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Faculty of Health Sciences

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Identifying circulating biomarkers in a cohort of hypercholesterolaemic patients with statin intolerance from Gauteng, South Africa

Dissertation submitted in fulfillment of the requirements for the degree,
Doctor of Philosophy in the Department of Physiology
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"Here's to those who inspire you and don't even know it." —Anonymous

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Declaration of Originality

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Articles submitted for publication

1. R Pienaar, P Soma, MN Phasha, A Phulukdaree. **Evaluation of miR-133, creatine kinase and aspartate aminotransferase in hypercholesterolaemic patients in South Africa – A pilot study**. Molecular and Cellular Biochemistry. Submitted on 09 Oct 2024.

2. R Pienaar, P Soma, A Phulukdaree. **Evaluating miR-33a and apolipoprotein A – 1 in a hypercholesterolaemic patients in South Africa – A pilot study.** Journal of Cellular Physiology. Submitted on 09 Oct 2024.

Summary

Despite recent improvements in the diagnosis and treatment of risk factors of cardiovascular disease, it remains one of the leading causes of death worldwide. Dyslipidaemia, defined as elevation of plasma cholesterol, and/or triglycerides, and low levels of high-density lipoprotein (HDL), is a key risk factor for cardiovascular disease morbidity and mortality. Simvastatin and atorvastatin are regarded as the first line treatment for the management of hypercholesterolemia, especially in public health care institutions. Statins are generally regarded as effective, especially when combined with lifestyle interventions, however, ~20% of patients experience mild to severe side effects, or statin intolerance, characterized by side effects such as muscle pain or myopathy. However, the mechanisms behind these side effects remain poorly understood.

This study aimed to quantify three circulating microRNAs (miRNAs) and protein biomarkers associated with statin intolerance in a cohort of hypercholesterolemic patients from Gauteng, South Africa. The study included 100 healthy controls, 100 hypercholesterolemic patients not on statins, and 100 statin-treated hypercholesterolemic patients. A questionnaire adapted from the American College of Cardiology's Statin Intolerance Application was used to assess statin-treated hypercholesterolemic participants' possible risk of statin intolerance. Of the statin-treated group, 15% had a high risk, 49% a moderate risk, and 36% a low risk of statin intolerance.

The study focused on miRNAs-33a, -133a, and -499a, due to their potential influence on lipid metabolism and muscle health. Using *in silico* screening tools, such as TargetScan, several target genes regulated by these miRNAs were identified. MicroRNA-33a was found to regulate genes associated with cholesterol efflux such as, ABCA1 and ABCG1, while miR-499a was associated with gene involved in muscle cells proliferation and differentiation, including FOXO4, PDCD4 and SOX6. The list of target genes for miR-133a included target genes of the SRF transcription factor, such as AOX1 and POLH.

Quantitative polymerase chain reaction (qPCR) was used to measure miRNA levels,

and enzyme-linked immunosorbent assay (ELISA) was used to quantify protein markers, such as creatine kinase (CK) and aspartate aminotransferase (AST). The study found no significant difference in ApoA-1 and AST levels across the three participant groups. However, CK levels were significantly higher among statin users compared to non-users.

Using qPCR, the miRNA levels of miR-33a and miR-133a was detectable. A significant difference in the relative fold change of miR-33a was noted, with an inverse relationship between miR-33a expression and ApoA-1 levels. A significant difference in the relative fold change of miR-133 expression was noted between the control group and statin-users, and the statin-users and the statin naïve patients. Although, no relationship was observed between miR-133a expression and AST levels, and CK levels and miR-133a expression for the statin-users, a positive relationship was observed between CK levels and miR-133a expression in the combined hypercholesterolaemic group.

This study's findings contribute to a greater understanding of epigenetics and its impact on the pharmacokinetics of statins. The knowledge gained from this study, combined with future findings, may over time contribute to a personalized approach to statin therapy, which is particularly relevant for the diverse population in South Africa. The study's outcomes suggest a necessity to explore a larger sample size, especially in examining the potential correlation between miR-499a and CK levels and determining if patients on different statin doses experience a differential effect.

Key words: MiR-33a, MiR-133a, MiR-499, Epigenetics, Statin-intolerance, Hypercholesterolemia, Statin-induced muscle injury, Apolipoprotein A 1, Creatine Kinase, Alanine Aminotransferase

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List of Abbreviations

ABC	Adenosine triphosphate binding cassette
ACC	American College of Cardiology
AE	Adverse event
ALT	Alanine aminotransferase
AMI	Acute myocardial infarction
AOX1	Aldehyde oxidase 1
ApoA-1	Apolipoprotein A1
ApoB	Apolipoprotein B
ApoB-100	Apolipoprotein B100
ApoE	Apolipoprotein E
AST	Aspartate aminotransferase
ATP	Adenosine Triphosphat
ATP8B1	ATPase phospholipid transporting 8B1 gene
AUC	Area Under the Curve
bHLH	Basic-helix-loop-helix
BMI	Body Mass Index
CCNDBP1	Cyclin D-type binding-protein 1
CCWG	Canadian Consensus Working Group
CK	Creatine Kinase
CK-MB	Creatine-kinase myocardial band
Cmax	Maximum Concentration
CPK	Creatine phosphokinase
CRP	C-Reactive Protein
cTnC	Cardiac troponin C
cTnI	Cardiac troponin I
cTnT	Cardiac troponin T
CVD	Cardiovascular Disease
CYP450	Cytochrome P450
DNA	Deoxyribonucleic acid
DMAPP	Dimethylallyl pyrophosphate
ECG	Electrocardiogram
ESC	European Society of Cardiology
EAS	European Atherosclerosis Society
Farnesyl-PP	Farnesyl pyrophosphate
FH	Familial hypercholesterolemia
GPP	Geranyl Pyrophosphate
GGPP	Geranyl-Geranyl Pyrophosphate
HDAC4	Histone Deacetylase 4
HDL	High Density Lipoprotein
HSPG	Heparan sulfate proteoglycans (HSPG)
HMG-CoA	3-hydroxy-3-methyl-glutaryl Coenzyme A
HMGCR	3-hydroxy-3-methyl-glutaryl Coenzyme A reductase
hs-CRP	High Sensitivity C-Reactive Protein

ICF	Insulin-like growth factor
IDL	Intermediate Density Lipoprotein
ILEP	International Lipid Expert Panel
ISCA2	Iron-sulfur cluster assembly 2
IPP	Isopentenyl pyrophosphate
LCAT	Lecithin–cholesterol acyltransferase
LDL	Low-Density Lipoprotein
LDL-R	Low-Density Lipoprotein Receptor
LDLRAP1	Low-density lipoprotein receptor adapter protein 1
LRP	Low-density lipoprotein receptor-related protein
LGALS8	Lectin galactoside-binding soluble 8
LXR	Liver X Receptor
MADS	MCM1, Agamous, Deficiens, Serum response factor
MEF2	Myocyte Enhancer Factor 2
MHC	Myosin heavy chain
MI	Myocardial infarction
MiR-133	MicroRNA-133
MiR-33	MicroRNA-33
MiR-499	MicroRNA-499
miRNA	microRNA
MMP2	Matrix metalloproteinase
mRNA	messenger RNA
MRPL44	Mitochondrial ribosomal protein L44
Myh7b	Myosin heavy chain 7B
MyoD	Myoblast determination protein 1
NFAT	Nuclear Factor of activated T-cells
NHI	National Health Insurance
NLA	National Lipid Association
NPC1	Neimann-Pick C1
NSTEMI	Non-ST elevation myocardial infarction
OATP	Organic anion transporter polypeptide
Pax	Paired box protein
PCNA	Proliferating cell nuclear antigen
PCSK9	Proprotein Convertase Subtilisin-Kexin Type 9
P-gp	P-glycoprotein
POLH	Polymerase (DNA directed) eta
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SAMS	Statin associated muscle symptoms
SR-B1	Scavenger Receptor Class B Type 1
SLCO1B1	Solute Carrier Organic Anion Transporter Family Member 1B1
SNP	Single nucleotide polymorphism
SOX	Sry-box

SREBP	Sterol regulatory element-binding protein
SRF	Serum Response Factor
t½	Half-life
T2DM	Type 2 Diabetes Mellitus
TGF	Transforming growth factor
Tmax	Time taken to reach the maximum concentration
ULN	Upper Normal Limit
VD	Volume of Distribution
VLDL	Very Low-Density Lipoprotein
WB	Whole Blood
WHO	World Health Organization

Abstract

Cardiovascular disease is one of the leading causes of death worldwide. Dyslipidemia, defined by high circulating levels of atherogenic apoB-containing lipoproteins, contributes significantly to the increasing burden of cardiovascular morbidity and mortality.

At public health care institutions, in South Africa, simvastatin and atorvastatin are the standard therapy for hypercholesterolemia. In combination with dietary and lifestyle changes statins are the gold standard therapy to effectively reduce LDL levels to lower the risk of cardiovascular disease, e.g. myocardial infarction or stroke. However, a proportion of patients do experience statin associated adverse effects, especially muscle related symptoms.

Studies suggest that statins affect hundreds of miRNAs, as one miRNA has the potential of binding to hundreds of mRNAs, the consequences may be enormous. However, whether these epigenetic changes lead to statin related adverse effects in South African statin users is still unknown.

The aim of this pilot study was therefore to identify potential circulating biomarkers of statin intolerance in a cohort of hypercholesterolaemic patients from Gauteng, South Africa. Following institutional ethical clearance, healthy participants (n=100), hypercholesterolemic participants not taking statins (n=100) and statin-treated hypercholesterolemic participants (n=100) were recruited. Informed Consent was obtained from all participants prior to study enrollment.

The median age (range) for participants included in the study was 49 (19 - 78), 47 (22 – 79) and 49 (19 – 78), for the control group, statin users and statin naïve group, respectively. Sampling and enrolment were based on South Africa's population ratios, with an average of ~80% black South Africans and ~20% white South African included in the study in each group. Of note is the higher prevalence of comorbidities in the statin naïve compared to the statin users. However, this is likely attributed to statin intolerance, delayed treatment initiation, socio-economic disparities, healthcare

access and treatment adherence.

All of the statin-treated hypercholesterolaemic participants were requested to complete a questionnaire adapted from the American College of Cardiology (ACC) Statin Intolerance Application. The ACC's Statin Intolerance Application was developed to assist clinicians to manage cholesterol, as well as managing and treating patients who present statin related adverse events. Of the 100 statin-treated hypercholesterolemic patients enrolled, 15 presented with a high risk of statin intolerance (15%), 49 presented with a moderate risk of statin intolerance (49%) and 36 presented with a low risk of statin intolerance (36%).

In silico screening of miR-33a, miR133a and miR-499a using TargetScan was used to assess the conserved mRNA targets of miR-33a (hsa-miR-33a), miR-133a (hsa-miR-133a) and miR-499a (hsa-miR-499a). The targets generated from TargetScan were filtered and sorted based on a predefined criteria for each miRNA. For miR-33a a total of 545 transcripts with conserved sites were identified using the TargetScan. The table generated from TargetScan was sorted according to the aggregate P_{CT} score, and targets were filtered based on their association to cholesterol homeostasis and atherosclerosis. The list of target genes with conserved binding sites for miR-33a/b identified, includes genes involved in regulating cholesterol homeostasis, specifically ABCA1 and ABCG1, fatty acid metabolism, cyclin dependent kinases and mitogen-activate protein kinases. The *in silico* screening performed to identify possible target genes for miR-133a using TargetScan generated 703 possible targets, The list of target genes included target genes of the SRF transcription factor, such as AOX1, CCNDBP1, ISCA2, LGALS8, MRPL44 and POLH. From the conserved targets identified for miR-499a using TargetScan of note is FOXO4, PDCD4 and SOX6, which all are genes associated in pathways related to regulation of smooth and skeletal muscle cell differentiation.

Quantitative polymerase chain reaction (qPCR) was used to determine the levels of miR-33a, miR-133a and miR-499a, enzyme-linked immunosorbent assay (ELISA) was used to quantify serum creatine kinase (CK) and aspartate aminotransferase (AST) levels in plasma. Quantitative analysis showed no significant difference in the ApoA-1

levels between participants in the control, statin-users, or statin naïve groups, (p= 0,4117). A significant difference between the CK levels between the different patient groups, p= <0,0001, was however noted with the average CK level for the statin-users being significantly higher compared to the other 2 groups. The miRNA levels of miR-33a and miR-133a were detectable. A significant difference (p= 0,0011*)* in the relative fold change of miR-33 was noted between statin-users and statin naïve patients compared to the control group. A significant difference in the relative fold change of miR-133 expression was noted between the control group and statin-users (p= 0,0006), and the statin-users and the statin naïve patients (p= 0,0239). MicroRNA-499a, however, was not detectable in any of the samples, likely due to low abundance and biological variation.

