction
PCSK9	Proprotein convertase subtilisin/kexin type 9
P-gp	P-glycoprotein
PTVD	Premature triple-vessel disease
ROS	Reactive oxygen species
RR	Relative risk
RT	Room temperature
S	Seconds
SAMS	Statin associated muscle symptoms
SBAH	Steve Biko Academic Hospital
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
STATE	Statin Adverse Treatment Experience
($t_{\frac{1}{2}}$)	Half-life
T2DM	Type 2 diabetes mellitus
UGT	Uridine 5'-diphospho-glucuronosyltransferases
UNL	Upper normal limit
USAGE	Understanding Statin Use in America and Gaps in Education
VD	Volume of distribution
VLDL	Very low-density lipoprotein
WB	Whole blood
WHO	World Health Organization

Chapter 1: Overview and Literature Review

1.1 Identifying the research problem

Statins are the preferred treatment for hypercholesterolaemia due to their demonstrated potency in reducing low-density lipoprotein (LDL) levels while they have a favourable safety profile. By blocking the enzyme HMGCR, statins prevent the creation of mevalonate, which is a key precursor in the cholesterol synthesis pathway in the liver while also having described anti-inflammatory effects in endothelial cells (1-4).

The metabolism of medications (like simvastatin and atorvastatin) plays a vital and rate-limiting role in drug pharmacokinetics. Genetic differences, including single nucleotide variants (SNVs) in genes related to drug metabolism (5, 6), can significantly influence drug metabolism resulting in impeded drug clearance, increased drug concentrations, and potential toxicity.

Such increased drug concentrations and associated toxicity may be causing adverse effects such as muscle inflammation, weakness, and other less common effects.

Therefore, genetic testing could potentially identify hypercholesterolaemic patients who are more susceptible to developing statin intolerance due to activity-altering variants in metabolic genes. This information could also be used to personalise lipid-lowering therapy decisions for these individuals resulting in improved outcomes.

1.2 Background

Although there have been significant strides in prevention, early diagnosis, and management, cardiovascular diseases (CVDs) still exert substantial socioeconomic pressures on healthcare and communities. In the last thirty years, the worldwide prevalence of CVDs has increased by 93% (rising from 271 million in 1990 to 523 million in 2019), and the death rate from these diseases has risen by approximately 54% (increasing to 18.6 million in 2019 from 12.1 million deaths in 1990). This represents approximately a third of all deaths worldwide each year (7) because increased levels of blood cholesterol pose a significant risk for CVDs. Hence reducing LDL levels can significantly mitigate this risk, particularly in patients with

hypercholesterolaemia (1).

While various medications, including HMGCR inhibitors, cholesterol absorption inhibitors, fibrates, nicotinic acid, and bile-acid binding resins are utilised to decrease LDL levels, (8-10) statins are the most frequently prescribed (1, 11).

1.3 Hypercholesterolaemia Therapy

1.3.1 Hypercholesterolaemia

Cholesterol in the body comes from two main sources: either synthesized within our cells or acquired from consuming high-cholesterol foods like eggs, dairy, poultry, and meat (5). Cholesterol is a crucial molecule for the survival of most life forms, serving as a fundamental structural component of eukaryotic cells. It also serves as a precursor to various steroid hormones and is essential for the production of bile salts and vitamin D (5).

Cholesterol is not soluble in water (hydrophobic), so it must be carried through the circulatory system by specialised transporter lipoproteins. The functions of lipoproteins depend on the lipoprotein density, for example, very low-density lipoproteins (VLDL) deliver lipids and cholesterol from the intestines to surrounding tissues whereas cholesterol is transported back to the liver for metabolism by high-density lipoproteins (HDLs) (12, 13).

Unhealthy lifestyle habits, such as being physically inactive, consuming a high-fat diet, and being obese, can increase the risk of elevated LDL levels and decreased HDL levels. Additional risk factors encompass smoking, type 2 diabetes mellitus (T2DM), high blood pressure, and a family history of heart disease or stroke (14).

Hypercholesterolaemia is classified as familial (genetic) and non-familial (acquired). In individuals with familial hypercholesterolaemia, high LDL cholesterol (LDL-C) levels are mainly caused by a delay in removing LDL from the bloodstream. (15-18).

1.3.2 Role of cholesterol in the pathogenesis of cardiovascular disease

Elevated levels of LDL-C contribute to the accumulation of cholesterol within the walls of arteries, a key factor in the development of atherosclerosis. Atherosclerosis is a progressive condition where fatty deposits (plaques) build up on the arterial walls, narrowing the arteries and reducing blood flow. Over time, this can lead to the formation of blood clots, which may result in heart attacks, strokes, or other cardiovascular events. The process begins when elevated LDL-C levels circulate in the bloodstream and penetrate the endothelial lining of blood vessels. This triggers an inflammatory response, with immune cells (like macrophages) attempting to clear the cholesterol. However, these immune cells can become overwhelmed and form foam cells, which contribute to plaque formation. As plaques grow, they may rupture, leading to the release of clot-promoting substances, and increasing the risk of severe cardiovascular events. High LDL-C levels lead to the accumulation of LDL particles in the endothelial cell layer lining blood vessels. This disrupts the normal function of endothelial cells, which are responsible for regulating vascular tone and promoting smooth blood flow. Dysfunctional endothelial cells increase the permeability of the arterial wall, allowing LDL particles to enter and accumulate in the intima. Through targeted interventions such as statin therapy, lowering LDL-C can significantly reduce the risk of cardiovascular events, underscoring the importance of managing lipid levels in the prevention and treatment of CVD (19-21).

Table 1: Mutations in three genes that cause familial hypercholesterolaemia.

Genes	**Effects of mutation**
LDL-receptor gene (LDLR)	Impaired hepatic clearance of circulating LDL particles (22).
Apolipoprotein B-100 gene (APOB)	Reduction in ligand binding to the LDL receptor.
Proprotein convertase subtilisin/kexin type 9 (PCSK9)	An increased affinity of PCSK9 for the LDLR leads to quicker removal of the LDLR by directing it to the lysosome for degradation in the liver, which consequently results in elevated plasma LDL-C levels (15-17).

1.4 Pharmacology

1.4.1 Molecular Composition of Statins

At the molecular level, statins consist of three components: a structure similar to the target enzyme (HMGCR), a complex hydrophobic ring system, and various side groups attached to the rings. The hydrophobic ring structures help statins bind to the enzyme, while the side groups influence their solubility (1). The chemical structures of simvastatin and atorvastatin, the two most commonly prescribed statin drugs in South Africa, are illustrated in Figure 1. Simvastatin is a type 1 inhibitor containing a decalin ring. Atorvastatin is a type 2 inhibitor containing a fluorophenyl group and methylethyl group (23, 24).

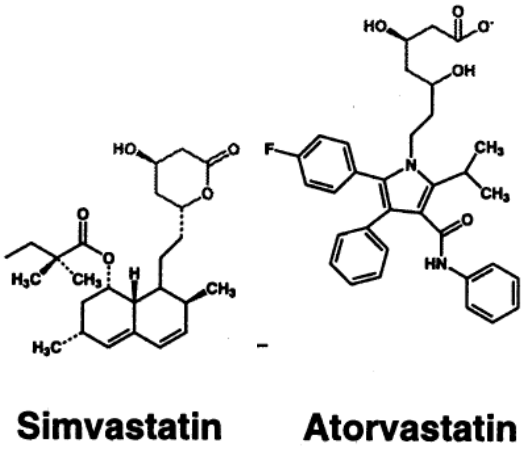

Figure 1: Chemical structures of simvastatin and atorvastatin (1, 25-27)

Simvastatin has a core structure based on a hexahydronaphthalene ring with a side chain containing a lactone ring, while atorvastatin features a more complex structure with a pyrrole ring. Both statins share a similar region that mimics the HMG-CoA molecule, allowing them to inhibit the HMGCR enzyme.

1.4.2 Action pathway

Statins work by blocking HMGCR, an essential enzyme for cholesterol production, through competitive inhibition. (Figure 2) (25, 28-30). Without the presence of statins, HMG-CoA is metabolised into mevalonate, which then leads to the production of farnesyl pyrophosphate (Farnesyl-PP). Farnesyl-PP is then used to produce several substances, including cholesterol. However, when statins are present, they inhibit HMGCR, preventing the conversion of HMG-CoA to mevalonate, a critical step in cholesterol biosynthesis. Statins block the binding of HMG-CoA to HMGCR by fitting into the enzyme's substrate-binding sites, inducing structural changes due to their rigid hydrophobic rings. Specifically, statins interact with a "cis-loop" structure in the

enzyme's active site, competitively inhibiting cholesterol synthesis in hepatocytes and thereby reducing LDL levels (1, 23).

As a consequence of lowering LDL levels, hepatocytes compensate by increasing the expression of LDL receptors, leading them to internalise more LDL from the plasma, further reducing circulating LDL levels (1, 31).

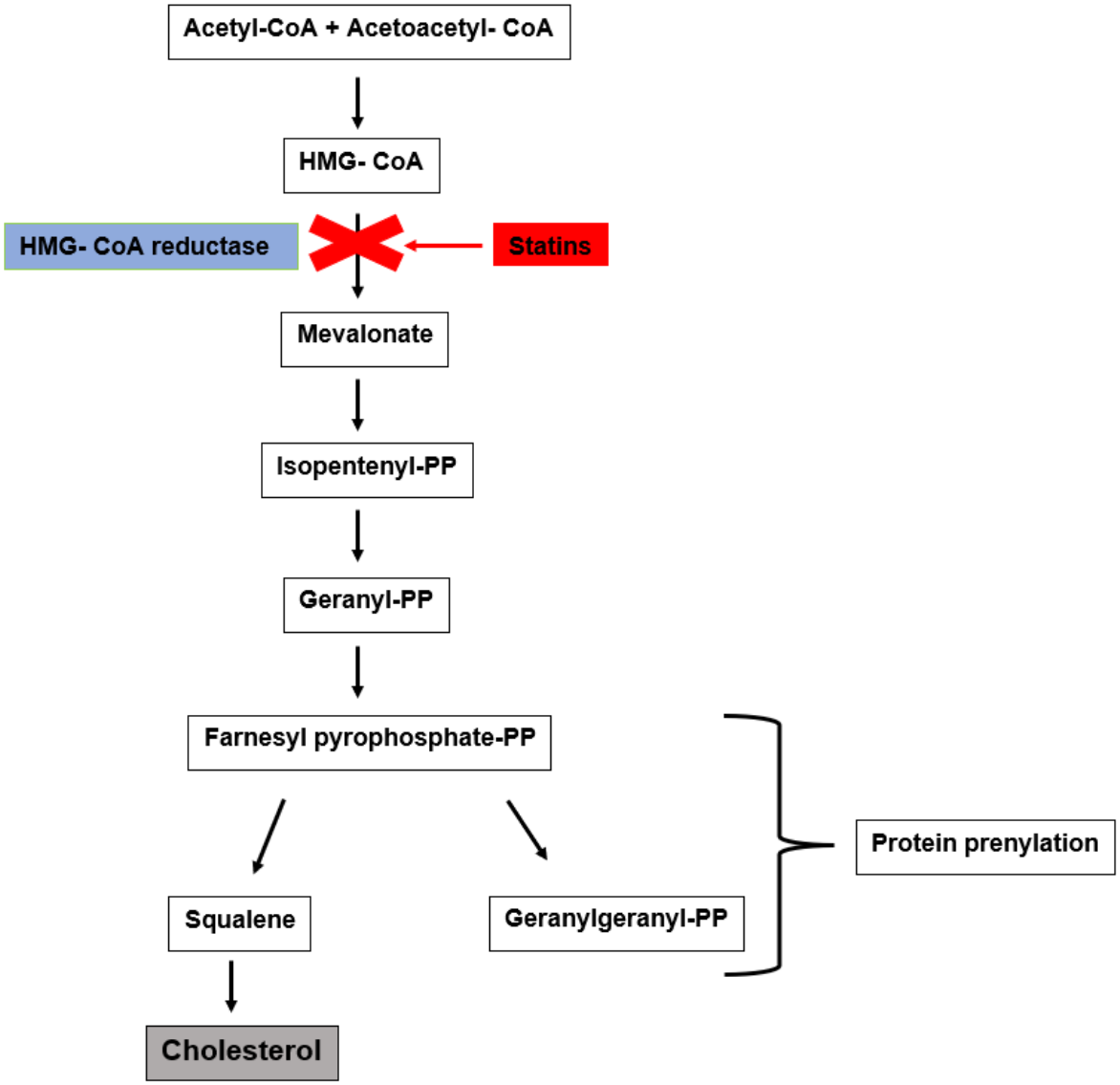

Figure 2: Mechanism of action of statins (25, 28-30)