A linear regression and Spearman R correlation analysis was conducted on matched samples between miR-33a and ApoA-1 levels. The Spearman's rank correlation coefficient was used to assess a possible correlation between the miR-33a expression and ApoA-1 levels. An inverse correlation between miR-33a and ApoA-1, was observed, with the r = -0,3266 (95% confidence interval -0,5885 to -0,002740) and p= 0,0424. Despite the lack of a significant difference in ApoA1 levels between the control, statin users, and statin-naïve groups, the inverse correlation between miR-33a expression and ApoA1 levels observed in the study supports the notion that miR-33a has a regulatory influence on cholesterol metabolism.

Following the quantification of AST and the expression of miR-133a, a linear regression and Spearman R correlation analysis was conducted on matched samples between miR-133a and AST levels to determine miR-133a's effect on AST levels. No correlation was observed between the miR-133a expression and AST levels for either the hypercholesterolaemic participants on statin therapy or the combined hypercholesterolaemic group, with r = -0,1088 (95% confidence interval -0,6336 to 0,4845) and p= 0,72 and r= 0,07492 (95% confidence interval -0,3326 to 0,4588) and p= 0,7161, respectively.

As there is evidence which supports the notion that miR-levels can influence muscle integrity, an alternative biomarker was also used to assess the possible association

between miR-133a and muscle damage. Creatine kinase is the gold-standard biomarker used to assess cardiac- and skeletal muscle damage. The Spearman's rank correlation coefficient was used to assess a possible correlation between the miR-133a levels and CK levels in statin-users and subsequently in a combined hypercholesterolaemic group (i.e. all the hypercholesterolaemic patient enrolled in the study, statin-treated and statin naïve patients). A positive correlation was observed between the CK levels and miR-133a levels in the combined hypercholesterolaemic group, with r= 0,4567 (95% confidence interval 0,1601 to 0,6777) and p= 0,0031. Although, no correlation was observed between the CK levels and miR-133a levels for the statin-users, a positive correlation was observed between the CK levels and miR-133a levels in the combined hypercholesterolaemic group. It was determined that there was a significant difference in the CK levels between the different patient groups, with the average CK level for the statin-users being significantly higher compared to the other two groups.

The findings of this study contribute to a growing body of evidence which supports the notion that of the three miR's assessed, miR-33a may serve as a drug target for managing hypercholesterolemia and cardiometabolic diseases, such as atherosclerosis. miR-33a inhibits genes involved in cholesterol efflux, fatty acid oxidation and insulin signaling thus aiding in cholesterol regulation and lipid metabolism. Statin intolerance, particularly myopathy, is associated with disruptions in lipid metabolism and oxidative stress. Statins act as an activator of SREBP2, which can lead to increased miR-33a levels. As miR-33a negatively regulates ApoA-1, increased levels of miR-33a can reduce ApoA-1 levels. This can lead to reduced activity of ApoA-1 which hinders the removal of cholesterol from tissues. Decreased ApoA-1 levels can also exacerbate systemic inflammation, potentially worsening muscle symptoms in statin intolerant patients.

Understanding the role of miRNAs and its potential connection to statin intolerance could provide valuable insights into the adverse effects experienced by statin users. This study helps gain a better understanding of statin intolerance and these findings will allow a more personalized approach to statin therapy, e.g. miR-33a inhibitors which could lead to restored ApoA-1 and HDL levels, which in turn could improve

cholesterol efflux and mitigate muscle-related symptoms.

Chapter 1: Introduction and literature review

Despite recent improvements in the diagnosis and treatment of risk factors of cardiovascular disease (CVD), it remains one of the leading causes of death worldwide. Around 17.9 million people died of CVD in 2019, accounting for more than 32% of deaths worldwide. (1, 2)

Dyslipidemia, defined by high circulating levels of total cholesterol and/or low-density lipoprotein (LDL), contributes significantly to the increasing burden of cardiovascular morbidity and mortality. Effectively reducing levels of LDL has improved since earlier years, however, even after lowering LDL to the desired target, residual risk for atherosclerotic cardiovascular disease still remains. (3-5) Elevated remnant cholesterol, defined as total cholesterol minus LDL-C and HDL-C, and lipoprotein (a) have been causally linked to increased risk for atherosclerotic cardiovascular disease. (6-8)

At public health care institutions, in South Africa, a low- to moderate dose statin (10- to 40 mg simvastatin or 10- to 20 mg atorvastatin) is considered standard therapy for hypercholesterolemia. Combined with dietary and lifestyle changes statins are the gold standard therapy to effectively reduce LDL levels and the risk for CVD. (9) However, they may be associated with side effects, especially statin intolerance. (10)

Statins are administered orally and undergo significant first-pass metabolism. (11-13) Statins are mainly metabolised in the liver by the cytochrome P450 (CYP450) enzymes. (14) Statins are regarded as safe and well-tolerated. In some cases, adverse effects have been reported, however, these usually resolve with discontinuation of statin therapy or when changing to another statin regimen. Despite the low incidence of general adverse events (AEs) associated with statin treatment, approximately 20% of patients may develop statin intolerance. (15-22)

Most of the reported statin intolerance incidences is related to myalgia, or other muscle related events. The incidence of statin intolerance is a widely debated topic and depend mainly on how the intolerance was assessed. Ordinarily, it should be proven

that a drug exerts a specific effect. However, the burden for the medical community is to establish that adverse events, whether over reported or not, are absent or exist at minimal levels, but also to prove or disprove causality and convince patients to adhere to the medication despite potential concerns. (23, 24)

In recent studies, it is suggested that the muscle related adverse events reported with statin use is greatly influenced by e.g. the media, warning about adverse effects by clinicians, information included in the informed consent form of clinical trials, etc., eliciting a nocebo effect. (25) Although, adverse events associated with statin use were reported within the first few years after introduction of statins for clinical use, the incidence of adverse events reported in large, randomized control trials have not shown significant differences in the number of patients reporting adverse events related to statins compared to placebo. (24)

Well performed double blinded randomized controlled trials are widely accepted as the most reliable way to evaluate the benefits, risk, safety, and tolerability of a drug. (26, 27) In the Anglo-Scandinavian Cardiac Outcomes Trial—Lipid-Lowering Arm, the researchers assessed adverse events reported by participants on 10mg atorvastatin first in the double blinded placebo controlled arm of the study, and then as part of a non-blinded non-randomized extension phase. During the blinded phase, participants assigned to the atorvastatin group reported adverse events in a similar rate to the placebo group, and no significant difference was observed in the rates of all reported AEs compared to the placebo group. However, in contrast, in the non-blinded phase, muscle related symptoms were reported significantly more by participants who were taking a statin compared to those who were not. This supports the notion of a nocebo effect, with an increase in reports of statin-related adverse events when patients were aware that they were on a statin therapy. (28)

Differential drug responses stem from a variety of factors, including age, gender, race, comorbidities, concomitant medications, lifestyle factors and genetic factors. Genetic factors contribute to contribute to around 90% of interindividual drug responses. (29-31) The increasing number of differential drug responses reported begs the need for investigation into potential contributing epigenetic factors. Epigenetic changes occur due to changes in external and environmental factors, e.g. age, temperature, stress,

diet, exposure to different toxins, and may even be hereditary. (32) Due to the strong association between epigenetic changes and different diseases, drugs targeting epigenetic changes have become an important topic in medical research. (33-35)

Research suggests that statins can interact directly with the epigenome, due to their ability to upregulate LDL receptor (LDL-R) production and cause significant changes in gene expression. This includes the expression of microRNAs (miRNAs). Up- or down-regulation of a single miRNA has the potential to affect hundreds of messenger RNAs (mRNAs), possibly leading to an array of symptoms associated with statin intolerance. (36, 37)

Studies suggest that statins affect hundreds of miRNAs in various cell lines and *in vitro* studies, as one miRNA has the potential of binding to hundreds of mRNAs, the consequences may be enormous. However, whether these epigenetic changes lead to or are related to the adverse effects widely identified with statin users are still unknown. (32, 34, 36, 38-41)

MicroRNA-33a, miR-133 and miR-499 are some of the miRNAs most frequently implicated in differential responses to statin treatment, specifically statin intolerance. In South Africa, the healthcare system is unevenly split between the private and public sectors, with a small portion of the population serviced by the private sector, which consumes a large scale of the healthcare resources. This uneven distribution of resources makes it challenging for South Africa to advance in personalised medicine, such as genetic testing, due to limited access to these services, a lack of representation in genomic databases, and the increased strain caused by communicable diseases. Additionally, financial and resource limitations prevent most South Africans from accessing advanced diagnostics and treatments. Thus, this study aimed to identify to identify potential circulating biomarkers of statin intolerance in a cohort of hypercholesterolaemic patients from Gauteng, South Africa. The results generated from this study, along with future research will help improve the understanding of epigenetics and its impact on statin treatments, potentially offering a more personalized approach to lipid-lowering therapy.

This chapter highlights the pharmacology of statins, different epigenetic mechanisms

that may explain the symptoms associated with statin intolerance and potential biomarkers associated with statin intolerance.

1.1 Physiology of cholesterol

Cholesterol is a 27-carbon compound comprised of a unique structure with a hydrocarbon tail. The centre sterol ring is common to all steroid hormones, Figure 1. (42) Cholesterol is insoluble in water and is thus primarily found in lipid and/ or lipid-protein complexes, such as cell membranes. (42, 43)

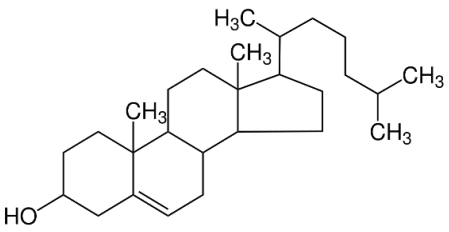

Figure 1: Chemical structure of cholesterol showing a polar head group (hydroxyl group), steroid nucleus (i.e., four fused rings, three six-carbon rings And one five-carbon ring) and alkyl or hydrocarbon side chain.

Cholesterol plays an important role during production and maintenance of cell membranes, steroid hormone, Vitamin D and bile acids synthesis. (44-46) Cholesterol is also responsible for bilayer stiffness, membrane biogenesis, permeability of cell membranes to water and ions, and forms an integral part of specialised lipid-protein membrane microdomains responsible for intracellular signaling cascades. (42, 47)

Cholesterol is mainly synthesised *de novo* by hepatocytes. *De novo* synthesis occurs in the endoplasmic reticulum as part of the mevalonate pathway. During this process, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) is converted to mevalonate by an enzyme, known as 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR), to ultimately yield 2 products, cholesterol and geranyl-geranyl pyrophosphate which is further processed into 3 products, prenylated proteins, dolichols and ubiquinone. (47, 48)

Once synthesised, cholesterol is transported in circulation, along with triglycerides, cholesterol esters and phospholipids, within specialised lipoprotein particles due to the hydrophilic nature of plasma e.g. HDL, intermediate density lipoprotein (IDL), LDL and very low-density lipoprotein (VLDL), although chylomicrons are not present in fasting plasma. (45, 46, 49).