The upstream steps of the mevalonate pathway begin with acetyl-CoA, which undergoes condensation reactions to form HMG-CoA. This is then converted into mevalonate by HMG-CoA reductase, the key regulatory enzyme of the pathway. Mevalonate undergoes a series of phosphorylation and decarboxylation reactions, leading to the formation of isopentenyl pyrophosphate (IPP) and

dimethylallyl pyrophosphate (DMAPP), which serve as essential isoprenoid precursors.

In the intermediate steps, IPP and DMAPP combine to form geranyl pyrophosphate (GPP), which subsequently converts into farnesyl pyrophosphate (FPP). FPP serves as a crucial branching point for multiple biological pathways. It contributes to squalene synthesis, ultimately leading to cholesterol and steroid biosynthesis. Additionally, FPP plays a role in protein prenylation, a modification essential for cell signaling and function. Another important derivative is dolichol, which is necessary for glycoprotein synthesis.

The downstream effects of the pathway extend beyond cholesterol production. Cholesterol is a precursor for essential molecules such as steroid hormones, bile acids, and vitamin D. Furthermore, the pathway generates ubiquinone (coenzyme Q), a vital component of the mitochondrial electron transport chain, ensuring efficient energy production. Lastly, prenylated proteins derived from this pathway regulate key cellular processes, including cell growth, differentiation, and membrane anchoring.

1.4.3 Pharmacokinetics of statins

a. Absorption

Taken orally, simvastatin and atorvastatin are primarily ingested in the duodenum through specialised mucosal surfaces and the transporter P-glycoprotein (P-gp). This process allows these drugs to enter the bloodstream and exert their cholesterol-lowering effects systemically (32-34). The range of absorption is 30% to 98% (30, 35). Lactone prodrugs such as simvastatin need to be hydrolysed by carboxylesterases to its functional state, β-hydroxy-acid, to produce their pharmacological effects (27, 36). The lactone form of atorvastatin is metabolised into two distinct byproducts: 2-hydroxy- and 4-hydroxy-atorvastatin acid (37). Most statins are first metabolised in the liver during their initial passage, which results in lower levels throughout the body (1, 28).

Table 2: Pharmacokinetic profiles of simvastatin and atorvastatin (14, 25, 27, 29, 30, 38, 39)

	Simvastatin	Atorvastatin
Solubility	Lipophilic	Lipophilic
IC_{50} HMGCR (nM)	1 – 2 (active metabolite)	1.16
Oral absorption (%) (30)	60 – 85 (food has no effect)	30 (decreased by food intake)
Bioavailability (%)	< 5	12
Cmax (ng/mL)	10 – 34	27 – 66
Tmax (hours) (40)	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 Cytochrome P450 (CYP450)	CYP 3A4	CYP 3A4
Transporter involved	Organic anion transporting polypeptide 1B1 (OATP1B1)	OAT1B1
Standard daily dose (mg)	10 – 40	10 – 80
Prodrug	Yes	No
Origin	Semi-synthetic	Synthetic

IC_{50}: indicator of a drug's effectiveness, this measure shows the amount of drug required to reduce a biological process by 50%, thereby reflecting its potency.

Cmax: peak concentration of a drug in the blood or target organ following administration of a dose.

Tmax: duration required for a drug to achieve its peak concentration after administration, for drugs that need to be absorbed.

Half-life: duration required for the amount of a drug's active ingredient in the body to drop to half.

Volume of distribution: represents the volume of plasma required to account for the entire amount of drug present in the patient's body.

b. Distribution

As lipophilic drugs, simvastatin and atorvastatin are transported across membranes through passive diffusion (27). The moderate volume of distribution (VD ~5.4 L/kg) of atorvastatin suggests widespread distribution throughout tissues (36). Additionally, both drugs are extensively bound to proteins, with over 90% of each drug attached to plasma proteins. Therefore, only a low percentage of the drug is unbound or pharmacologically active. This high protein-binding potential leads to a prolonged elimination half-life ($t_{\frac{1}{2}}$) of 0.5–3 hours (36).

c. Metabolism

Atorvastatin and simvastatin undergo metabolism primarily by the CYP3A4 isoenzyme in the liver (phase 1 metabolism). Its resulting hydroxylated acid metabolites for atorvastatin (2-hydroxyatorvastatin and 4-hydroxy-atorvastatin) and simvastatin (ß-hydroxy acid along with its 6'-hydroxy, 6'-hydroxymethyl, and 6'-exomethylene

derivatives) exert inhibition on 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), consequently leading to a reduction in circulating levels of LDL-C.

Of these metabolites, 2-hydroxy-atorvastatin is the most prevalent. Lactone metabolites, produced from atorvastatin's acid forms through the action of uridine 5'-diphospho-glucuronosyltransferases (UGTs), lack inhibitory activity against HMGCR. Instead, these lactone metabolites are linked to statin-related myopathy (41).

The UGT family, which includes subfamilies including UGT1A, UGT2A, and UGT2B, is involved in phase II drug metabolism. The glucuronidation process, which transforms small lipophilic compounds into more hydrophilic metabolites to aid in their easy excretion from the body, is facilitated by the UGTs' enzymes. Species of statin lactone are thought to be more myotoxic than the acid versions of the same compounds (38).

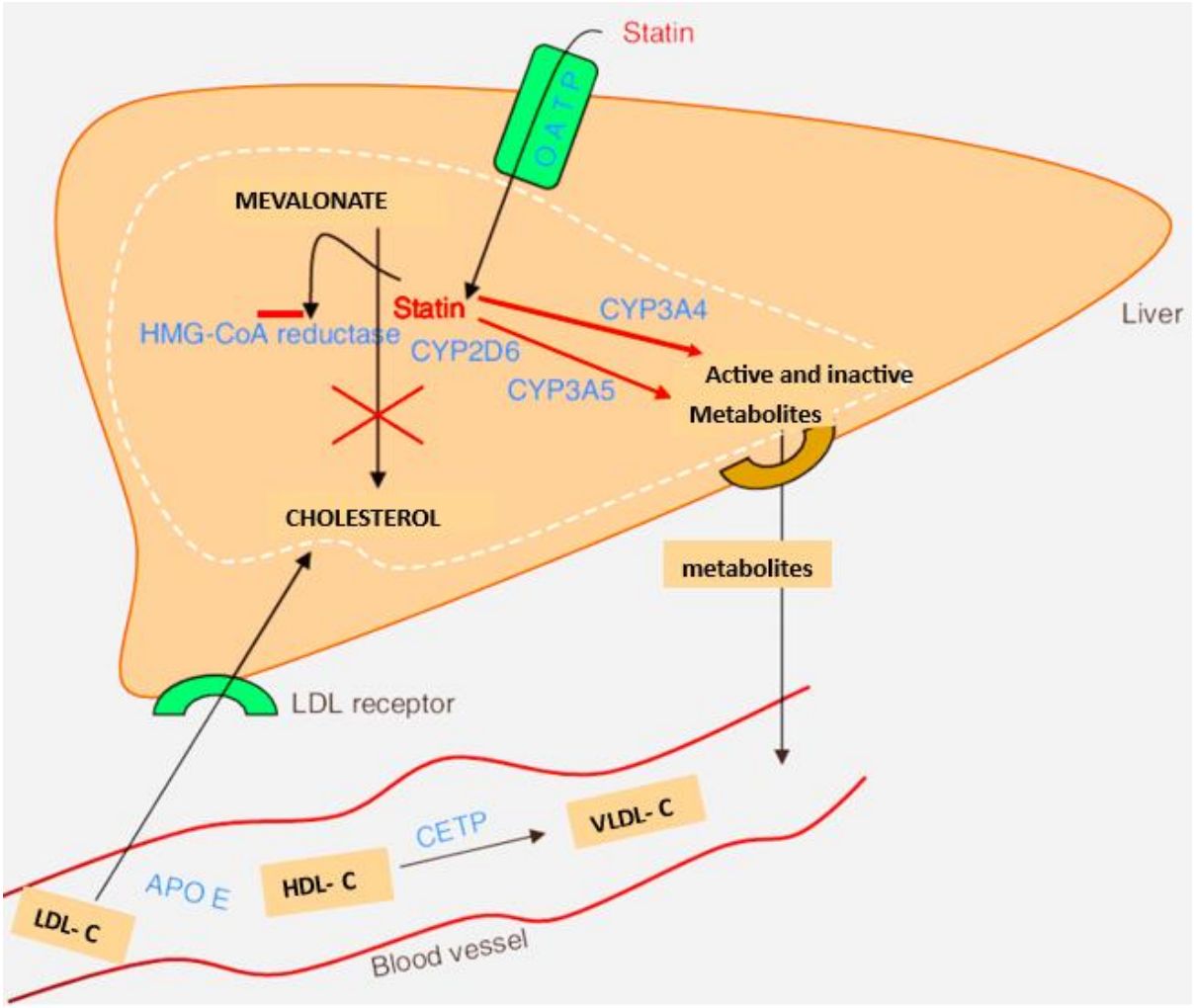

Figure 3: Metabolic pathways of statins (42)

Key molecular pathways important for cholesterol production, as well as the metabolism and distribution of statins are illustrated. Enzymes and transporters affected by genetic variations that impact the effectiveness of statins are shown in blue. Atorvastatin and simvastatin, the represented statins, are predominantly metabolised by CYP3A4, with minor involvement of CYP3A5 and CYP2D6 pathways. The CYP enzymatic pathways comprise the polymorphic genes, a single nucleotide polymorphism (SNP) found to be linked with muscle damage caused by statins. An illustration of a blood vessel beneath the liver highlights the impact of genetic variations on two key proteins apolipoprotein E (APO E) and cholesteryl ester transfer protein (CETP). These proteins play crucial roles in lipid transport, particularly in returning cholesterol and other lipids to the liver for processing. Variations in the genes encoding APO E and CETP can influence how effectively lipids are transferred, potentially affecting cholesterol metabolism and cardiovascular health.

d. Excretion

Around 75-85% (36) of the statin metabolites are excreted via the bile, mediated by ATP-binding cassette (ABC) transporters such as P-gp and multidrug resistance-associated protein 2 (MDR1), (1, 27), (35) while the water-soluble metabolites such as atorvastatin acyl glucuronide are eliminated through the kidneys. (36) Except for atorvastatin, the elimination $t_{\frac{1}{2}}$ of statins appears to be short (0.5–3 hr), this short half-life minimises the potential for drug buildup in the bloodstream with continuous dosing, thereby lowering the likelihood of severe toxic effects (36, 40).

1.4.4 Statin Intolerance

Statin intolerance refers to the incapacity to endure the necessary dose of statin medication to effectively lower cholesterol production and the level of LDL (43). This intolerance may stem from various adverse effects associated with statin use, such as muscle weakness, pain, headaches, sleep disturbances, dyspepsia, erectile dysfunction, arthritis, alopecia, nausea, gynecomastia and rashes (44).

Warden *et al.* (2023) (45) estimated that statin-associated muscle symptoms (SAMS) affect roughly 10% of individuals undergoing statin treatment, with a range of occurrence from 5% to 25%.

Muscle-related adverse events associated with statin use account for up to 72% of all reported statin adverse events (46). Creatine kinase is an enzyme critical in energy metabolism, particularly in muscle tissues. It catalyses the conversion of creatine and adenosine triphosphate (ATP) into creatine phosphate and adenosine diphosphate

(ADP). This reaction is essential for maintaining a readily available reserve of high-energy phosphate in cells, especially in tissues with high energy demands like skeletal muscles, the heart, and the brain. CK levels increase in the bloodstream when muscle cells are damaged, making CK a useful marker in blood tests for detecting muscle injuries or conditions such as muscular dystrophy, rhabdomyolysis, or a heart attack. Adverse muscle events can take many different forms. For example, asymptomatic myopathy (an increase in creatine kinase (CK) levels without any symptoms) can occur, as can myositis (a condition where CK levels exceed the upper normal limit [UNL] by more than five times) and, in more severe cases, rhabdomyolysis (a condition where CK levels rise to more than ten times the UNL). Clinical rhabdomyolysis is typified by a notable increase in serum creatinine of at least 0.5 mg/dl, along with myoglobinuria or substantial myonecrosis, as per the guidelines set forth by the National Lipid Association (NLA) (44).

In addition, the use of statins has been linked to a higher risk of developing T2DM, liver toxicity, and possible hemorrhagic stroke (46-49).

Several factors can contribute to or exacerbate statin intolerance, including intense physical activity, (50) genetic predisposition such as hypothyroidism, chronic kidney disease, drug-drug interactions, smaller body size, and being of female gender. Nonetheless, the most crucial factor contributing to this adverse effect is the dosage of the statin (51, 52) (Table 3). Despite its effective lipid-lowering potential, simvastatin is the most widely associated with adverse effects (53, 54) (55). Simvastatin is regarded as the first-line treatment for hypercholesterolaemia and in cases where simvastatin is contra-indicated atorvastatin is prescribed. At present, plasma statin levels are not routinely monitored. If a patient exhibits intolerance to the prescribed dose, healthcare providers may consider lowering the amount administered of the current statin or switching to an alternative statin (partial intolerance). In more severe cases, discontinuing statin therapy altogether may be necessary (complete intolerance) (50, 51).

Table 3: The comparative dosage between simvastatin and atorvastatin

Percentage decrease of low-density lipoprotein	Simvastatin	Atorvastatin
< 20 %	-	-
20-30%	10 mg	-
30-40%	20 mg	10 mg
< 40%	40 mg	20 mg

1.4.5 Prevalence of Statin Associated Muscle Symptoms

One of the main reasons why patients stop taking statins results from muscle-related side effects. The Statin Adverse Treatment Experience (STATE) study looked into how statins were used and why people stopped taking them. The causes of statin discontinuation in 1,500 high-cholesterol patients who had taken a statin within the previous 24 months had at least one statin-related symptom within the previous six months. 332 respondents (22.1%) discontinued their statin treatment, primarily because they were intolerant of the adverse effects or were unhappy with them. Comparing those who stopped taking their statins with those who continued, the former group had more severe symptoms, mostly musculoskeletal (56).

The Understanding Statin Use in America and Gaps in Education (USAGE) study examined the practices, attitudes, beliefs, and behaviors of individuals currently using or who have previously used statins. This study involved 10,138 participants, including 8918 present users and 1220 former users of statins. 60% of individuals who had previously used statins reported experiencing muscle-related side effects and 35% of those who were currently using them which is higher than that reported in the study by Warden *et al.* (2023) (45). Applying these findings to the entire USAGE participant population suggests that 7.5% (756 out of 10,138) of respondents discontinued statin treatment because of its side effects (56).

Statin-induced myopathy has been found to occur at 1.5–5% of subjects in randomised clinical studies. Real-world clinical circumstances, on the other hand, point to a higher frequency, suggesting that some detrimental effects may go unnoticed in trials due to specific exclusion criteria. For example, in the PROSISA trial, which included lipid specialists from 23 carefully chosen Italian lipid clinics, 9.6% of the individuals had SAMS. This frequency was substantially greater among younger people (10.5%

compared to 8.2% in those 65 and older, $p < 0.0001$), women (10.7% compared to 8.7% in men, $p < 0.0001$), and physically active people (11.5% compared to 9.8% in those who were sedentary, $p = 0.0127$).

In a six-month double-blind, randomised controlled trial with 420 healthy volunteers, 9.4% of those taking daily atorvastatin 80 mg reported experiencing myalgia, while only 4.6% of participants in the placebo group reported similar symptoms.

In the Anglo-Scandinavian Cardiac Outcomes Trial-Lipid Lowering Arm (ASCOT-LLA), a double-blind randomised controlled trial, muscle-related adverse events occurred at similar rates between patients receiving daily atorvastatin 10 mg (1.26%) and those on a placebo (1%). These rates were significantly lower compared to those reported in other studies (57).

An estimated 30% of people receiving statin therapy are thought to have some degree of SAMS. 10.5% of participants in the Prediction of Muscular Risk in Observational Conditions (PRIMO) study—which comprised 7,924 patients on high-dose statins for a minimum of three months—reported having symptoms related to their muscles.

Conversely, the National Health and Nutrition Examination Survey reported a 23% prevalence of muscle-related symptoms among statin users. Other registries have reported prevalence rates as high as 29%. A meta-analysis including 37,939 statin patients found that myalgia occurred at a rate of about 9.5%, though some observational studies suggest that the rate of SAMS may approach 20% (52).

From October 1, 2018, to January 31, 2021, a study was carried out in Jordan at the National Center for Diabetes, Endocrinology, and Genetics (NCDEG) involving 400 patients prescribed statins. The study found that the rate of statin-induced myopathy was 27.4% as stated by Abed *et al.* (2022) (58).

This data suggest that SAMS significantly contributes to statin discontinuation and non-adherence in real-world settings. This is evident from the STATE and USAGE surveys, where a substantial proportion of patients discontinued therapy due to muscle-related side effects. The real-world impact is further highlighted by studies like the one conducted by Abed *et al.* (2022) (58), which reported a 27.4% occurrence rate of statin-induced myopathy, emphasising the clinical relevance of SAMS in everyday practice.

1.4.6 Statin Drug Interaction

A drug-drug interaction (DDI) occurs when one medication affects another in a way that deviates from the expected effects of each medication when taken separately. Such interactions can lead to alterations in the efficacy or toxicity of one or both of the involved medications (59).

The occurrence of potential drug-drug interactions varies widely, ranging from 15% to 80%, depending on the clinical context and the quantity of prescribed medications (60).

The primary metabolic pathway for statins involves hepatic cytochrome P450 (CYP450) enzymes, specifically CYP3A4 (1, 27, 36). Therefore, statins are susceptible to inhibition by drugs with a greater binding affinity for CYP3A4, which can result in impaired breakdown of statins and an increased risk of intolerance (61). Examples of drugs that act as CYP3A4 inhibitors, include cyclosporin, amiodarone, itraconazole, and clarithromycin. These inhibitors reliably increase the systemic exposure of statins such as atorvastatin and simvastatin (38).

1.5 Mechanism of Statin-Induced Myopathy

Although the specific mechanism behind SAMS are not clear, mitochondrial dysfunction is perhaps the leading mechanism contributing to statin-induced myopathy (62).

Myofiber death, or myopathy, can manifest as muscle weakness, pain, or even rhabdomyolysis, a severe condition characterised by muscle breakdown. Lactone metabolites, do not contribute to HMGCR inhibition but are implicated in statin-induced muscle toxicity (41). The mitochondria generate the majority of cellular ATP especially in tissues such as muscle that demand high levels (63-66). Mitochondria produce ATP via oxidative phosphorylation using the electron transport chain (ETC), which produces reactive oxygen species (ROS) like superoxide (O_2^-) as a by-product. While moderate ROS levels play a crucial role in myofiber processes such as repair, an imbalance in favour of ROS can lead to oxidative stress and cell damage (63-67). Statins have been found to decrease mitochondrial respiration, which results in a higher generation of ROS and decreased ATP production (63-67). As a result, coenzyme Q10 (CoQ10) supplementation has been suggested as a possible treatment for SAMS (63-67).

1.6 Genetic polymorphisms

With the aid of genotyping technologies and the development of detailed SNV maps of the human genome, we are able to investigate complex genetic traits such as drug response and multi-factorial diseases. Mutations create hereditary variation, which is vital for the survival and adaptability of the human species. Unrelated people share 99.5% of their deoxyribonucleic acid (DNA) sequence, while the differences in the remaining genetic sequence within a population are referred to as polymorphisms (68, 69).

Pharmacogenetic research has identified numerous polymorphisms that contribute to the varied responses to statins, influencing their pharmacodynamics by altering the molecular drug target (70).

1.6.1 HMG-CoA Reductase versus statin efficiency

Despite statin treatment, a significant number of cardiovascular events continue to occur, suggesting ongoing underlying pathophysiological factors. Differences in how people respond to statins are affected by both genetic and non-genetic factors. Alternative splicing is regarded as a key mechanism that contributes to differences in drug response. Research on this mechanism might uncover new pathways affecting cholesterol balance and statin effectiveness, potentially lowering the risk of CVD (3).

Given its crucial role in hepatic regulation of plasma cholesterol, HMGCR is a principal candidate gene for investigating genetic sequence variations. The *HMGCR* gene which encodes HMGCR, plays a crucial role in the pharmacogenomics of statin (2).

HMGCR is located on chromosome 5q13.3-q14 (71) and comprises 20 exons, each roughly 25 kb in length. This gene produces a 4475 bp transcript encoding 88 amino acids and is expressed widely across the body (72).

Haplotype 7 is linked to alternative splicing that excludes exon 13 (31). It includes two intronic SNPs (*rs17244841* and *rs3846662*) that do not affect a typical coding SNV but rather influence the expression of the *HMGCR* gene. HMGCR is directly inhibited by statins and experiences alternative splicing of exon 13, which encodes part of the enzyme's statin-binding domain (73). The altered catalytic site of HMGCR makes the binding of statins difficult (74).