These transporter lipoproteins are comprised of a liquid core, containing cholesterol esters and triglycerides, with a hydrophilic outer layer containing phospholipids, apolipoprotein, and free cholesterol. (45, 46, 49, 50) There are several types of transporter lipoproteins, with their nomenclature depending on the density of the lipoprotein, which is also a reflection of lipoprotein function. (Table 1; Table 2) (45, 50, 51)

Table 1: Functions of lipoproteins (51-56)

Lipoprotein	**Function**
Chylomicrons	Lowest density lipoproteins with a size of ≥ 100nm. Transport lipids from the small intestine to the liver and peripheral tissue. ApoB48 is the main structural apolipoprotein which aids in the transport of dietary lipids from the small intestine to the peripheral tissue.
VLDL	Triglyceride rich lipoproteins, containing apo C-II, apo E and apo B-100. Transport lipids from the liver to peripheral tissue
VLDL remnant	As lipids are removed from the liver, an alteration in the density of lipoprotein occurs, resulting in the transformation of VLDL to IDL. The transition of VLDL to IDL is accompanied by the transfer of apo C-II to HDL.
LDL	This is the ultimate stage of transformation of VLDL, LDL transports lipids from the liver. LDL acts as the major transporter of cholesterol. (45)
HDL	Important for the transport of cholesterol and other lipids from the peripheral tissues, e.g. cardiovascular system, either (1) back to the liver during reverse cholesterol transport, (2) to steroidogenic tissues/organs to aid in hormone production or (3) exchange of lipid with apoB-containing lipoproteins, e.g. LDL and VLDL.
VLDL Very low-density lipoprotein, **IDL** Intermediate density lipoprotein, **LDL** Low-density lipoprotein, **HDL** High-density lipoprotein	

Table 2: Density and composition of lipoproteins (51-54)

Lipoprotein Fraction	**Size (nm)**	**Density (g/mL)**	**Cholesterol (%)**	**Triglyceride (%)**	**Total lipid (%)**	**Protein (%)**
Chylomicron	100-500	<0.960	4	88	99	1
VLDL	30-80	0.960 – 1.006	23	56	90-93	8
IDL	25-50	1.006-1.019	43	29	89	11
LDL	18-28	1.019-1.063	58	13	79	21
HDL	5-15	1.063-1.125	41	16	67	33
VLDL Very low-density lipoprotein, **IDL** Intermediate density lipoprotein, **LDL** Low-density lipoprotein, **HDL** High-density lipoprotein						

Low-density lipoprotein acts as the major transporter for cholesterol in humans. The

lowest density class of lipoproteins are chylomicrons, synthesised in the intestinal mucosa from dietary fats, i.e., triglycerides, cholesterol, and phospholipids, along with apolipoproteins such as apoB48. Chylomicrons attain their low-density to their large size and high triglycerides content, which prohibit it from crossing the capillary barrier. Thus, chylomicrons are directly transported into the lymphatic system from the intestinal mucosa and ultimately enter the circulation via the thoracic duct. (51-54)

Lipoprotein lipases, bound to the capillary endothelium, hydrolyse triglycerides from chylomicrons to free fatty acids which are deposited into adipose tissues. This action leaves the chylomicron depleted. The depleted chylomicron referred to as a remnant is then taken up by hepatocytes via LDL-Rs, e.g. Low-density lipoprotein receptor-related protein (LRP), and heparan sulfate proteoglycans (HSPG). (46, 51-54, 57) The heparan sulfate proteoglycans, are cell surface molecules, that regulate cholesterol homeostasis by interacting with lipoproteins. Heparan sulfate proteoglycans bind to apoB-containing lipoproteins and apoE-rich molecules, anchoring them to the cell surface, aiding lipoprotein receptors to interact with these molecules. (58-61) Apolipoprotein E is the critical ligand responsible for remnant lipoprotein clearance, remnants are captured by interaction with HSPG, which is mediated by ApoE. (61) Low-density lipoprotein receptor-related protein, a multifunctional receptor, mainly expressed in the neurons, epithelial and muscle cells, fibroblasts, monocytes, macrophages, hepatocytes, adipocytes, and vascular smooth muscle cells, play a critical role in lipid metabolism and cholesterol homeostasis. (62) Low-density lipoprotein receptor-related protein aids in lipoprotein transport, regulation of cell surface protease activity and protection against atherosclerosis by modulation of platelet-derived growth factor receptor-β. (63) Low-density lipoprotein receptor-related protein mediates the endocytosis of chylomicron remnants, VLDL remnants, and other apoE-rich lipoproteins. Due to the wide range of ligands recognized by LRP, LRP plays a diverse role in lipid metabolism, binding to lipoproteins mainly through ApoE. (62, 63)

Very low-density lipoproteins are triglyceride rich containing apolipoprotein B100 (apoB-100) which serves as a ligand for the LDL-R. Lipoprotein lipases, reduces two thirds of the VLDL to ultimately terminate as LDL and the remaining one third is cleared by hepatic LDL-R, scavenger receptors, e.g. Scavenger Receptor Class B Type 1 (SR-

B1), and apoE receptors. (51-54, 64, 65)

High density lipoproteins contain a small number of phospholipids and apolipoprotein A1 (apoA-1) and serves as an apoprotein reservoir. High density lipoproteins are responsible for the reverse transport of cholesterol which aid in the metabolic balance of cholesterol. (52, 66-70)

1.2 Pathophysiology – hypercholesterolaemia

Overproduction and/or inadequate clearance of apoB containing lipoproteins can have severe pathophysiological effects on the human body. Dyslipidemia, is defined as a total cholesterol of > 5 mmol/L, LDL of > 3 mmol/L and/or an HDL of < 1.2 mmol/L for women and 1.0 mmol/L for men, fasting triglycerides of > 1.7 mmol/L. (Table 3) (71, 72)

Hypercholesterolemia can be caused by a number of known hereditary (5, 8), environmental and lifestyle factors, e.g. gene mutations of the LDL-R, apoB, low-density lipoprotein receptor adapter protein 1 (LDLRAP1) and proprotein convertase subtilisin-kexin type 9 (PCSK9) (73), smoking, excessive dietary intake, hepatic over production and/or inadequate clearance or usage of cholesterol in the circulatory or lymphatic system. (74)

Table 3: Normal values of lipogram parameters (71, 72, 75, 76)

Lipid	**Normal range**
Total cholesterol	2.59 – 5.18 mmol/L
Triglyceride	0.57-1.70 mmol/L
HDL - Cholesterol	Male: > 1.0 mmol/L Female: > 1.2 mmol/L
Non-HDL Cholesterol	2.07-4.14 mmol/L
LDL – Cholesterol	1.30 – 3.37 mmol/L
ApoA-1	1.10 – 2.05 g/L
ApoB	0.55 – 1.05 g/L
ApoB/ApoA-1 Ratio	0 – 0.77
Lipoprotein (a)	0.01 – 0.30 g/L

Familial hypercholesterolemia (FH) affects approximately 1 in every 202 people

globally. (77) Familial hypercholesterolemia is defined by a life-long elevation of LDL which can lead to CVD if left untreated or not treated appropriately. (77) The most common cause of FH is a mutation in the LDL-R gene, which encodes LDL-R proteins. Low-density lipoprotein receptor mutations attributes to 80 to 90% of genetically confirmed FH globally. (78, 79) During normal functioning of the gene, LDL binds to these receptors and is subsequently removed from the circulation, however, mutations of the LDL-R gene result in decreased transcription of LDL-R proteins, resulting in abnormally high levels of plasma LDL, due to decreased clearance from the circulation. (5) There are currently 5 classes of LDLR mutations, (I) mutations are most commonly nonsense mutations, large deletions and promotor mutations which results in no detectable LDL-R protein, (II) these mutations cause a (IIa) completed, or (IIb) partial block of the transport of LDL to the LDLR, (III) results in reduced LDL binding to LDL-R, (IV) results in a deficiency in the internalization of LDL while (V) mutations affects the recycling of the LDL-R, the receptor is synthesised traffics to the cell surface and binds LDL normally, however, instead of the receptor being recycled back to the cell surface, the receptor is degraded. (80) Other pathogenic mutations include mutations in the apoB, LDLRAP1 or PCSK9. (5, 8, 73) The familial defective apoB-100 mutation affects the LDL-R binding domain of ApoB, while LDLRAP1 is an autosomal recessive cause of hypercholesterolemia. Meanwhile, the gain of function PCSK9 mutation leads to an increase in the degradation of LDL-R, and thus hypercholesterolaemia. (81)

Among the hereditary causes for hypercholesterolemia, it is important to differentiate between monogenic and polygenic hypercholesterolemia. Polygenic hypercholesterolemia is a common cause of elevated serum cholesterol. Polygenic hypercholesterolemia refers to a condition where the elevated levels of LDL-C cannot be explained by a single gene mutation, and thus a combined effect of multiple genetic variations is postulated. (79, 82, 83) Polygenic hypercholesterolemia is estimated to account for 20% - 30% patients diagnosed with clinical FH. (83) Polygenic hypercholesterolemia is confirmed using the polygenic risk score (PRS) that estimates the combined LDL elevating effect of common genetic variants. (83) Talmud *et al.* (2013) proposed that in patients with familial hypercholesterolemia without a known mutations to attribute to their elevated LDL-C levels, a polygenic cause should be considered. (84)

Unhealthy lifestyle choices can also result in hypercholesterolemia. Examples include poor dietary choices, lack of exercise, cigarette or tobacco smoking, gender, age, obesity, hypothyroidism, nephrotic syndrome, cholestasis and type 2 diabetes mellitus (T2DM). (73, 85) Certain medications can also lead to an increased risk of hypercholesterolemia, e.g. cyclosporine and thiazide diuretics. (85)

Age is a strong and common risk factor, research suggest that an advanced age is a higher risk for hypercholesterolemia, however, according to the National Cholesterol Education Program, adults 20 years and older should be screened for hypercholesterolemia at least once every 5 years as hypercholesterolemia can go undetected in younger adults. (86-88)

Sex-specific difference also significantly influences the risk and progression of hypercholesterolemia and cardiovascular disease. (89) This could be due to the reduced LDL and VLDL and increased HDL in premenopausal women due to the protective effect seen with higher estrogen levels. (89, 90) The Framingham Study, which analysed frozen plasma samples from 1574 men and 1692 women, showed that the sex differences in lipid metabolism was most pronounced for HDL, where women presented with a two-fold higher concentration of large HDL particles compared to men. (91) However, in postmenopausal women, the risk of hypercholesterolemia and CVD significantly increases compared to premenopausal women due to the decline in estrogen levels, leading to increased LDL, reduced HDL and increase total cholesterol. (89, 90)

High-fat diets are one of the most common factors that lead to elevated cholesterol levels. When there is an increase in dietary fat intake there is a rapid increase in chylomicron remnants taken up by the liver. Chylomicron remnants are converted to lipoproteins within hepatocytes, thus an increase in chylomicron remnants in the liver lead to an increase in production of lipoprotein, resulting in abnormally high levels of lipoproteins in the blood. (51, 52, 92)

When LDL levels in the circulation rise, an increased amount of LDL penetrate the endothelial lining of the arteries, where LDL tends to start accumulating in the artery walls, leading to plaque formation. (51, 52, 92)

Vessel walls consists of a monolayer of endothelial cells that border luminal blood flow. The intima, consisting of glycosaminoglycans and collagen, is located underneath the layer of endothelial cell. This is followed by layers of smooth muscle, namely the media, and lastly, a fibrous layers, namely the adventitia. Atherosclerosis tends to occur in regions of arteries that exhibited disturbed blood flow, e.g. at bifurcations. The disturbed blood flow result in changes in the alignment of the endothelial cells, resulting in increased permeability of larger molecules, e.g. plasma LDL and triglyceride rich lipoproteins, by either trans-endothelial transport or diffusion at the cell-cell junctions. (46, 93, 94) Oxidative modification of the LDL molecules trapped in the vessel wall take place, resulting in damage to the endothelium by the LDL particle. (93, 94) During this oxidative modification, LDL is oxidised due to an increase in free radicals and oxidant species. (93, 95) Oxidised LDL triggers the activation of innate and complement immune systems, leading to an increased production of reactive oxidant species and the recruitment of macrophages. Oxidative changes to the LDL molecules leave the molecule susceptible to uptake by macrophages. Macrophages phagocytise oxidised LDL and form foam cells. Due to the rapid increase of LDL uptake, it results in a buildup of cholesterol esters in the macrophages which form lipid droplets, giving the macrophages the appearance of foam cells, (52) This results in activation of a cascade of proinflammatory cytokines, eventually cause smooth muscles to proliferate and migrate from the intima to the endothelium. As a result of the rapid increase of growth and inflammatory factors, smooth muscle cell produce collagen, promoting foam cell formation and a fatty streak below the endothelium. This, in turn, leads to increased absorption of LDL by macrophages, resulting in lipotoxicity of the endoplasmic reticulum, leading to macrophage apoptosis and plaque necrosis. The necrotic plaque triggers additional inflammation and chemotaxis of neutrophils. Process is illustrated in Figure 2. (93, 94)

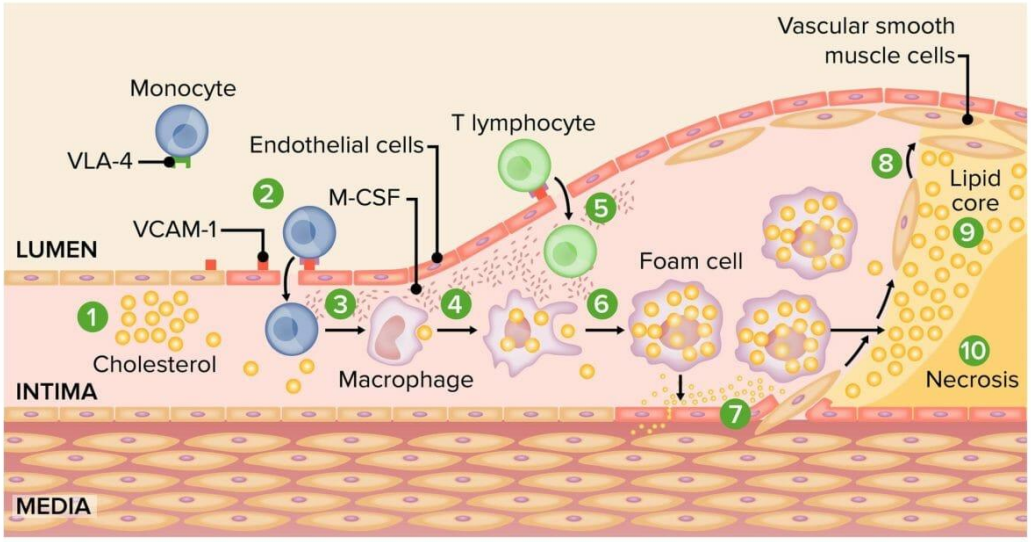

Figure 2: Pathogenesis of atherosclerosis (96, 97)
(1) The process begin by activation of endothelial cells (inner lining of the arteries) by factors like oxidised LDLs, high blood pressure and smoking. Leading to increased permeability of the endothelial layer.
(2, 3) Damaged endothelial cells express leukocyte adhesion molecules, i.e. vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), Alpha4beta1 integrin (VLA-4), P-selectin and E-selectin, which attract circulating leukocytes, particularly monocytes, which leads to monocyte adhesion and transmigration into the intima.
(4) Once in the intima, monocytes differentiate into macrophages, the process is mediated by macrophage colony-stimulating factor (MCS-F).
(5) Plaque formation by joining of the T lymphocytes and macrophages.
(6) Macrophages then engulf oxidised LDL, forming of lipid-rich foam cells.
(7) Migration and replication of vascular smooth muscle cells.
(8) Accumulation of smooth muscle cells in the plaque, forming a fibroproliferative lesion.
(9, 10) Death of foam cells and formation of necrotic core.

High density lipoprotein opposes this reaction by removing excess cholesterol from peripheral tissue and transporting it to the liver for excretion. Cholesterol esters are formed within the HDL particles with assistance of lecithin–cholesterol acyltransferase (LCAT) activity. Cholesterol esters are then transported back to the liver where it is metabolised, utilized as substrates for cholesterol derived-molecules or excreted. (49) However, when an imbalance occurs between HDL and LDL cholesterol (such as low levels of HDL and high levels of LDL), it leads to an accumulation of LDL in the periphery, causing abnormal buildup and resulting in dyslipidemia. (49)

Elevated cholesterol levels together with endothelial damage are primarily responsible for the development and progression of atherosclerosis, (3, 7) which plays a key role in the onset of coronary artery disease and peripheral artery disease, all of which can

lead to increased morbidity, mortality, and treatment expenses. (4-6)

1.3 Treatment of hypercholesterolemia

Dietary and lifestyle changes are the starting point when exploring treatments for lowering of LDL in patients diagnosed with hypercholesterolemia, e.g. healthy diet, physical activity, exercise, and counselling on maintenance of a healthy body weight. (81) However, in most instances, dietary and lifestyle changes are not sufficient and lipid lowering therapy is often required to effectively reduce LDL levels. (48, 81)

Several lipid-modifying drugs are available for the treatment of hypercholesterolemia. These include bile-acid binding resins, e.g., cholestyramine, fibrates, e.g., nicotinic acid, e.g., niacin, cholesterol absorption inhibitors, e.g., ezetimibe, HMG-CoA reductase inhibitors, e.g., statins, and more recently a concomitant therapy known as PCSK9 inhibitors are being added to the therapy regimen for patients who do not achieve lipid-lowering targets despite being on the maximum tolerated statin dose. (48, 81, 98)

Statins are most frequently prescribed due to their efficacy and cost-effectiveness. (99) The American Heart Association and American College of Cardiology (ACC) recommend treating patients diagnosed with hypercholesterolemia with a low to moderate dose statin as first line therapy, in the event that there is not adequate LDL lowering, the dose should be increased to a high intensity statin, shown in Figure 3. (98, 100)

The South African public sector's approach to statin therapy differs from international recommendations, primarily due to factors such as medication availability, cost considerations, and healthcare infrastructure challenges. International guidelines often advise to initially prescribe a high-intensity statin therapy in patients at high cardiovascular risk or to titrate statins based on LDL targets. However, in South Africa's public healthcare system, access to high-intensity statins is often limited. Most patients receive low- to moderate-intensity statins, such as simvastatin at doses of 10–40 mg, with higher doses typically available only through referral to tertiary-level hospitals. (9)

South Africa, however, adopts the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS) guidelines for the management of dyslipidemia. The ESC/EAS recommends prescribing a high-intensity statin at the maximum tolerated dose to achieve LDL-C targets as first-line treatment. If LDL-C targets are not achieved on statin alone, combination therapy with ezetimibe should be considered. For patients who do not reach their LDL-C targets with statins and ezetimibe, a PCSK9 inhibitor should be considered in addition to the statin/ezetimibe combination. (101) Simvastatin and atorvastatin are currently the first-line treatment at tertiary institutions. (99) The Cholesterol Treatment Trialists (CTT) collaboration performed a meta-analysis of 26 randomized trials involving at least 100 participants and at least 2 years of treatment. For each trial, the researchers calculated the risk reductions and the average risk reduction per 1.0 mmol/L LDL cholesterol reduction at 1 year after randomization. In the trials comparing more intensive to less intensive statin therapy, the mean additional reduction in LDL at 1 year was 0.51 mmol/l. More intensive statin regimens however, should have a significant 15% reduction of major cardiovascular events compared to less intensive statins. (13% reductions in coronary events or non-fatal myocardial infarction, 19% reduction in coronary revascularization, and a 16% reduction in ischemic stroke). Across the 26 trials analysed as part of the meta-analysis, all-cause mortality was reduced by 1% per 1.0 mmol/L LDL reduction, which was largely driven by deaths due to coronary heart disease and other cardiac causes. (102)

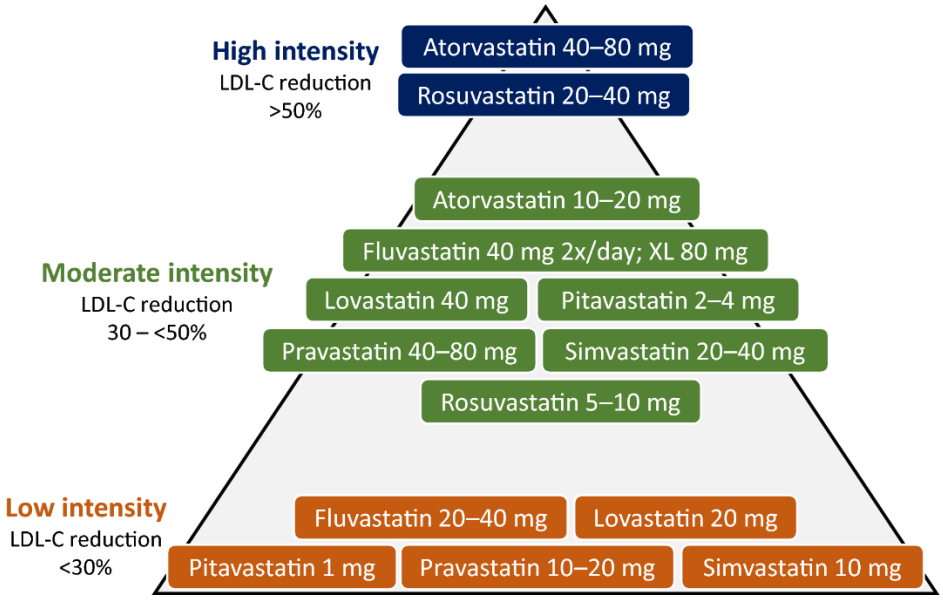

Figure 3: Statin dosing according to the American College of Cardiology and American Heart Association classification of intensity. (98)

1.3.1 Statins mechanism of action

Statins are a class of drugs commonly used to lower cholesterol levels in the body. Statins are the most efficient agents for reducing LDL. (103) Studies show that statin reduces the progression of atherosclerosis and in some instances may even lead to the regression of already diagnosed atherosclerosis. (104) The mechanism of action of statins involves inhibiting the enzyme HMGCR, which is responsible for the *de novo* synthesis of cholesterol in the liver. (105)

Statins work by competitively inhibiting HMGCR which plays an essential role in the *de novo* synthesis of cholesterol, presented in Figure 4. (103, 105) 3-Hydroxy-3-methylglutaryl-coenzyme A reductase is the rate-limiting enzyme for cholesterol synthesis in the liver and other tissues. (104) In the absence of statins, HMG-CoA is converted to mevalonate (or mevalonic acid), mevalonate is then phosphorylated by mevalonate kinase to form mevalonate-5-phosphate. Mevalonate-5-phasphate is further phosphorylated to phosphomevalonate kinase to form mevalonate-5-diphosphate, which is decarboxylated by mevalonate-5-diphosphate decarboxylases to form isopentenyl pyrophosphate (IPP), releasing CO_2. Isopentenyl pyrophosphate isomerase converts IPP to dimethylallyl pyrophosphate (DMAPP). Dimethylallyl pyrophosphate and IPP condense to form geranyl pyrophosphate (GPP) via geranyl pyrophosphate synthase, GPP then condenses with another IPP molecule to form

farnesyl pyrophosphate (Farnesyl-PP) vis farnesyl pyrophosphate synthase. Farnesyl-pyrophosphate yields two products, one of which is cholesterol. However, in the presence of statins, HMGCR is inhibited and cannot catalyse the conversion of HMG-CoA to mevalonate, which is the rate limiting step in cholesterol biosynthesis, thus resulting is reduction of synthesis of cholesterol in the liver. (104, 106)

Statins sterically inhibit substrates from binding to the enzyme. To accommodate the rigid hydrophobic rings of statins, the substrate-binding sites on the enzyme undergo conformational changes. This competitive inhibition results in decreased cholesterol synthesis in hepatocytes and subsequently lower levels of LDL. (14) However, by inhibiting the functioning of HMGCR, statin don't only inhibit the *de novo* of cholesterol but also increase HDL concentration, decreases triglycerides (14) and aid in increasing the expression of LDL-R in hepatocytes thus increasing the uptake of cholesterol from the circulation. (14, 104)

Statins work by inhibiting HMG-COA reductase, reducing cholesterol synthesis, thus activating SREBP processing, thus increasing the expression of genes encoding of LDL-R and ultimately increasing the number of LDL-R on the hepatocytes. The increased expression of LDL-R leads to increased uptake of LDL from the circulation. Sterol-regulated membrane-bound transcription factors (SREBPs) are synthesized as membrane proteins attached to the endoplasmic reticulum. However, in cholesterol-depleted cells, the SREBPs are transported to the Golgi complex where they are processed by proteases to release a soluble fragment which enters the nucleus and stimulates transcription of genes encoding for HMG-CoA and other genes involved in cholesterol homeostasis, including LDL-R. Sterol-regulated membrane-bound transcription factors mediated regulation of LDL-R is a critical step for the mechanism of action of statins. (107)