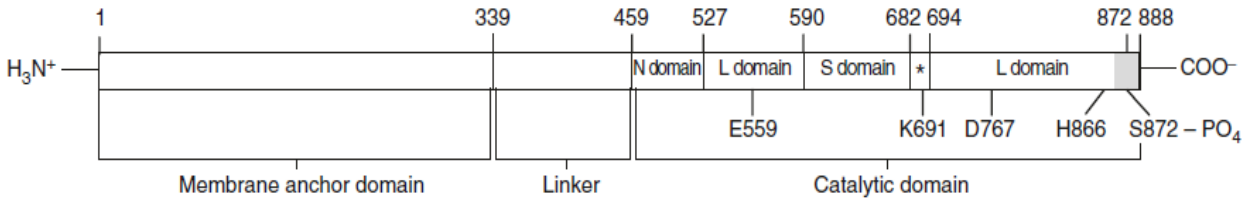

Figure 4: A schematic representation of the Human HMGCR enzyme (75)

The linker domain, membrane-anchor domain, and the catalytic domain are the three unique domains that make up the human HMGCR protein. The L domain and the linker domain are connected by the N domain. A portion of the L domain is involved in binding HMG-CoA, whereas the S domain is in charge of binding NADP(H). An * indicates a cis-loop connects the NADPH-binding region and the HMG-CoA-binding region.

As illustrated in Figure 4, the enzyme HMGCR enzyme is anchored to the endoplasmic reticulum by its membrane domain. Its catalytic domain contains active residues, while the L domain contributes to the substrate binding site and acts as a lid-like structure covering the active site when the statin is bound. When statins bind, they create a shallow hydrophobic pocket, which displaces the L domain and inhibits the enzyme. The HMG-like part of the statin molecule extends into the catalytic site (75).

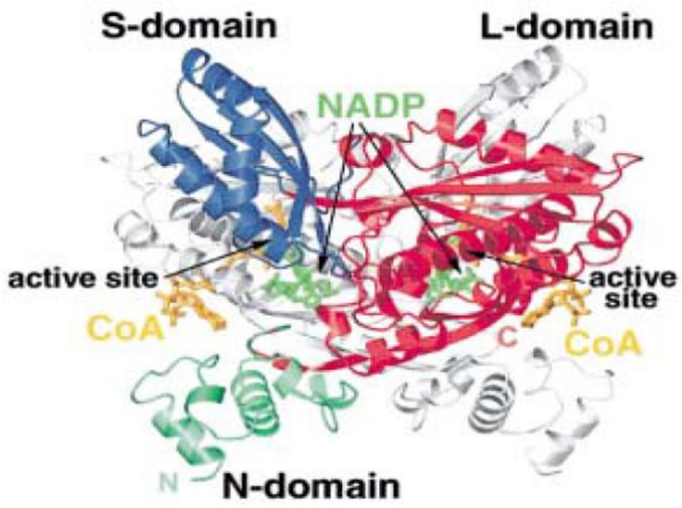

Figure 5: Ribbon diagram of the Human HMGCR monomer structure (76)

The large central L-domain (red), small S-domain (blue), the small, helical amino-terminal N-domain (green), CoA (orange), and NADP(H) (green). The two active sites are marked, where the statin binds to one of these active sites, inhibiting its function. A monomer's three domains all work together to actively form a dimer. The equivalent amino acids in the neighbouring monomer are encircled by the N-terminal residues of the L-domain, creating a β-sheet that is characterised by nine main chain hydrogen bonds between the two monomers.

If the active site of HMGCR (Figure 5) is altered by the presence of variants, this will make statin binding more difficult.

Research on genetic variations in the *HMGCR* gene among the South African population is limited. Therefore, this study aimed to assess their occurrence in both healthy individuals and hypercholesterolaemic patients receiving statin therapy in Gauteng, South Africa. The study specifically investigated the prevalence of HMGCR variants (*rs12916* and *rs17244841*) in a randomly selected cohort of healthy individuals and explored potential associations between these variants and statin intolerance, as well as CK levels, in hypercholesterolaemic patients treated with statins.

The aims and objectives of this study were to:

1. To assess the occurrence of HMGCR *rs12916* and *rs17244841* variants in (a) a healthy control group, and (b) patients diagnosed with hypercholesterolaemia who have been on a stable dose of simvastatin or atorvastatin for at least 12 weeks, using Polymerase Chain Reaction (PCR) with TaqMan® SNV genotyping assays.
2. To assess the severity of statin intolerance in hypercholesterolaemic patients, the Statin Intolerance Application from the American College of Cardiology (ACC) was utilised. The study aimed to investigate potential correlations between symptoms and signs of statin intolerance and specific genetic variants.
3. To measure CK levels using Enzyme-Linked Immunosorbent Assay (ELISA) and analyse potential correlations between symptoms and signs of statin intolerance, alongside assessing serum CK levels in hypercholesterolaemic patients with specific genetic variants.

Chapter 2: Materials and Methods

This clinical study sought to establish the baseline prevalence of HMGCR *rs12916* and *rs17244841* in a randomly chosen sample of the general population in Gauteng, South Africa. Additionally, it sought to explore potential associations between these HMGCR genetic variants and statin intolerance among patients diagnosed with hypercholesterolaemia.

2.1 Ethics

This protocol underwent review by the Ethics Committee (Faculty of Health Sciences) and was approved by the Committee on 02nd February 2024 (Ethics Reference No.: 489/2023, Appendix E). Prior to the collection of samples or data (Appendices C and D), each participant provided signed informed consent. Confidentiality and anonymity were ensured through the use of numerical codes as patient identifiers. Healthcare professionals collected the blood samples used for analysis. While this specific research project does not provide direct therapeutic benefit to participants, it aims to enhance understanding of hypercholesterolaemia treatment in the varied South African population by determining and examining the frequency of *rs12916* and *rs17244841* HMGCR variations.

2.2 Methodological approach

This cross-sectional, observational study was designed to examine the incidence of HMGCR variants (*rs12916* and *rs17244841*) in Gauteng, South Africa, and to investigate possible connections between the treatment group's statin intolerance and these variations.

2.3 Setting

This study was set at the University of Pretoria, in the Gauteng Province of South Africa. Patients with hypercholesterolaemia were enrolled from the Clinical Research Unit (CRU) at the University of Pretoria and other medical centers in Gauteng. These public institutions are typically funded by the government, catering to a socioeconomically disadvantaged population reliant on public healthcare services.

Healthy individuals or controls were recruited from the community, shopping malls, clinics, university students, the university community (personnel) and volunteers from public domains including but not limited to local gymnasiums. Flyers or posters with

details about the study were displayed within local gymnasiums. These included information on the purpose of the study, eligibility criteria (such as being a healthy individual), and how to sign up or participate.

2.4 Participant Recruitment

Sample size and recruitment

Eighty-six hypercholesterolaemic patients receiving statin treatment for *rs12916* at CRU were enrolled during their follow-up visits. Additionally, one hundred individuals from the general population served as controls.

Ninety-two hypercholesterolaemic patients receiving statin treatment for *rs17244841* at CRU were enrolled during their follow-up visits. Similarly, one hundred individuals from the public served as controls.

Controls were defined as individuals from the public who had neither a diagnosis of hypercholesterolaemia nor elevated cholesterol levels. Patients with hypercholesterolaemia were diagnosed by qualified clinicians to be included in the study group. The control group had no prior exposure to statins. Cholesterol and CK levels were not assessed in the control group, as their role was solely to provide background prevalence information on the gene variants.

Ancestry is an essential factor in understanding genetic predispositions, particularly in pharmacogenomics and disease susceptibility. This study, conducted in Tshwane, Gauteng, considers the unique diversity of the South African population, which includes individuals of African and European descent. Given the known population-specific genetic variations, ancestry was factored into the data analysis to ensure accurate interpretation of findings. By stratifying participants according to ancestry, the study aimed to identify potential correlations between genetic variants (HMGCR *rs12916* and *rs17244841*) and statin intolerance, thereby improving the relevance of the results to the local population (77, 78).

Inclusion criteria for participants with hypercholesterolaemia undergoing statin treatment

A patient with hypercholesterolaemia could participate if they met all of the following

eligibility criteria:

1. Written informed consent for participation in the study was required before any study-related procedures could begin (Appendix C)
2. Patient ≥ 18 years
3. Diagnosed with hypercholesterolaemia by a clinician
4. On a consistent and stable dose of atorvastatin or simvastatin for minimum of 12 weeks

Exclusion criteria for individuals with hypercholesterolaemia undergoing statin therapy

A hypercholesterolaemia patient was excluded from participation if they met any of the following criteria:

1. Reluctant to provide consent
2. Patient ≤ 18 years
3. Any disruption of atorvastatin or simvastatin therapy within the preceding 12-week period
4. Patient with liver disease

Inclusion criteria for control participants

A participant was deemed eligible if they fulfilled all of the following criteria:

1. Written informed consent for participation in the study was required before any study-related procedures could begin (Appendix D)
2. Subject ≥ 18 years

Exclusion Criteria for control participants

A participant was deemed ineligible if any of the following exclusion criteria applied:

1. Reluctant to provide consent
2. Subject ≤ 18 years
3. Subject with abnormal cholesterol levels or have been previously diagnosed with hypercholesterolaemia
4. Subject on any lipid-lowering therapy

2.5 Assessment and Laboratory Analysis

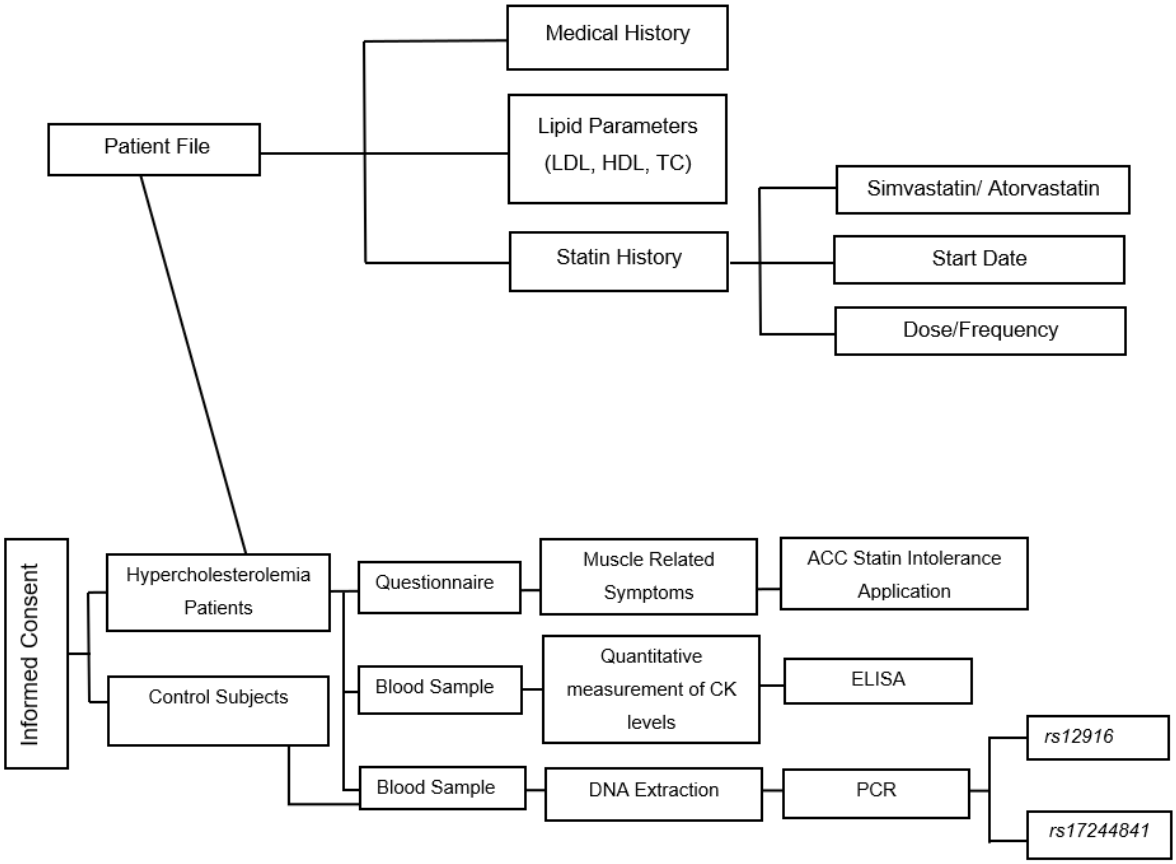

Figure 6: Diagrammatic overview of the study's experimental design

2.5.1 Questionnaire:

The ACCs Statin Intolerance Application represents a significant advancement in cardiovascular care by addressing the complex issue of statin intolerance. This innovative tool is designed to assist healthcare professionals in identifying and managing patients who encounter difficulties with statin therapy. The application provides a comprehensive and individualised approach, guiding clinicians through a systematic assessment of patient symptoms, medical history, and concurrent medications. It offers evidence-based recommendations for statin rechallenge when appropriate and outlines alternative lipid-lowering strategies for patients unable to tolerate statins. With a focus on shared decision-making, the application includes educational resources for patients, fostering better communication between healthcare providers and individuals facing statin intolerance. Continuous updates ensure that the ACCs Statin Intolerance Application remains aligned with the latest research and guidelines, offering a dynamic and invaluable resource in optimising