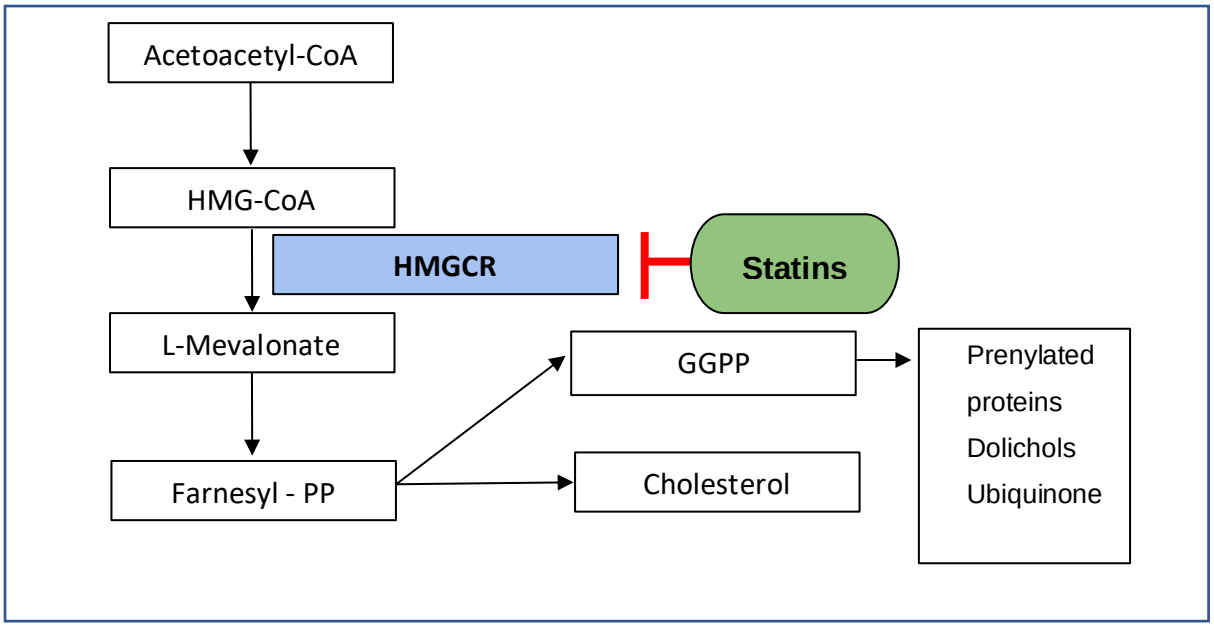

Figure 4: Mechanism of action of statins

HMG-CoA Hydroxymethylglutaryl coenzyme A, **HMGCR** 3-Hydroxy-3-methylglutaryl-coenzyme A, **Farnesyl–PP** Farnesyl pyrophosphate, **GGPP** Geranyl-geranyl pyrophosphate

1.3.2 Pharmacokinetics of statins

Statins are complex drugs and are divided into groups based on their synthesis and chemical structure. Three classes related to synthesis exist: natural, semisynthetic, and synthetic. Simvastatin is a fungal derived inhibitor of HMG-CoA, synthesised by the alkylation of lovastatin, making simvastatin a semisynthetic statin. During this process, the 2-methylbutyrate moiety at the C-8 position is replaced with a 2,2,-dimethylbutyrate. (108) Atorvastatin is a fully synthetic compound. (109) Synthetic statins are chemically synthesised, and only shares the HMG-CoA-like moiety, which mimics the HMG-CoA substrate and HMGCR binding. (108)

All chemical structures of statins work on a similar basis, (I) an analogue of HMG-CoA, (II) a complex hydrophobic ring structure involved in binding to HMGCR and (III) side-groups that determine the solubility of the drug and the pharmacokinetic properties. Chemical structures of simvastatin and atorvastatin are illustrated in Figure 5 and 6 below. Statins are also divided into 2 groups, namely, Type 1 and Type 2, based on their chemical structure. (108) This structural classification is done based on the ring structure in the statin. Type 1 statins have a naphthalene ring, and the Type 2 statins have a hydrophobic ring that covalently bind to HMG-CoA like moiety. Simvastatin and

atorvastatin are classified as Type 1 and Type 2, respectively. (108)

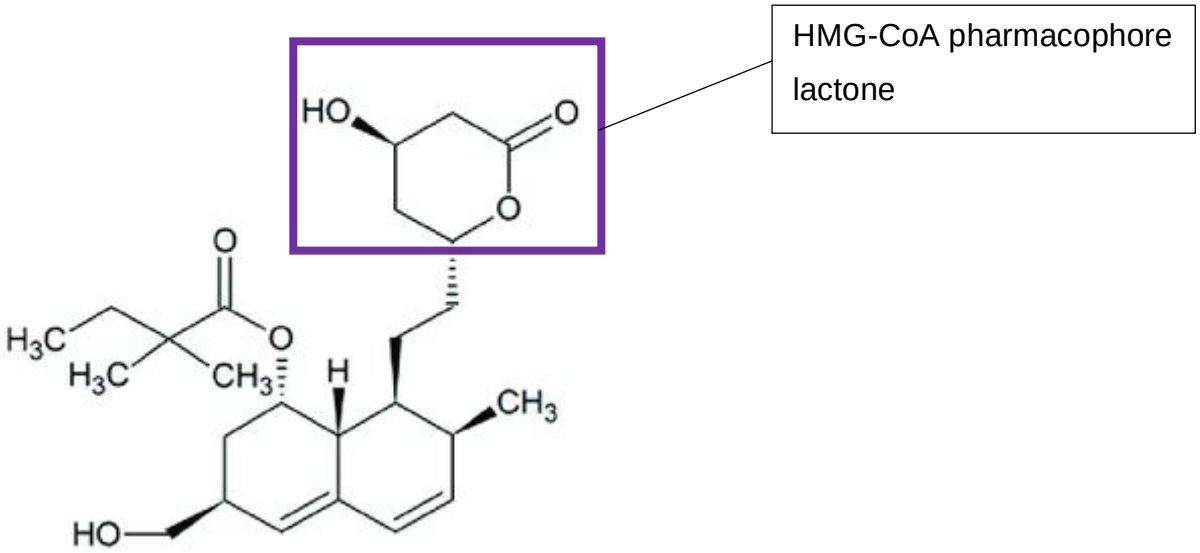

Figure 5: Simvastatin chemical structure, showing a methyl butyrate appendage, hexahydronaphthalene ring system and HMG-CoA pharmacophore lactone

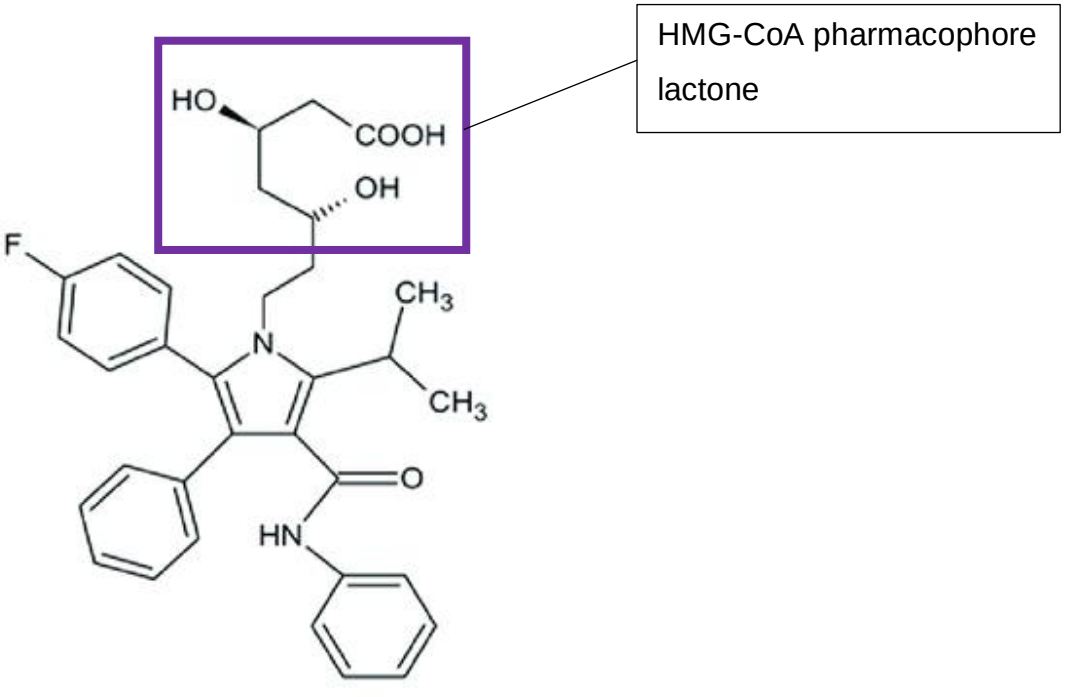

Figure 6: Atorvastatin chemical structure, showing a penta-substituted pyrrole ring system and fluorophenyl ring system with HMG-CoA pharmacophore lactone

a. Absorption of statins

Simvastatin is administered orally as a lactone prodrug meaning it is administered in an inactive form and has to be reversibly hydrolysed by carboxyesterases, in the liver, intestinal wall, and plasma *in vivo* to its active metabolite, β-hydroxy-acid, to achieve pharmacological activity. (108) Atorvastatin, however, is pharmacologically active, and is administered in the active form. After administration atorvastatin is hydrolysed into its two active metabolites, 2-hydroxy- and 4-hydroxy-atorvastatin acid. Statins and its acid metabolites appear to exist in equilibrium with their inactive lactone forms *in-vivo, a*s both the lactone and acid forms are detected in circulation post oral administration, which introduces several layers of complexity when assessing clinically relevant pharmacokinetic parameters. (110)

Gastrointestinal absorption percentages ranging from 60–85% for simvastatin, unaffected by food, compared to 30% absorption of atorvastatin which is decreased by food intake, Table 4. (111) Atorvastatin has high solubility, high permeability characteristics, making it a case 1 drug according to the biopharmaceutic drug classes, meaning the rate of absorption of the drug is dependent on gastric emptying. (14)

Statins are absorbed rapidly following administration, reaching their peak concentrations ($t_{\frac{1}{2}}$) with 2 – 5 or 7 – 20 hours for simvastatin and atorvastatin respectively. (12, 112, 113). Statins are mainly absorbed in the small intestine aided by specialised mucosa and the transporter, P-glycoprotein (p-gp). (11-13)

All statins are mostly metabolised by cytochrome P450 (CYP450) enzymes, whether located in the enterocytes or hepatocytes. After absorption, simvastatin and atorvastatin are partially metabolised to their active metabolites by intestinal enterocyte CYP450 isoenzymes, specifically CYP3A4, which is responsible for the metabolism of the greatest number of drugs in humans. Simvastatin and atorvastatin are extracted by P-gp from the gut wall that is extensively distributed along the epithelial lining of the small intestine. (113). From here, simvastatin and atorvastatin are transported to the liver via the hepatic portal vein, where they undergo further first-pass metabolism by CYP450 enzymes. (14, 108)

Due to the extensive first pass metabolism in the gut and liver, there is a substantial decrease in the concertation of the parent drug in the systemic circulation, resulting in a maximum systemic plasma concentration (Cmax) of 10–34 ng/mL for 20 mg simvastatin and atorvastatin in comparison to 27-66 ng/mL Consequently resulting in decreased bioavailabilities of < 5% and 12%, respectively. (14, 111) It takes approximately 2-4 hours (Tmax) to reach Cmax. (16) (Table 4)

During the extensive presystemic metabolism in the liver, both simvastatin and atorvastatin are metabolised into pharmacologically active metabolites. These metabolites prevent the *de novo* synthesis of cholesterol by hepatocytes, and then either get eliminated through bile, or enter the systemic circulation via the hepatic veins or inferior vena cava. The systemic plasma concentrations of atorvastatin acid and simvastatin lactone may not be significant, but the concentrations of their active metabolites in the liver could better correlate with the therapeutic effects on plasma LDL, as the liver is their primary site of action. (14, 111, 114)

Table 4: Basic pharmacokinetic profile of simvastatin and atorvastatin (14, 111, 114). Retrieved from De Beer *et al.(115)*

	Simvastatin	**Atorvastatin**
Solubility	Lipophilic	Lipophilic
IC_{50} HMGCR (nM)	1 – 2 (active metabolite)	1.16
Oral absorption (%)(111)	60 – 85 food has no effect	30 decreased by food intake
Bioavailability (%)	< 5	12
Cmax (ng/mL)	10 – 34	27 – 66
Tmax (hours) (114)	2-4	2-4
Liver extraction (%)	≥ 80	70
Protein binding (%)	> 95	> 98
Half-life ($t_{\frac{1}{2}}$) (h)	2 – 5	7 - 20
VD (L/kg)	-	~5.4
Metabolism (CYP450)	CYP 3A4	CYP 3A4
Metabolites	Active	Active
Hepatic extraction (%)	78 - 87	>70
Transporter involved	OATP1B1	OAT1B1

Lipophilicity (*C* log *P*) (octanol/water)	4.68 (47.860)	4.6 (1.482)
Standard daily dose (mg)	10 – 40	10 – 80
Clearance (L/hr/kg)	0.45	0.25

IC_{50} Half-maximal inhibitory concentration, **HMGCR** 3-hydroxy-3-methyl-glutaryl Coenzyme A reductase, **Cmax** Maximum Concentration, **Tmax** Time taken to reach the maximum concentration, **$t_{\frac{1}{2}}$** Half-life, **VD** Volume of Distribution, **CYP450** Cytochrome P450

b. Distribution of statins

The permeability of cell membranes is determined by the composition of their lipid bilayer. For a drug to pass through the apical- and basolateral membranes, a drug must possess certain physiochemical characteristics, e.g. charge, lipophilicity, size, and hydrogen bond potential. (12) Lipophilic statins are transported through passive diffusion while hydrophilic statins are transported by facilitated diffusion through and across membrane barriers. (14, 116) Lipophilic statins are transported through extrahepatic cell membranes, resulting in a lower hepatoselectivity compared to hydrophilic statins. (117)

The volume of distribution (VD) is used to access the distribution and tissue binding of a specific drug, using variables such as (I) protein binding, (II) tissue binding and (III) membrane permeability. (112, 118) In layman's terms, the VD measures how much of the statins are tissue-bound and no longer in the systemic circulation. The VD for simvastatin and atorvastatin is 232.57 ± 132.54 L/kg (*232.57 L/kg x 70 kg (weight of the average person)= 16279.9 L)* (119) and 381 L/kg (*381 L/kg x 70 kg (weight of the average person)= 26670 L*)(determined after administration of 5 mg of drug as intravenous infusion), respectively. (110) The high VD seen with both simvastatin and atorvastatin may be due to their favourable physiochemical characteristics: (I) favourable charge, (II) size and (III) hydrogen bond potential. (110, 120)

Both simvastatin and atorvastatin are highly protein bound once they enter the systemic circulation, > 95% and > 98%, respectively. From the systemic circulation, they are redistributed to tissue and other organs. The distribution of statins is heavily affected by their high affinity for binding to plasma proteins, specifically albumin. Atorvastatin and simvastatin, for instance, have less than 10% of drug left in free,

unbound form after entering the systemic circulation. (112) Due to the high affinity in which simvastatin and atorvastatin bind to plasma proteins, they have elimination half-lives ($t\frac{1}{2}$) of 15 – 30 hours, which contributes to their greater efficacy in lowering LDL levels compared to other statins, for example fluvastatin and lovastatin. (14, 114)

After entering the liver, statins can effectively lower LDL levels by inhibiting cholesterol synthesis. However, due to their ability to hinder the production of mevalonate, statins inhibit cholesterol synthesis in any cell responsible for synthesising cholesterol. (121, 122)

The efficient extraction of statins and their active metabolites by the liver is crucial for their therapeutic effectiveness as well as their metabolism, either into active metabolites or inactive byproducts.

c. Metabolism of statins

One thing all statins have in common is their main therapeutic target, the liver. (103) More than 80% of the drug is retained by the liver, aiding to their low concentration, 12% and 5% for atorvastatin and simvastatin respectively. (103) Enterocyte CYP450 enzymes aid in the initial metabolism (113), of most statins before they enter the portal circulation. (13) Statins are mainly metabolised by hepatic CYP450 enzymes (phase I metabolism (13), specifically CYP3A4. (14, 123) Atorvastatin and simvastatin are metabolised into active metabolites, by CYP3A4, which include 2-hydroxy- and 4-hydroxy-atorvastatin acid, and *b*-hydroxy acid and its 6¢-hydroxy, 6¢-hydroxymethyl and 6¢-exomethylene derivatives, respectively. (14) Most drug interactions involving statins take place during this metabolic phase. (124) However, their active metabolites may also undergo further phase II metabolism by uridinediphosphoglucuronyltransferases to inactive compounds that are excreted. (14) Lipophilic statins bind to the CYP3A4 isoenzyme with less affinity, which means drugs that have a stronger binding affinity for CYP3A4 will inhibit the final breakdown of statins, leading to increased risk for statin intolerance. (124)

d. Excretion of statins

The major (75-85%) (123) route of excretion of statins is via bile after metabolism by

CYP3A4 isoenzymes in the liver, (14) with the remaining metabolites excreted by the kidneys. (123) Statins and their metabolites reach the bile canaliculi either through passive diffusion or via unidirectional efflux transporters located in the bile canalicular membrane, e.g. breast cancer resistance protein, bile salt export pump, multi-drug resistance associated protein and p-glycoprotein. (14) Water soluble metabolites that are not excreted through the bile are eliminated through the kidneys through urinary excretion. (112) The elimination half-life for simvastatin, appears to be short meaning there is a less likelihood of drug accumulation in the systemic circulation after continuous dosing, decreasing the probability of severe toxic effects. (114, 123)

1.3.3 Statin adverse effects

The potential adverse effects associated with statin use remains a subject of significant interest in academic literature and social media worldwide. (125) Statins are recognised as safe and well-tolerated medications. However, statin adverse effects, including muscle-related symptoms, are reported in approximately a third of patients on statin therapy. (126) Adverse effects include nausea, vomiting, diarrhoea, abdominal pain and skin rash. However, the only adverse events that have been reliably demonstrated to be associated with statin therapy are myopathy and diabetes mellitus. (27) Discontinuing statin therapy or switching to an alternative statin regimen typically resolves these effects. (127) However, the benefit, still outweighs the limited risk associated with statin therapy. (27)

The most commonly reported AEs associated with statin use is muscle related symptoms, which range from mild muscle aches to rhabdomyolysis. In observational studies and clinical settings, 10% to 29% of patients report muscle-related AEs. (128)

A multicenter, randomised, double-blind, parallel-dose study conducted by Ballantyne *et al.* (129) on 917 hypercholesterolemia patients to compare the efficacy of 80 mg/d simvastatin compared to 80 mg/d atorvastatin reported the most common AEs experienced by participants were gastrointestinal events, e.g., Diarrhea (simvastatin 1.3%; atorvastatin 3.0%), constipation (simvastatin 1.3%; atorvastatin 1.5%), nausea (simvastatin 1.8%; atorvastatin 0.9%), and muscle related events, e.g. myalgia, arthralgia, muscular weakness, muscular cramp, musculoskeletal stiffness, and body

ache (however no patients presented with a creatine kinase (CK) >5).

Contrary to the study conducted by Ballantyne *et al.* (129), Newman *et al.* (130) reported arthritis (0.15% in patients who received atorvastatin and 0.21% in other statin groups), cholecystitis and cholelithiasis (0.18% and 0.17% in atorvastatin group respectively). Other hepatic AEs included: abnormal liver function tests, which included all parameters, (i.e., ALT, AST, alkaline phosphatase (ASL), gamma-glutamyl transferase (GGT), serum bilirubin, prothrombin time (PT), the international normalized ratio (INR), total protein and albumin) (0.06%), cholestatic jaundice (0.02%), enzymatic abnormality (0.01%), and increases in alanine aminotransferase (ALT) (0.02%) and aspartate aminotransferase (AST) (0.01%). Discontinuations considered related to hepatic and musculoskeletal AEs were rare (<1%). The study reported myalgia in a very small sample of the study population (0.03% in atorvastatin and 0.02% in other statins). (130)

The Heart Protection Study by the Heart Protection Study Collaborative Group (131) aimed at resolving the remaining uncertainties, including the long-term effects of lipid-lowering therapy, the impact of lipid-lowering therapy on non-vascular mortality, the effect of lipid-lowering therapy or different populations of lipid-lowering therapy by assessing its long-term effects, optimal LD-C reduction, safety and tolerability of long term statin use, impact of LDL-C reduction, with statins, on other non-cardiovascular diseases. The study included 20 536 participants randomised to either 40 mg simvastatin daily or matching placebo. The following AEs were reported throughout the duration of the trial: New cancer incidences (7.9% in simvastatin group, 7.8% in placebo group), non-melanoma skin cancer (2.4% in simvastatin group, 2% in placebo), elevated liver enzymes (0.5% in simvastatin group and 0.3% in placebo group) and myopathy (32.9% in simvastatin group and 33.2% in placebo group). The relative risk for any form of myalgia in the simvastatin group (with or without elevated CK levels) was 0.99, compared to 1.7 in patients with a CK concentration of 4 times the ULN. (131) Despite the number of adverse events reported in both the simvastatin and placebo group, no significant difference between the groups were observed. There was also no significant difference in the discontinuation rates between the statin treated and placebo groups secondary to muscle related adverse events. (131)

The STELLAR Trial, reported on by Jones *et al.* (132) had a primary objective to compare rosuvastatin with simvastatin, pravastatin and atorvastatin at different dose ranges for their efficacy in reducing LDL levels. A total of 2 431 adults were randomised to different treatment groups, however only 94% of participants completed the trial. The trial participants reported the following AEs: myalgia (4% overall, highest number (>5%) of patients reporting myalgia were rosuvastatin 80 mg (7.3%), atorvastatin 20 mg (6.4%), atorvastatin 80 mg (5.4%), or pravastatin 20 mg (5.4%)), and headache (3%). Five patients (2 on atorvastatin 80 mg; 1on atorvastatin 20 mg; 1 on simvastatin 40mg; and 1 on simvastatin 80 mg) had clinically important ALT elevations (>3 times the upper limit of normal). Three patients (1 on rosuvastatin 80 mg; and 2 on simvastatin 10 mg) had a clinically important elevation of>10 x the upper normal limit (UNL) of CK. A more than 10 fold higher rate of myopathy was reported in patients who were treated with simvastatin 80mg daily compared to 20 mg daily. (132)

The most commonly reported adverse event associated with statin therapy is muscle related symptoms, defined as muscle pain, tenderness, or weakness, that is not accompanied by elevated CK levels. Statin therapy has been associated with a relatively high risk of myopathy in both observational and randomized studies, however the actual number of reported cases remain low, with about 1 in 10 000 patients treated per year presenting with myopathy and even fewer presenting with rhabdomyolysis (2 in 100 000 patients treated per year). (27)

Different studies have provided varying insights into specific AEs associated with statins, however, discontinuations due to hepatic and musculoskeletal AEs are rare when compared to the benefits of statin therapy. Overall, the adverse effects of statin use vary among individuals and studies, highlighting the importance of monitoring and managing these effects on a case-by-case basis**.**

1.4 Statin intolerance

a. Background and definition

Statins are generally well tolerated but not all hypercholesterolaemic patients are able to use statins. Despite the low incidence of general AEs associated with statin

treatment as highlighted in the section 1.3.3., the discontinuation of statins remains high, even in patients with CVD. Non-compliance to statin therapy lead to an increase in the risk of myocardial infarction and coronary artery/heart disease. (22)

The mechanism by which statins cause statin intolerance is still widely debated. (133) Sakamoto *et al.* (2013) proposed that the reduction in the products yielded in the mevalonate pathway during the synthesis of cholesterol may contribute to the prevalence of statin induced myopathy and muscle weakness. This is based on the premise that statins inhibit HMGCR resulting not only in decrease cholesterol synthesis but also the product yielded during this process. Geranyl-geranyl pyrophosphate, a precursor for GG-Rab, which plays a role in membrane trafficking, vesicle movement, vesicle formation and membrane fusion. Reduced production of GGPP, may result in reduced GG-Rab, ultimately affecting membrane trafficking and decreases mitochondrial membrane potential. This decrease could decrease Adenosine Triphosphate (ATP) production and promote eventual muscle weakness and necrosis. (122)

Hanai *et al*. (2007) however proposed that the muscle-specific ubiquitin protein ligase, atrogin-1, which promotes protein breakdown and muscle atrophy could lead to statin intolerance. (134) Real time PCR was performed to determine the atrogin-1 levels in 3 groups of patients, a control group, patients experience myopathy and patients experiencing myopathy while treated with HMG-CoA reductase inhibitor. Significantly higher levels of atrogin-1 was seen in patients treated with HMG-CoA reductase inhibitor compared to the other groups. (134, 135)