cardiovascular risk management for patients with diverse needs. Within this application, muscle symptoms are categorised on a scale from 0 to 10, where 0-2 indicates mild, 3-5 signifies moderate, and 6–10 denotes severe intolerance. Patients' statin intolerance on their current therapy was assessed using a validated questionnaire, utilising the ACCs Statin Intolerance Application. The risk of statin intolerance was determined using the calculator available in the ACC Statin Intolerance app (Appendix A).

2.5.2 Sample collection (blood):

Following informed consent, 2 x 5ml whole blood (WB) samples were collected in a citrate tube by venipuncture from each patient and control participant. One tube was used to prepare plasma by centrifugation. A total of 1 ml of WB was transferred into a sterile microcentrifuge tube and stored at -80ºC until deoxyribonucleic acid (DNA) extraction.

2.5.3 DNA extraction:

Genomic DNA was isolated from 150 µl of WB using the Quick-DNA™ Miniprep Plus Kit (Zymo Research D3025).

To the 150 µl of WB, 200 µl of cell lysis buffer and 20 µl of Proteinase K were added in a 1.5 ml microcentrifuge tube. The tubes were mixed thoroughly for 10-15 seconds (s) and were incubated at 55ºC for 10 minutes (min).

Equivolume (370 µl) genomic binding buffer was added to the sample and vortexed for 10-15s. The homogenized mixture was transferred into a Zymo-Spin™ IIC-XL column in a collection tube and was centrifuged at 12 000 x g for 1 min.

The flow-through from the collection tube was discarded following standard biohazard waste disposal protocols, ensuring proper handling and containment of biological material, and the column was placed into a new collection tube. A total volume of 400 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 700 µl g-DNA wash buffer was added to the spin column and was centrifuged at 12 000 x g for 1 min. The collection tube was emptied, 200 µl g-DNA wash buffer was added to the spin columns and it was 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, 75 µl DNA

elution buffer was added directly on to the matrix. The sample was incubated for 5 min at room temperature (RT) and was centrifuged at 12,000 x g for 1 min to elute the DNA. The eluted DNA was stored at -80ºC and quantified using spectrophotometry (Nanodrop 2000c, Thermo-Fisher, South Africa). The DNA was standardized to a concentration of 10 ng/µl and was used for TaqMan® single nucleotide variant (SNV) genotyping assays2.5.4 TaqMan® SNV.

Genotyping Assays

In the Polymerase Chain Reaction (PCR) process using TaqMan® SNV genotyping assays from Thermo-Fisher, South Africa, probe-based assays were employed for the detection of gene variations in HMGCR (*rs12916* and *rs17244841*).

These probes differ from regular primers in two key ways. Firstly, they lack a free hydroxyl group required for DNA polymerase extension (79). Secondly, they are covalently linked to two molecules: a fluorescent reporter at the 5' end and a quencher at the 3' end (79). These molecules are joined by a DNA sequence (79). The reporter molecule indicates the amount of product generated during the reaction, while the quencher molecule suppresses the fluorescence of the reporter (79). As long as the reporter and quencher molecules remain near, the reporter does not fluoresce. This state facilitates fluorescence resonance energy transfer (FRET), where the energy from the excited reporter is transferred to the quencher (79).

Quality Assurance of TaqMan® SNV genotyping assay

TaqMan® assays used allele-specific oligonucleotide probes that were designed to specifically bind to the target allele. This design ensured high specificity and reduced the likelihood of false positives, making TaqMan® SNV genotyping highly reliable.
The assays allowed for discrimination between different alleles of an SNV. By using two different fluorophores on the probes, one for each allele, the presence of specific alleles was accurately detected and differentiated.

TaqMan® assays detected even low amounts of target DNA. The real-time PCR technology used, allowed for precise quantification of the target DNA, making them suitable for applications where high sensitivity is crucial.

TaqMan® SNV genotyping assays offer high reproducibility due to their robust and standardised methodology and are commonly used in clinical settings for diagnostic purposes, such as identifying disease-associated mutations or predicting drug responses based on an individual's genetic makeup. In addition, two samples from each genotype (wildtype homozygous, variant homozygous and heterozygous) were randomly selected, and the PCR product was sequenced at Inqaba Biotechnology for validation of the genotypic change.

Table 4: HMGCR variants

SNV Identifier	Nucleotide base change	Sequence
rs12916	**C/T**	CAGTGCAATTGACCTTCTCCCTCAC**[C/T]**CCTGCC AGTTGAAAATGGATTTTTA[47]
rs17244841	**T/A**	ACTACATCTCAAAAAAAAATTTTTT**[T/A]**AAATCCTT TATATTACAATCATACT[47]

HMGCR variants include two key SNVs *rs12916* and *rs17244841* within the *HMGCR* gene. These variants have been studied for their potential role in statin intolerance.

Polymerase Chain Reaction cycling conditions for TaqMan® genotyping involved three steps, the process began with a polymerase activation step, where the reaction mixture was heated to 95ºC for 10 min for 1 cycle. Following polymerase activation, the denaturation step took place. This step was repeated for 40 cycles, with each cycle consisting of heating the reaction mixture to 95ºC for 15 seconds. After denaturation, the annealing and extension step occurred. Similar to denaturation, this step was also repeated for 40 cycles. During each cycle, the reaction mixture was maintained at 60ºC for 1 min.

2.5.5 Quantitative measurement of Creatine Kinase in plasma (*CKM Human SingleStep ELISA® Kit*)

Creatine kinase levels was estimated using the CKM Human SingleStep ELISA® Kit (Elab Science, catalogue number: E-EL-H1433). Plasma samples were thawed at RT and vortexed for 5 min. Samples were then diluted into Sample Diluent NS and assayed. A total volume of 50 µl of each sample was added to the appropriate wells

in the 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 x *g*. 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. 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 – 10 min on a plate shaker set at 400 x *g*. 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 intensity was recorded at 450 nm. A standard curve was derived from the known concentration of serially diluted CK vs the optical density (OD). A linear equation was generated and used to solve for the unknown CK concentrations in patient plasma.

2.5.6 Statistics and data management

The frequency of HMGCR variants and their influence on statin toxicity were assessed using stepwise multiple regression models. The processed data, detailed below, will be archived for at least 15 years on the Research Data Repository Platform at the University of Pretoria. Additionally, both raw and statistically analysed data will be stored on Prof. A. Phulukdaree's Google Drive and in the online repository Mendely Data. A biostatistician was consulted that is endorsed by the University of Pretoria (Appendix F).

A power analysis was performed using the disease prevalence in the specific region, with a sample size of at least 100 per test group and 1.5 times that number for the control group, given the absence of previous studies on this gene variant in our population. Descriptive statistics were used, including mean, standard deviation, and range for continuous data, and frequency. Proportions with 95% confidence intervals (CIs) were used for categorical variables.

Proportions were compared to characterise genes and SNVs across various patient groups. The Hardy-Weinberg equilibrium (HWE) was assessed using the Court-lab calculator, and association analysis was performed using Fisher’s Exact Test (80).

The questionnaire data enabled stratification of hypercholesterolaemic patients into categories of mild, moderate, and severe adverse toxicity. The biochemical marker CK served as an indication of muscle toxicity, which was measured using an ELISA. The data generated from the ELISA were conducted in duplicate for each sample and the absolute quantification was obtained using a standard curve as described in the methods. The severity of toxicity was subsequently correlated with CK values. Variations in CK levels among statin-intolerant patients, categorised by severity, were assessed using a two-tailed non-parametric Mann Whitney test in GraphPad Prism v 9.0 software.

Logistic regression and proportion comparison tests were implemented to assess qualitative metrics, including the severity of statin side effects. A significance level of 0.05 was established, and the analytical methodology was the same as that employed for quantitative measurements.

Chapter 3: Results

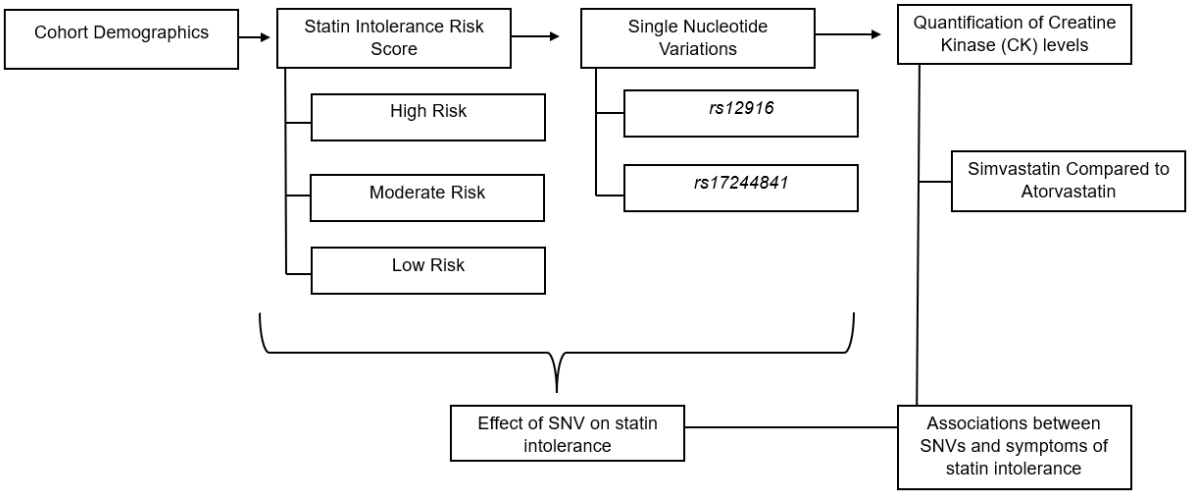

Figure 7: Graphical representation of the results

3.1 Cohort demographics (53, 81)

The cohort demographics for the patient group differed slightly for each variant based on the number of successful reactions and reproducible data obtained in the genotyping assay (Table 5). The median age for participants in the control group is 49 years and the median age for hypercholesterolaemic participants is 51 years. The male to female ratio was kept consistent among the 3 groups at a 1:1 ration, however the simvastatin to atorvastatin users were in a 1:2 ratio.