Despite the number of debated mechanisms, statin intolerance results from a combination of factors, including mitochondrial dysfunction, genetic predisposition, oxidative stress, and miRNA dysfunction. (10, 32, 135, 136)

Although statins are the cornerstone treatment for elevated LDL levels and atherosclerotic diseases, 20% of individuals are not taking an adequate statin dose. (17-22) Various organisations and experts present divergent interpretations of statin intolerance within academic discourse. The National Lipid Association (NLA), International Lipid Expert Panel (ILEP), and the Canadian Consensus Working Group

(CCWG) however, all share a consensus that statin intolerance represents a clinical syndrome. It is characterised by the inability to tolerate at least two statins, one of which is administered at its minimum daily dose. This intolerance manifests through symptoms and indications linked to statin treatment, such as an elevation in laboratory biomarkers or myopathy. (13, 22, 137) Typically, these symptoms and signs subside upon discontinuation of statin therapy. (22, 138-140) No standardised diagnostic criterion exists for the diagnosis of statin intolerance. Statin intolerance loosely refers to the inability to use a statin due to significant symptoms or elevated biomarkers, e.g., CK levels. However, the biggest challenge is to clearly link these symptoms experienced to statin use. The general rule of thumb however is, if the symptoms start within 3 months of treatment and resolve upon treatment cessation, the symptoms are most likely linked to statin use. (22, 138, 141)

It is however of importance to note that not all participants who meet the criteria for statin intolerance as highlighted above, have reproducible statin intolerance on a double-blinded rechallenge. The GAUSS-3 (Goal Achievement After Utilizing an AntiPCSK9Antibody in Statin Intolerant Subjects 3) (142) was designed as a 2-stage randomized clinical trial to identify patients with statin-induced muscle symptoms during a placebo controlled rechallenge procedure and then to compare the effectiveness and tolerability of ezetimibe and a PCSK9 inhibitor. In phase A of the trial, patients with a history of statin-related muscle symptoms were given both atorvastatin and placebo in a crossover design. During this phase, 42.6% of patients stopped taking atorvastatin due to intolerable muscle symptoms, which is slightly higher compared to the 36% of patients who reported statin-related myalgia after statin rechallenge with a history of statin-induced myalgia, in the study performed by Taylor *et al.* (2016). (143) Interestingly, 26.5% of patients reported similar muscle-related symptoms with placebo but not with atorvastatin, indicating that not all muscle -related symptoms are related to statins. (142)

In a study by Wood *et al.* (2020) (144) the enrolled patients, with previously reported statin intolerance, received 4 bottles containing 20mg atorvastatin and 4 bottles containing placebo to be used in a random and blinded sequence for a 1-month period. A total of 49 patients completed the 12 months of the trial. The primary study end point was derived using the nocebo ration, which is the ratio of the symptom intensity

induced by taking the placebo to the symptom intensity induced by taking a statin. The researchers noted a nocebo ratio of 2.2 (95% confidence interval (CI), −62.3 to 66.7). Among the participants in the trial, the mean symptom intensity was 8.0 during no-tablet months 95% (CI, 4.7 to 11.3), 15.4 while taking placebo (95% CI, 12.1 to 18.7; p<0.001 compared to no-tablet months) and 16.3 while taking statins (95% CI, 13.0 to 19.6; p<0.001 when compared to no-tablet months and p = 0.39 when compared with placebo months). (144)

No standardised and confirmed biomarkers currently exist to measure statin intolerance. Only elevated serum CK is used is an indirect biomarker of skeletal muscle damage. The CCWG proposed specific terminology to give consistency when diagnosing and describing muscle related AEs. The term myopathy is an umbrella term used to describe any disease associated with muscle, whether it be acquired or hereditary diseases. (126, 141) Consequently, common categorisations of statin intolerance hinge upon the levels of CK and include a spectrum ranging from asymptomatic myopathy to myalgia, myositis, and exceptionally rare, yet potentially life-threatening rhabdomyolysis. (Table 5). (145-147)

Type	Laboratory Characteristics	Symptoms
Myalgia	CK ≤ ULN	Symptoms of muscle aches and/or weakness (muscle tenderness, muscle weakness, cramps, fatigue) but no elevation of CK levels
Myopathy	CK < 5x ULN	Umbrella term is used for any disease related to muscle which is subcategorised depending on participant symptoms. A CK elevation of < 5 times the ULN
Myositis	CK > 5x ULN	Muscle-related symptoms with a CK elevation of > 5 times the ULN
Rhabdomyolysis	CK > 10x ULN	Severe muscle-related symptoms with a CK elevation of > 10 times the ULN, are often associated with myoglobinuria. Rhabdomyolysis occurs with rapid destruction of skeletal muscle resulting in release of myoglobin which may cause severe renal damage and failure.
ULN Upper Limit Normal, **CK** Creatine Kinase		

Table 5: Biochemical categorisation of CK levels (148, 149)

The rates of statin intolerance in clinical practice is higher than events reported in randomised clinical trials. (150) In certain cases, manifestations of muscle fatigue and weakness do not coincide with elevated levels of CK. (22) Tragni *et al*. (2007) (151) conducted a study involving approximately 14,120 individuals who were on newly prescribed statins. The study revealed that only three patients exhibited CK levels exceeding ten times the upper normal limit (UNL), while four patients demonstrated an AST/ALT ratio surpassing ten times the UNL. (151) Ballantyne *et al*. (2003) (129) documented a wide range of muscle-related AEs in a substantial cohort, resulting in treatment discontinuation for 3 out of 435 patients taking simvastatin and 15 out of 464 patients taking atorvastatin. These events included myalgia, arthralgia, muscular weakness, muscular cramps, musculoskeletal stiffness, and general bodily discomfort. However, none of these symptoms were accompanied by CK levels exceeding five times the ULN.(129) .

Randomised controlled trials rarely report on the tolerability of statin but rather on their

primary end points which generally include efficacy and safety. Tolerability of statin is measured by observational and post-marketing surveillance studies. However, there is still a need for a standardised and validated scale for the assessment of statin tolerability. (24)

Recently the CCWG proposed 6 key factors to consider when managing statin intolerance in participants. These include; (I) Ensuring the participant has a valid indication for the statin therapy, (II) Identifying risk factors that may preclude the use of statins, (III) Ensuring the participant is informed of all the risks and benefits of taking statin therapy, (IV) Encourage other lifestyle interventions, such as dietary and exercise, to lower cardiovascular risk, (V) Use a systematic/ challenge/rechallenge/dechallenge approach to patients with goal-inhibiting statin intolerance, (VI) If needed, suggest the use of a non-statin alternative to achieve the therapeutic goal, e.g., PCSK9 inhibitor or ezetimibe. However, usually the symptoms experienced are mild and can be easily managed by prescribing an alternative statin at the same dose equivalence while monitoring associated biomarkers to ensure the muscle-related symptoms do not worsen. (13, 126)

Due to its cost-effectiveness, simvastatin is the most frequently prescribed statin within the public sector, in South Africa. (152, 153) Despite the substantial lipid-lowering efficacy observed with simvastatin therapy, it is most frequently associated with reports of intolerance. In the event a patient is intolerant to the minimum therapeutic simvastatin dose, the second-line therapy is the equivalent therapeutic dose of atorvastatin, in the event that the patient can tolerate another statin dose with sufficient therapeutic effect, the patient presents with partial intolerance. (154, 155) Partial intolerance refers to a condition where an individual can tolerate a lower dosage or an alternative statin, while complete intolerance is characterised by severe symptoms that necessitate the discontinuation of all statin medications. Complete intolerance, however, refers to the inability to tolerate any minimum statin dose (usually at their usual lowest daily dose.) (155, 156) It is theorised that the muscle-associated side effects experienced with statin intolerance are due to the effects of statins on energy metabolism or due to the decreased levels of coenzyme Q10. (157)

Statin intolerance can be managed through various strategies, these include;

Intermittent or non-daily dosing, switching the statin, combination therapy with non-statin lipid lowering therapies and lifestyle modifications. (158, 159) Intermittent dosing strategies have also emerged as a promising approach to lower LDL levels while moderating SAMS. (159) The guidance provided to date on mitigation strategies to manage and treat statin intolerance differ, however the common theme among these are that most patients can tolerate statins. (159) In 2015, the EAS consensus panel released recommendations on the assessment, evaluation and management of statin associated muscle symptoms (SAMS). The panel further differentiated SAMS according to CK levels. Most of the patient reporting any muscle symptoms have a normal or mildly elevated CK (i.e. a CK level of less that 4x ULN), if these participants have a low risk of atherosclerotic CVD, these patients may not require statin therapy and counselling on lifestyle changes, e.g. low-fat diet, can be explored. However, for patients with a normal or mildly elevated CK with risk of atherosclerotic CVD, the patient should be educated on the importance of the statin therapy and an assessment should be done on the benefits of continuing statin therapy despite experiencing muscle symptoms. (159) If patients are found to be statin intolerant, additional therapies should be employed, e.g. ezetimibe and PCSK9 inhibitors. (159, 160)

b. Risk factors for statin intolerance

Randomised clinical trials and observational studies (including post-marketing surveillance studies) have identified a wide range of risk factors associated with statin induced myopathy. (141) These include, patient characteristics, e.g., age, sex (females are more likely to develop AEs), small body frame and fragility, hereditary conditions; excessive grapefruit juice consumption (which functions as a CYP3A4 inhibitor); co-morbid conditions, e.g., hypothyroidism, chronic kidney disease, T2DM, alcoholism or previously diagnosed muscle disease; genetic predispositions such as polymorphisms of drug transporters or CYP isoenzymes; or concurrent use of medications, including, antidepressants, illicit drug use and high dose statins. Sex-specific differences in responses to treatments, specifically statin treatment, can be attributed to multiple factors, including hormonal regulation, pharmacokinetics, body composition, and genetic variations. (161)

Treatment related factors, e.g., high dose statin therapy, drug-drug interactions,

metabolic activity of hepatic CYP and variance in transportation mechanism, may also result in increased levels of statin plasma concentration. (156) Despite the fact that there are various factors that can influence the plasma concentrations of statins, there is no routine monitoring of plasma statin concentrations. (156)

Statin plasma concentrations are mostly affected by drug-drug interactions. The most common interactions with statins take place during metabolism. Drug-Drug interactions can either be beneficial or destructive, it can either result in increased efficacy or increased risk of intolerance or AEs. (124, 162)

As described in Section 1.3.2c, CYP3A4 is the primary enzyme responsible for statin metabolism, lipophilic statins bind to the CYP3A4 isoenzyme with a lower binding affinity, meaning drug with a strong binding affinity that lipophilic statins will displace the statin from the isoenzyme, resulting in reduced metabolism and ultimately increased plasma concentrations, (163) examples of these drugs include, erythromycin, itraconazole, ritonavir, cyclosporine, amiodarone, gemfibrozil and niacin (>1 g/day). (164, 165) One of the most potent CYP3A4 inhibitors is grapefruit juice, (166) resulting in a 240% increase in statin plasma concentration when statins are taken concurrently with grapefruit juice. (167)

Conversely, just like certain drugs inhibit the binding of statin to CYP3A4 inhibiting their metabolism, certain drugs induce the CYP3A4 isoenzyme, inducing statin metabolism, thus decreasing statin plasma concentrations, e.g., rifampicin, St John's Wart and Fosamprenavir, (168, 169) resulting in a rapid decrease of the peak plasma concentration, area under the curve (AUC) and total exposure of statins which may ultimately lead to reduced effectiveness and a lower risk to statin intolerance. (168-170)

1.4.1 Pharmacogenomics

Differential drug responses stem from an array of factors, encompassing age, gender, comorbid conditions, polypharmacy, and genetic influences. Extensive evidence supports the notion that genetic variations underlie approximately 90% of the interindividual variations in drug responses, leading to notable discrepancies

exceeding a ten-fold disparity in drug metabolism and elimination rates among individuals. (29-31) Over 30 genetic variations have been identified thus far, which may contribute to varied responses among individuals receiving statin therapy. These variations are categorised into two groups: those affecting the way the drug is processed in the body (pharmacokinetics) and those influencing its effects on the body (pharmacodynamics). Of these genetic variations, specific focus has been placed on candidate genes related to the transport, export, and hepatic metabolism of simvastatin and atorvastatin. (155)