Table 5: Participant (cohort) demographics for *rs12916*

	Control (n= 100)	**Patients (rs12916 study) (n= 86)**	**Patients (rs17244841 study) (n= 92)**
Median age (range) in years	49 (20 – 75)	51 (19 – 75)	51 (19 – 75)
Gender			
Male (%)	49 (49%)	42 (49%)	44 (48%)
Female (%)	51 (51%)	44 (51%)	48 (52%)
Ethnicity			
African (n)	83	72	79
European (n)	17	14	13
Co-existing conditions			
Rheumatoid arthritis (n)	0	2	2
Psoriatic arthritis (n)	0	3	4
Osteoarthritis (n)	0	7	8
Type 2 Diabetes Mellitus (n)	0	11	12
Hypothyroidism (n)	0	2	2
Low body mass index (n)	0	2	2
Treatment			
Atorvastatin (n)	0	28 (33%)	26 (28%)
Simvastatin (n)	0	58 (67%)	66 (72%)

3.2 Statin intolerance risk score

For this study, all patients diagnosed with hypercholesterolaemia on statin therapy completed an extensive questionnaire, which was modified from the ACC Statin Intolerance Application. (Appendix A). This survey gathered information on the intensity and frequency of muscle symptoms, patient features, and medical history that may contribute to the risk of developing statin intolerance.

Table 6: The risk distribution for statin intolerance for hypercholesterolaemia patients included in each genotype study

Risk	***rs12916* study**		***rs17244841* study**	
	Atorvastatin n *(%)*	**Simvastatin n *(%)***	**Atorvastatin n *(%)***	**Simvastatin n *(%)***
High Risk	2 *(2)*	10 *(11)*	1 *(1)*	12 *(13)*
Moderate Risk	14 *(15)*	27 *(29)*	13 *(14)*	31 *(34)*
Low Risk	12 *(13)*	21 *(23)*	12 *(13)*	23 *(25)*

The distribution of patients receiving atorvastatin and simvastatin were stratified based on the outcomes generated from the application as high, moderate, or low risk for intolerance, with the highest percentage of patients indicating symptoms related to moderate risk of toxicity for both treatments (Table 6).

In the *rs12916* study (Table 7), 17 % of patients treated with atorvastatin were identified as moderate to high risk for statin intolerance, and 13% at low risk. For those on simvastatin, the risk distribution was 40% moderate to high risk and 23% low risk. Four of the 86 participants with comorbidities (arthritis and T2DM) showed higher risk for statin sensitivity. All four individuals were determined to have a moderate risk of acquiring statin intolerance after undergoing additional evaluation using the statin intolerance questionnaire. These four patients also included two who had the *rs12916* variation.

In the *rs17244841* study (Table 7), 13% of patients treated with atorvastatin were identified as moderate to high risk for statin intolerance, and 13% at low risk. For those on simvastatin, the risk distribution was 45% moderate to high, and 25% low risk. In essence, the highest percentages of statin intolerance risk were found in patients on simvastatin. Four of the 92 participants had conditions like arthritis and T2DM that could worsen or cause statin sensitivity. All four individuals were determined to have a moderate risk of acquiring statin intolerance after undergoing additional evaluation using the statin intolerance questionnaire. These four patients also included three who carried the *rs17244841* variation.

3.3. Single nucleotide variations

Hardy-Weinberg Population genetics' equilibrium principle defines the steady frequency of genotypes and alleles in a population across generations when selection, mutation, migration, and genetic drift are not present. It asserts that under specific circumstances, genotype and allele frequencies within a population will hold steady across successive generations. Random mating, a sizable population, the absence of mutation, migration, and natural selection are some of these requirements. Deviations from HWE may be a sign of population-level evolutionary processes.

The genotype distribution for HMGCR *rs12916* in the patient group and for *rs17244841* in both the patient and control groups did not align with HWE ($p< 0.05$). The genotype distribution for HMGCR *rs12916* in the control group conformed to HWE ($p> 0.05$).

The *rs12916* polymorphic variant was found to be significantly more prevalent among individuals in the control group, with the odds of a healthy individual having a 1.88-fold risk of carrying the variant compared to hypercholesterolaemia patients: odds ratio (OR) of 0.5318 (95% CI: 0.3543 to 0.8081, $p= 0.0034$). The 95% confidence interval (CI) for the OR ranged from 0.3543 to 0.8081, indicating that this finding is statistically significant, and the *p*-value of 0.0034 suggests that the difference is unlikely to have occurred by chance. The presence of the *rs17244841* variant was similar in the control group (Table 9) and in hypercholesterolemia patients (OR: 0.7846, 95% CI: 0.5093 to 1.204, $p= 0.2774$).

Table 7: Genotype and allele distribution for healthy controls and hypercholesterolaemia

rs12916		
Genotype	**Control n (%)**	**Patients n (%)**
CC	19 *(19)*	38 *(44)*
CT	39 *(39)*	17 *(20)*
TT	42 *(42)*	31 *(36)*
Allele	**n (*frequency*)**	**n (*frequency*)**
C	77 *(0.39)*	93 *(0.54)*
T	123 *(0.61)***	79 *(0.46)*
rs17244841		

Genotype	**Control n (%)**	**Patients n (%)**
TT	21 *(21)*	19 *(21)*
AT	18 *(18)*	27 *(29)*
AA	61 *(61)*	46 *(50)*
Allele	**n (*frequency*)**	**n (*frequency*)**
T	60 *(0.30)*	65 *(0.35)*
A	140 *(0.70)*	119 *(0.65)*

Where CC: homozygous wildtype, CT: heterozygous, TT: homozygous variant *rs12916*; and where TT: homozygous wildtype, AT: heterozygous, AA: homozygous variant *rs17244841. Where *p<0.005 for the odds of being a carrier of the variant allele as calculated using the Fischer's exact test.*

3.4 Quantification of creatine kinase

An enzyme called creatine kinase, abundantly present in tissues and cells that need energy, such as the heart and skeletal muscles, is regarded as the gold-standard biomarker for detecting and monitoring muscle damage.

To measure serum CK levels in patients with the *rs12916* variant undergoing statin therapy, 39 patients were selected based on a pre-defined criteria. Creatine Kinase levels were computed as described in Chapter 2, section 2.5.5, and the data were recorded in pg/mL. The samples selected maintained a consistent ratio of patients on simvastatin to those on atorvastatin, with 13 patients on atorvastatin and 26 on simvastatin.

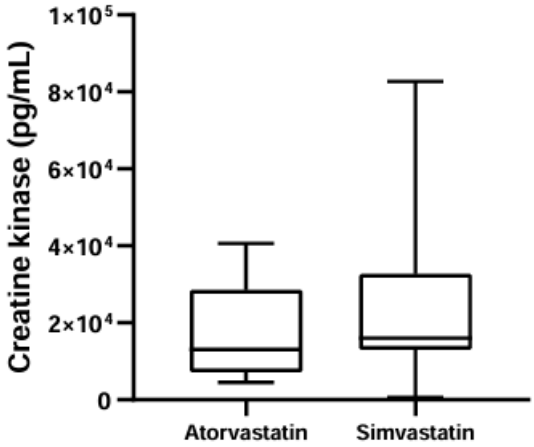

Figure 8: Levels of CK in patients taking atorvastatin and simvastatin

On average, patients on atorvastatin had 17650 pg/mL CK, while those on simvastatin had 25041 pg/mL CK, as depicted in the box and whisker plot (Figure 8, *p=0.2111*).

Stratification of statin intolerance risk and creatine kinase levels by genotype

Table 11 shows the distribution of statin intolerance risk stratified by genotypes. For *rs12916* the greatest difference was the high percentage of heterozygous individuals presented with a moderate to high risk of statin intolerance. For *rs17244841* the risk for intolerance was similar regardless of the genotype as homozygous wildtype, homozygous variant, or heterozygosity.

Table 8: Statin intolerance risk scores stratified by genotypes

rs12916	**Moderate to High Risk (%)**	**Low Risk (%)**
TT	50,0	50,0
CC	36,9	63,2
CT	83,4	16,7
rs17244841	**Moderate to High Risk (%)**	**Low Risk (%)**
AA	47,4	52,6
TT	44,4	55,6
AT	50,0	50,0

The distribution of statin intolerance risk and *rs17244841* genotypes is depicted in Table 11. For individuals with the AA genotype, 10,5% were considered high risk, 36,8% were moderate risk, and 52,6% were low risk. Those with the TT genotype had a similar distribution, with 11,1% at high risk, 33,3% at moderate risk, and 55,6% at low risk. In contrast, individuals with the TA genotype had no high-risk cases; instead, half were at moderate risk and the other half at low risk.

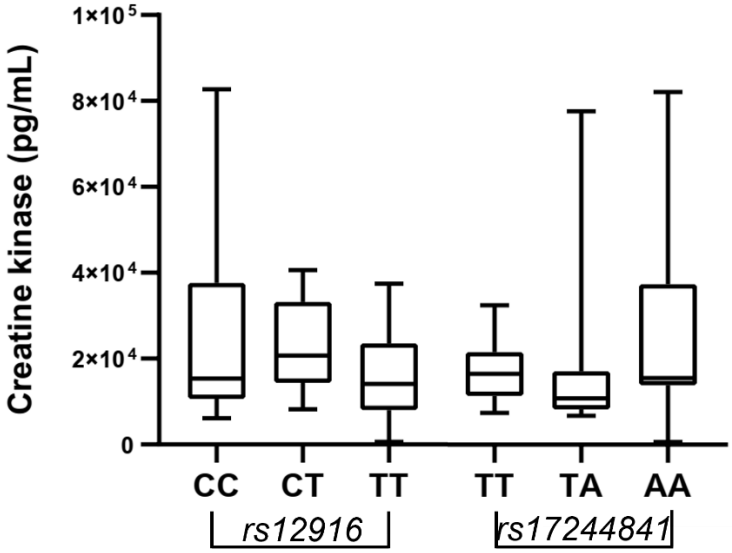

Figure 9: Creatine kinase levels stratified by genotype. Where CC: homozygous wildtype, CT: heterozygous, TT: homozygous variant *rs12916*; and where TT: homozygous wildtype, AT: heterozygous, AA: homozygous variant *rs17244841.*

Figure 9 illustrates the average CK levels among individuals stratified by *rs12916* genotypes did not differ significantly (TT: 16501 pg/mL, CT: 22914 pg/mL, and CC: 26948 pg/mL, p=0.3658). Creatine kinase levels stratified by *rs17244841* genotypes AA: 25891 pg/mL, TT: 17613 pg/mL, and TA: 19410 pg/mL) also did not vary significantly (p=0.2314).

Chapter 4: Discussion

Cardiovascular diseases continue to be a primary cause of death globally, highlighting the importance of effective strategies for managing cholesterol. Statins, a widely prescribed class of medications known for their ability to lower cholesterol, have transformed cardiovascular treatment.

Simvastatin and atorvastatin are two examples of statins that work by preventing the liver's enzyme HMGCR from performing its function. Statins efficiently lower blood cholesterol levels by altering this important route, especially LDL-C, or "bad cholesterol." This mechanism underpins their primary role in preventing atherosclerosis, a condition characterised by the accumulation of cholesterol-rich plaques in arteries (65, 82-84).

Simvastatin, one of the earliest statins, has demonstrated efficacy in lowering LDL-C and reducing the risk of cardiovascular events. However, its use is not without challenges. Some individuals may experience SAMS, ranging from mild muscle pain to the more severe rhabdomyolysis. Atorvastatin, another widely prescribed statin, shares these benefits and challenges. Although some individuals may tolerate statins better, statin intolerance is still a concern. Management strategies include dose adjustments, switching to a different statin, or exploring alternative lipid-lowering medications (45, 85).

Thus, this study examined the prevalence of two HMGCR variants, *rs12916* and *rs17244841*, in a South African cohort, and between healthy controls compared to patients with hypercholesterolemia. Two hundred seventy eight participants were included in the study, of which 100 were healthy controls, 86 were hypercholesterolaemic patients receiving statin treatment for *rs12916* and 92 hypercholesterolaemic patients receiving statin treatment for *rs17244841* (Table 5).

In 2013, the ACC and the American Heart Association (AHA) jointly formulated a guideline for managing blood cholesterol levels to mitigate atherosclerosis. All hypercholesterolaemic patients enrolled into the study was requested to complete the questionnaire adapted from the ACC’s Statin Intolerance application.

For the *rs12916* study, 29% of patients receiving simvastatin had a moderate risk to statin intolerance compared to only 11% showing a high risk to statin intolerance. For patients on atorvastatin, 2% were at high risk for statin intolerance and 15% at moderate risk. For the *rs17244841* study, 34% of patients receiving simvastatin had a moderate risk to statin intolerance compared to only 13% showing a high risk to statin intolerance. For patients on atorvastatin, 1% were at high risk for statin intolerance and 13% at moderate risk (Table 6).

This study examined the occurrence of two HMGCR variants, *rs12916* and *rs17244841*. It was found that the *rs12916* variant appeared more frequently in the control group, with 42% of participants carrying the variant, compared to only 36% in the group of patients with hypercholesterolaemia. This suggests that the *rs12916* variant might be less common among individuals with hypercholesterolaemia (Table 7).

Zhao *et al.* (2021) (86) identified *rs12916* as a significant genetic marker for the onset of premature triple-vessel disease (PTVD). This is notable because hypercholesterolaemia is a major factor in PTVD development, indicating that *rs12916* is a strong genetic marker for the risk of developing hypercholesterolaemia (86).

Abd El- Kader *et al*. (2019) (87) conducted research on Egyptian patients carrying the *rs12916* C/T. The C allele had a frequency of 56, while the T allele had a frequency of 136. There was no correlation between the *rs12916* C/T single nucleotide polymorphism (SNP) genotype in the *HMGCR* gene and the reduction in LDL-C levels.

The *rs17244841* variant was present in 61% of the control group and 50% of the test group (Table 7). In the largely Caucasian Pravastatin Information/CRP Evaluation (PRINCE) population, the estimated allele frequency for *rs17244841* variant was 0.965 (96.5%) for allele A and 0.035 (3%) for allele T. This suggests that in this population, the A allele is much more common, while the T allele is rare. Additionally, *rs17244841* was found to be significantly linked with decreased total and LDL-C response to simvastatin treatment in the Cholesterol and Pharmacogenomics (CAP) population, which included 335 African Americans and 596 Caucasians (72). Soko *et al*. (2016) (88) estimated the allele frequency for *rs17244841* T allele for the following

populations: African populations (Nigeria: 0.09 (9%), Kenya: 0.09 (9%), and African American: 0.10 (10%)), European populations (Finnish: 0.01 (1%) and British: 0.03 (3%)), and Asian population (Chinese: 0.00 (0%) and Japanese: 0.02 (2%)).

The *rs12916* variant of the *HMGCR* gene is located on chromosome five and belongs to the category of 3' untranslated region (3' UTR) variants (86). Kettunen *et al*. (2019) (89) showed that reducing *rs12916* expression in individuals of European descent could lower the risk of coronary heart disease (CHD), a finding that contradicts our results in the South African population. In the South African population, *rs12916* expression does not affect CHD. Statins, which inhibit HMGCR, are crucial for managing CHD. Additionally, Swerdlow *et al*. (2015) (90) found a link between *rs12916*, weight gain associated with statin use, and an increased risk of type 2 diabetes. These studies highlight the importance of *rs12916* in CHD, emphasising its connection to both the risk of CHD and the adverse effects related to statin therapy.

In the present study of the *rs12916* variant, 33% of patients were on atorvastatin and 67% on simvastatin (Table 5). Among these, 7% of atorvastatin users and 17% of simvastatin users had a high risk of statin intolerance. Among these patients, five had the TT genotype; four were taking simvastatin, and one was taking atorvastatin and no significant difference in the levels of plasma CK was found.

This aligns with findings from Abd El- Kader *et al*. (2019), (87) who determined that there is no notable connection between the SNP *rs12916* C/T and the response to atorvastatin. In the current study, individuals with the *rs12916* CC genotype had the highest average CK value of 26948 pg/mL followed by the CT genotype with 22914 pg/mL. The TT (variant) genotype had the lowest average CK value of 16501 pg/mL, all of which were not significantly different from each other. Newman *et al*. (2003) (91) found that patients on different doses of atorvastatin experienced a low incidence of adverse events in both clinical and placebo studies, which is consistent with our findings (Figure 9).

For *rs17244841*, 28% of patients were taking atorvastatin and 72% were taking simvastatin (Table 5). Of this 4% of patients had a high risk of statin intolerance for atorvastatin when compared to 18% of patients taking simvastatin. Of these, there

were four patients with the AA genotype and all four were taking simvastatin. The average CK level for simvastatin was 24813 pg/mL compared to 16855 pg/mL for atorvastatin, and although the difference was not statistically significant, this underscores and affirms that atorvastatin is a safer and better-tolerated choice for patients with high cholesterol levels (92). This assertion is supported by De Beer *et al*. (2021) (53) , Thompson *et al*. (2016) (54) and Nogueira *et al*. (2019) (55) who stated that SAMS are more common with simvastatin than any other statin.

In this study, the majority (72% and 74%, respectively) of hypercholesterolaemic patients carrying the *rs12916* and *rs17244841* variants reported experiencing muscle-related adverse effects. Among them, a large proportion (82.5% and 82.6%, respectively) described their muscle pain as mild to moderate, while a smaller percentage (17.3%) reported severe muscle pain.

In statin-treated patients with hypercholesterolaemia, the risk of developing statin intolerance associated with *rs12916* and *rs17244841* varied, classified as low (38%), moderate (48%), or high (14%), as reported in the questionnaires. The frequency within the latter two groups (62%) exhibited a strong correlation with the observed occurrence of muscle-related adverse effects of 72% as stated by Ward *et al*. (2019) (46). Warden *et al.* (2023) (45) estimated that SAMS occur in roughly 10% of individuals undergoing statin treatment within the general population, with a range of occurrence from 5% to 25%. Thompson *et al*. (2016) (54) and Nogueira *et al*. (2019) (55) stated that SAMS occur in 10%-25% of patients taking statins.

Stroes *et al*. (2015) (93) noted that 7–29% of patients experience symptoms associated with statin-induced muscle issues. Notably, in the majority of instances, SAMS does coincide with significant CK elevation. For cases of SAMS accompanied by CK levels exceeding 10 times the UNL, typically denoted as myopathy, the incidence is approximately 1 per 10,000 individuals per year with standard statin doses. Furthermore, the incidence of rhabdomyolysis linked to statin therapy stands at 1 in 100,000 individuals per year. This underscores a consistent correlation between CK levels and signs and symptoms of statin intolerance. This was confirmed by Berent *et al*. (2019) (94) who stated that in 96.5% of patients with muscle symptoms, there was no elevation of CK. Similarly, Magni *et al*. (2015) (92) stated that increased CK is

not necessarily indicative of statin-associated myopathy.

The maximum CK level observed in this study was 82733 pg/mL. The individual with this peak CK level had the *rs17244841* variant, yet experienced minimal or no symptoms related to muscle issues. This is correlated with studies by Magni *et al*. (2015) (92) who indicated that elevated CK levels do not necessarily signify statin-associated myopathy.

The study found no direct association between CK elevation and reported muscle symptoms in statin users. Some individuals had markedly elevated CK levels but remained asymptomatic, while others with muscle pain or weakness had normal CK levels. This aligns with broader clinical data suggesting that CK is an unreliable marker for diagnosing statin-induced myopathy. The *rs17244841* variant was associated with increased CK levels and higher risk of intolerance, while *rs12916* showed no significant correlation with either CK levels or symptoms. These findings suggest that genetic predisposition plays a crucial role in determining an individual's response to statins, supporting the emerging field of pharmacogenomics in lipid management.
Routine CK testing may be unnecessary in most statin users unless severe muscle symptoms or rhabdomyolysis are suspected. Physicians should not rely on CK levels alone to diagnose statin intolerance but rather consider a combination of patient-reported symptoms, genetic risk factors, and overall cardiovascular risk. Future research should investigate alternative biomarkers beyond CK for detecting muscle-related adverse effects and explore the molecular mechanisms underlying SAMS, particularly mitochondrial dysfunction and muscle membrane instability.

Type 2 diabetes mellitus has been linked to an increased risk of statin intolerance, primarily due to muscle metabolism alterations and mitochondrial dysfunction. T2DM is associated with impaired glucose uptake in muscle cells, which may predispose individuals to mitochondrial dysfunction and muscle fatigue. Statins, particularly lipophilic statins like simvastatin, may worsen this effect by reducing mitochondrial CoQ10 levels, further compromising muscle energy production (95, 96).

Hypothyroidism impairs the metabolism and clearance of certain drugs, including statins. This leads to higher circulating statin levels, increasing the risk of myopathy

and muscle toxicity. Patients with untreated hypothyroidism often have elevated CK levels, even without statin use. The addition of statins may exacerbate this effect, leading to muscle pain and weakness that could be misattributed to statin intolerance rather than the underlying thyroid disorder (44, 97, 98).

A total of 21% of patients had underlying conditions such as T2DM, rheumatoid arthritis, hypothyroidism, osteoarthritis, a low body mass index (BMI), and psoriatic arthritis all of which are risk factors that could provoke statin-induced myopathy. (99) Despite these factors, the questionnaire indicated that only a minority (10.5% and 9%) of patients with these comorbidities were considered at high risk for developing statin intolerance. This evaluation could have been impacted by the statin dosage, often viewed as the primary risk factor for statin intolerance, along with the limited size of the study sample (53, 100, 101).

Limitations:

Due to financial constraints, further research with larger sample sizes is recommended to validate these findings and refine strategies for individualised statin therapy, ultimately improving patient care and reducing the incidence of statin-induced myopathy and other related side effects.

Lack of CK measurement not being assessed in the control group. This could enhance the validity of the results and allow for a more comprehensive analysis of the variables being studied.

With limited participants, the findings may not be representative of the broader population, affecting generalisability. A study with a small cohort might only reflect the experiences of a particular subset, such as individuals from a specific geographic location. This reduced generalisability can lead to over or under representation of specific subgroups.

Selection bias limits the study's internal and external validity, making it difficult to generalise the findings to the broader population. Proper study design and diverse, representative sampling are essential to mitigate this limitation.

Chapter 5: Conclusion

In this study, the *rs12916* variant showed no association with statin intolerance or elevated CK levels, indicating that this genetic variant did not influence patients' likelihood of experiencing statin-related side effects. However, the *rs17244841* variant was found to have a significant connection to both increased severity of statin intolerance and higher CK levels. This suggests that individuals carrying the *rs17244841* variant may be more prone to experiencing muscle-related side effects when taking statins, highlighting the variant's potential role in influencing the body's response to these medications.

Patients with hypercholesterolaemia, especially those with a history of statin intolerance, should undergo genetic screening for the *rs17244841* variant**,** given its association with increased severity of statin intolerance and elevated CK levels.
For patients carrying the *rs17244841* variant, consider atorvastatin over simvastatin, as simvastatin has been linked to a higher incidence of muscle-related adverse effects. Monitor patients more frequently for symptoms of muscle toxicity.
For patients without the *rs17244841* variant, standard statin therapy protocols may be followed, but periodic monitoring should still be considered.

The findings of this study highlight the importance of recognising that genetic variants linked to statin toxicity in other populations may not be relevant to our population. Therefore, identifying SNVs specific to our diverse population is essential to determine whether these genetic differences influence patients' susceptibility to drug intolerance.

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Appendices

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

Demographics

Sex:

□ Female □ Male

Ancestry: _____

Age: _____ years

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 or 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? (Where 1-2 indicates mild, 3-4 indicates moderate and 5-7 indicates severe.)

□ 1 – 2

□ 3 – 4

□ 5 – 7

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. When did the muscle symptoms start?

_____ / _____ / _____

6. Factors that increase risk of statin intolerance

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

- 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
- Anaemia
- Hypoparathyroidism
- Viral illness

□ Anaemia

□ Peripheral arterial disease

8. Current statin and drug interactions

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

What is the name of the statin?

_____

Contraindicated medication

_____

Appendix B: Data capture sheets

Table 1: Observed genotype frequencies

rs12916

	Ancestry	Homozygous wild type	Heterozygous	Homozygous variant
White				
Black				

rs17244841

	Ancestry	Homozygous wild type	Heterozygous	Homozygous variant
White				
Black				

Table 2: Estimated CK levels of hypercholesterolaemic subjects

Patient number **(Hypercholesterolaemic)**	**CK level** **(Normal = < 250 U/L)**
001	
002	
003	
004	
005	
100	

Appendix C: Participant's Information & Informed Consent Document for Patients

STUDY TITLE:

The prevalence of 3-hydroxy-3-methylglutaryl-Coenzyme A reductase variants in hypercholesterolaemic patients with statin intolerance

Protocol no: 489/2023 Researcher: Ashley Gunas

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 cholesterol. 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 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 uptake does not happen as it should which means, 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 EXPECTED 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 levels, 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. The blood will be stored -80 degrees celsius for up to 5 years and then discarded in medical biohazard waste.

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

Are you older than 18 years?

Have you been diagnosed with high cholesterol?

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:

Are you younger than 18 years?

Did you have any disruptions in your atorvastatin or simvastatin therapy within the preceding 12-week period?

Are you diagnosed with any liver disease?

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 single nucleotide variants (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, Ashley Gunas, 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 penalised 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)	Date
_____	_____
Participant's signature	Date
_____	_____
Researcher's name (Please print)	Date
_____	_____
Researcher's signature	Date

Appendix D: Participant's Information & Informed Consent Document For Control Group

STUDY TITLE:

The prevalence of 3-hydroxy-3-methylglutaryl-Coenzyme A reductase variants in hypercholesterolaemic patients with statin intolerance

Protocol no: 489/2023 Researcher: Ashley Gunas

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.

You will be taking part in the control group of the study, which means you are currently not diagnosed with high cholesterol or using any anti-cholesterol medications.

2) THE NATURE AND PURPOSE OF THIS STUDY

Statins are normally taken up by our bodies where it is broken down so it can lower our cholesterol levels and be excreted as waste. However, in some cases this uptake process does not happen as it should which mean some patients are more likely to have severe adverse / side effects.

This generally happens due to a large number of reasons. In this study we will be testing for possible biomarkers in statin users that might cause the adverse / side effects experienced with statin use.

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

A 5ml (about a teaspoon full) tube of blood will be collected to determine whether you do have any of the biomarkers. The blood will be stored -80 degrees celsius for up to 5 years and then discarded in medical biohazard waste.

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

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:

Are you younger than 18 years?

Have you been diagnosed with abnormally high levels of cholesterol?

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) 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.

6) 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.

7) 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.

8) 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, Ashley Gunas will be able to identify you as participant. Results will be published or presented in such a fashion that participants 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 Physiology, the University of Pretoria.

9) 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 penalised 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) Date

_____ _____

Participant's signature Date

_____ _____

Researcher's name (Please print) Date

_____ _____

Researcher's signature Date

Appendix E: Ethics Approval

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 dd 18 March 2022 and Expires 18 March 2027.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through June 30, 2025 and Expires 07/28/2026.

Faculty of Health Sciences **Research Ethics Committee**

2 February 2024

Approval Certificate
New Application

Dear Mr A Gunas

Ethics Reference No.: 489/2023
Title: The prevalence of 3-hydroxy-3-methylglutaryl-Coenzyme A reductase variants in hypercholesterolaemic patients with statin intolerance

The **New Application** as supported by documents received between 2023-08-29 and 2024-01-31 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2024-01-31 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Ethics Approval is valid for 1 year and needs to be renewed annually by 2025-02-02.
- The Research Ethics Committee (REC) must monitor your research continuously. To this end, you must submit as may be applicable for your kind of research:
  a) annual reports;
  b) reports requested *ad hoc* by the REC;
  c) all visitation and audit reports by a regulatory body (e.g. the HPCSA, FDA, SAHPRA) within 10 days of receiving one;
  d) all routine monitoring reports compiled by the Clinical Research Associate or Site Manager within 10 days of receiving one.
- The REC may select your research study for an audit or a site visitation by the REC.
- The REC may require that you make amendments and take corrective actions.
- The REC may suspend or withdraw approval.
- Please remember to use your protocol number (489/2023) on any documents or correspondence with the Research Ethics Committee regarding your research.

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

On behalf of the FHS REC, Professor Werdie (CW) Van Staden
MBChB, MMed(Psych), MD, FCPsych(SA), FTCL, UPLM
***Chairperson**: Faculty of Health Sciences Research Ethics Committee*

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
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Gezina 0031, South Africa
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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

Appendix F: Statistical Clearance

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Letter of Statistical Clearance

Wednesday, 19 April 2023

This letter is to confirm that the MSc student: **Ashley Gunas**, student number: **23995158**, studying at the University of Pretoria, presented the project with the title: **The prevalence and effect of HMGCR genetic variants in a cohort of hypercholesterolaemic patients on statins in Gauteng, South Africa** to me.

I hereby confirm that I am aware of the project and the statistical analysis of the data generated from the project is adequate to achieve the aims and objectives of this study.

Yours sincerely

Prof. Pieter WA Meyer
Ass. Prof. and HOD

Prof PWA Meyer
Head of Department: Immunology
RasCom appointed Biostatistician
University Pretoria

Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

Appendix G: MSc Committee Approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

MSc Committee
School of Medicine
Faculty of Health Sciences

24 July 2023

Prof A Phulukdaree
Department of Physiology
Faculty of Health Sciences

Dear Prof,

Cpt A Gunas, Student no 23995158

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

Student name	Cpt A Gunas	**Student number**	23995158
Name of study leader	Prof A Phulukdaree		
Department	Physiology		
Title of MSc	**Approved title: The prevalence of 3-hydroxy-3-methylglutaryl-coenzyme A reductase variants in hypercholesterolaemic patients with statin intolerance** Revised title: The prevalence of 3-hydroxy-3-methylglutaryl-CoA reductase variants and the effect of statin intolerance in hypercholesterolaemic patients The prevalence and effect of HMGCR genetic variants in a cohort of hypercholesterolaemic patients on statins in Gauteng, South Africa		
Date of first submission	May 2023		

July 2023	• Thank you for submitting the revised protocol and requested documents. • Please rather refer to **Coenzyme A** than CoA in the title. Also, submit a recently dated MSc form. It is dated 29 April 2023. • Include a motivation section in the protocol where you briefly discuss the quality assurance of the SNV TaqMan® genotyping assay. You state that it will be done before synthesis, how is it confirmed afterwards? • Additionally, repeating a subset of samples to ensure reproducibility may add value. Please consider.
	• The committee has approved your protocol. • However, there is a concern about the title: **The prevalence of 3-hydroxy-3-methylglutaryl-coenzyme A reductase variants and the effect of statin intolerance in hypercholesteolaemic patients.** You will look at the prevalence of these variants and would like to compare the presence/absence of these variants with statin intolerance. That is not clear from the title. Please review.
	• Thank you for submitting a revised protocol and required documents.
Decision	This protocol has been provisionally approved. Please submit the protocol to ethics, and supply the MSc committee with proof of acceptance. 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

Appendix H: AHCA Approval Letter

GAUTENG PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

STEVE BIKO ACADEMIC HOSPITAL

Enquiries: Dr JS Mangwane
Tel No: +2712 3452018
Fax No: +2712 354 2151
E-mail: joseph.mangwane@gauteng.gov.za

For attention: Alisa Phulukdaree

NHRD Ref Number: GP_202306_056

Re: REQUEST FOR PERMISION TO CONDUCT RESEARCH AT STEVE BIKO ACADEMIC HOSPITAL

TITLE: The prevalence of 3-hydroxy-3-methylglutaryl-CoA reductase variants and the effect of statin intolerance in hypercholesterolaemic patients

Permission is hereby granted for the above-mentioned research to be conducted at Steve Biko Academic Hospital. This is done in accordance to the "Promotion of access to information act No 2 of 2000".

Please note that in addition to receiving approval from Hospital Research Committee, the researcher is expected to seek permission from all relevant department. Furthermore, collection of data and consent for participation remain the responsibility of the researcher.

The hospital will not incur extra cost as a result of the research being conducted within the hospital.

You are also required to submit your final report or summary of your findings and recommendations to the office of the CEO.

STATUS OF APPLICATION:
Approved

Date: 2024-03-11

Dr. J S. Mangwane
Manager: Medical Service

Appendix I: Research Article Proof of submission for publication

11/22/24, 5:00 PM University of Pretoria Mail - GENEJOURNAL-D-24-05837 - Confirming your submission to Gene

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Alisa Phulukdaree <alisa.phulukdaree@up.ac.za>

GENEJOURNAL-D-24-05837 - Confirming your submission to Gene

1 message

GENE <em@editorialmanager.com> 22 November 2024 at 11:40
Reply-To: GENE <support@elsevier.com>
To: Alisa Phulukdaree <alisa.phulukdaree@up.ac.za>

Re:%ARTICLe_TITLE%
by Ashley Gunas; Rene Pienaar; Sajee Alummoottil; Iman van den Bout; Alisa Phulukdaree
Research paper

Dear Prof Alisa Phulukdaree,

Your submission entitled "Profiling 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase rs12916 and rs17244841 gene variants and its relationship with creatine kinase in statin-treated hypercholesterolaemic patients" has been received by Gene. It has been assigned the following manuscript number: **GENEJOURNAL-D-24-05837**.

You may check on the progress of your paper by logging on to the Editorial Manager as an author. The URL is https://www.editorialmanager.com/genejournal/.

Your username is: alisa.phulukdaree@up.ac.za
If you need to retrieve password details, please go to: click here to reset your password.

Your manuscript will be given a reference number once an Editor has been assigned.

Thank you for submitting your work to this journal.

Kind regards,

Editorial Manager
Gene

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