Genetic variations in, e.g. CYP3A4, CYP3A5, ABCB1, HMGCR, LDLR, SLCOB1, etc. affect the pharmacogenomics of statins and have been individually linked to differential responses to hypercholesterolemia treatment, especially within the diverse South African population. (171) To date, more than 30 genetic variations were identified that may be associated with differential statin responses. (155) In African populations, especially South Africans, certain variants of these genetic variations are more prevalent than in other populations, necessitating the adjustment of statin dosing. (10, 155)

1.4.2 Epigenetic variation

The increasing number of reported AEs associated with statin intolerance, begs the need for investigations into potential contributing epigenetic factors which may provide insight to reduce or prevent these side effects. It is becoming increasingly evident that the use of pharmaceuticals can result in changes in gene expression that continues even after cessation of the drug. (172)

The definition of epigenetics has evolved in the literature over the years since 1940. (35) It was changed and developed into a term used to describe a molecular phenomenon in organisms. In modern medicine, epigenetics is defined as “heritable traits in cells and organisms that do not involve changes to the underlying deoxyribose nucleic acid (DNA) sequence”. These changes may persist through the organism’s life, despite the cessation of the factor that initiated the change observed. Epigenetic changes can be essential for normal functionality of an organism, however, in most cases, these changes can lead to adverse effects. (37)

There are various areas of epigenetics and epigenetic changes that can occur in the human genome. These epigenetic changes include DNA methylation, histone modification and chromatin remodeling; recently, changes in the expression of miRNAs were added to the epigenetic profile. (36, 37) There are over two thousand known human miRNAs, each with overlapping targets and functions. (32). MicroRNAs gained attention due to their involvement in many pathological processes, hence potential biomarkers for disease diagnosis, disease severity, prognosis, and therapeutic targets. However, currently, there is limited research and evidence on the mechanisms in which statins affect miRNA expression. (32, 136)

MicroRNAs are a family of non-coding single stranded ribonucleic acid (RNA) that regulate gene expression at a post-translational level. (173-175) MicroRNAs are generated by a two-step processing pathway to yield RNA molecules. (176) MicroRNAs comprise of approximately eighteen to twenty-seven nucleotides, which play an important role in regulating a wide variety of processes in humans, these include cell differentiation, development, and proliferation. (4-7) MicroRNAs regulate gene expression through messenger RNA (mRNA) degradation, blocking mRNA translation. (177)

MicroRNA is transcribed from the miRNA gene by RNA polymerase II, producing a primary transcript (pri-miR) of 100–1000 nucleotides. This transcript includes a hairpin structure, a 5´-cap, and a polyadenylated tail. Pri-miR is processed in one of two ways: the canonical pathway, involving the microprocessor complex (Drosha and DGCR8), or the non-canonical pathway, using the spliceosome apparatus. Both pathways produce a hair-pin precursor-miRNA (pre-miRNA) of 60–70 base pairs. (177)

The pre-miRNA is then exported from the nucleus via Exportin-5 and RanGTP. In the cytoplasm, Dicer processes the pre-miRNA into a mature miRNA, a double-stranded molecule of 19–25 nucleotides. (177) The mature miRNA associates with Argonaute 2 (Ago-2) in the RNA-induced silencing complex (RISC), where it is unwound and guided to its target mRNA. Binding of the miRNA to mRNA suppresses gene expression by either blocking translation or reducing mRNA stability. (177) Illustrated in Figure 7.

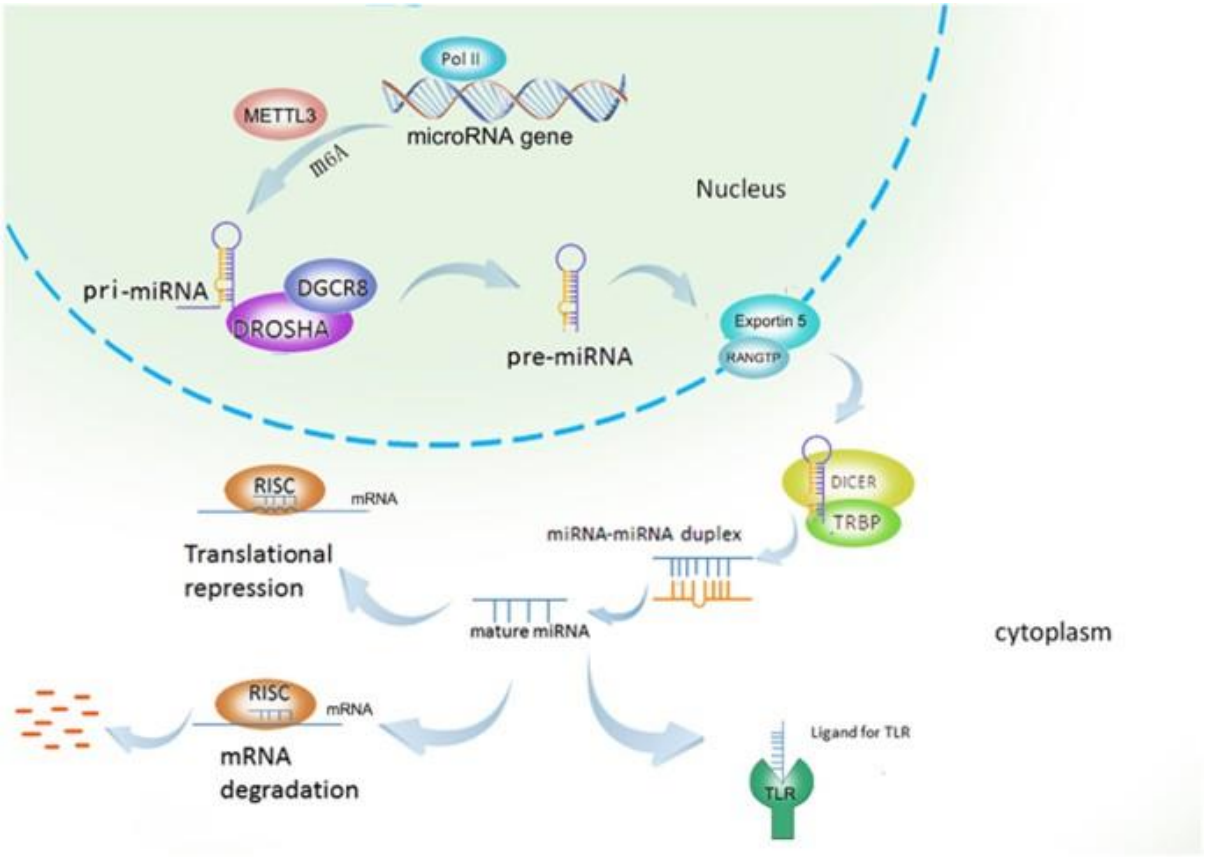

Figure 7: The biosynthesis of miRNAs through the canonical and non-canonical pathways. (178)

Non-coding RNAs (ncRNAs) are categorized into two main groups based on their length. (I) Small non-coding RNAs (sncRNAs), which are shorter than 200 nucleotides and play a role in gene expression regulation, including the activity of miRNAs and small interfering RNAs (siRNAs). (II) Long non-coding RNAs (lncRNAs) which are over 200 nucleotides in length. (179) Stable ncRNAs have been identified in plasma and are being explored as potential biomarkers for diseases such as cancer, lung disease, and cardiovascular disease. These ncRNAs include sncRNAs (e.g., miRNAs, ribosomal RNAs, and siRNAs) and lncRNAs (e.g., antisense RNAs and long intergenic ncRNAs, circular RNAs). (179) Up - or down regulation of different miRNAs have been described to play an important role in the pathophysiology of various diseases, especially CVDs. (6)

a. MicroRNA-33

MicroRNA-33 (miR-33a) is one of the most extensively studied miRNAs. (180) The

miR-33a family comprises of 2 members, miR-33a and miR-33b. MicroRNA-33a is located on intron 16 of the human sterol regulatory element-binding protein 2 (SREBP-2) host gene on chromosome 22 and miR-33b is located on intron 17 of the human SREBP-1 gene on chromosome 17. (181) Both isoforms are predicted to suppress the same subset of genes as they differ by only 2 nucleotides in the seed sequence, structures of hsa-mir-33a and has-mir-33b generated from miRbase as presented in Figure 8 and 9, respectively. (38)

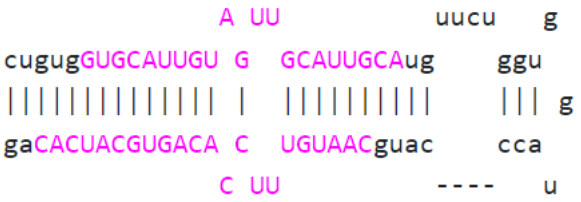

Figure 8: hsa-miR-33a structure
miR-33a is a miRNA encoded within intron 16 of the SREBF2 gene. MicroRNA-33a has a characteristic stem-loop structure, which, once in its mature form, consists of 22 nucleotides.

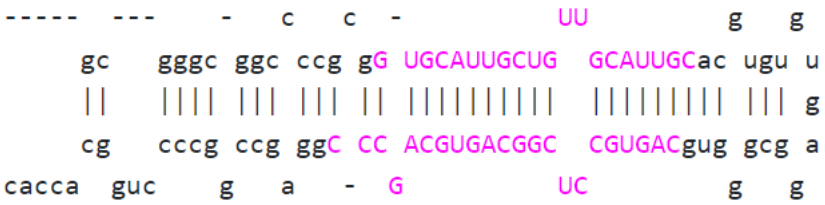

Figure 9: hsa-miR-33b structure.
miR-33b is a miRNA encoded within intron 17 of the SREBF1 gene. MicroRNA-33b is transcribed as a precursor molecule (pre-miR-33b) that folds into a hairpin loop structure, which, once in its mature form, consists of 22 nucleotides.

MicroRNA-33 and hypercholesterolemia

In a recent study, Martino *et al.* (2015), showed that both miR33a and miR-33b are upregulated in children diagnosed with familial hypercholesterolemia and that both miRs correlated with total cholesterol, LDL and Apo B-100. (182)

In a comprehensive review, Chen *et al.* (2013)(38) highlighted the aetiology and pathophysiology of the association between miR-33a and atherosclerosis. MicroRNA-33 plays an important role in lipid metabolism, specifically HDL biogenesis, cholesterol homeostasis and fatty acid, phospholipid, triglyceride, and bile acid metabolism. (38)

Increasing evidence suggest that miR-33a/b also play a pivotal role in cholesterol homeostasis in cooperation with the sterol regulatory element-binding protein (SREBP) gene, *Srebp-1* and *Srebp-2,* which modulates the transcription of a number of genes involved in the synthesis and receptor-mediated uptake of cholesterol and fatty acids. (183-189)

When intracellular cholesterol is decreased, miR-33a and *Srebp-2* are cotranscribed and aid to increase cellular cholesterol by reducing cholesterol export and by inhibiting various transporters involved in cholesterol transport, adenosine triphosphate binding cassette (ABC) transporters, ABCA1 and ABCG1 and the endolysosomal transport protein Niemann-Pick C1 (NPC1), thus suggesting that miR-33a might affect cholesterol homeostasis by down-regulating transmembrane cholesterol efflux transporters. Adenosine triphosphate binding cassette A1, a transmembranous protein, plays an important role in the reverse cholesterol transport by mediating the transport of cholesterol from the peripheral tissue to the liver for metabolism and excretion as well as the export of cholesterol from the peripheral tissues to ApoA-1. (190)

Niemann-Pick C1 is a lysosomal transporter that facilitates the transport, regulated by miR-33a, responsible for the transport of cholesterol from the lysosome to the part of the cell in need. Niemann-Pick C1 works in combination with ABCA1 to promote cellular cholesterol efflux to ApoA-1. (185, 191)

Considering the important role ABCA1 and ABCG1 play in mediating the active efflux of phospholipids to ApoA-1 and HDL and the regulator role miR-33a play in the expression of ABCA1 and ABCG1, researchers speculate that miR-33a may also play a pivotal role in regulating phospholipid efflux. ATPase phospholipid transporting 8B1 gene (ATP8B1), responsible for transporting phosphatidylserine from the outside of the inside of the plasma membrane, contains miR-33a binding sites, suggesting miR-33a may be involved in phospholipid homeostasis. Rayner *et al.* (2011) found that, inhibition of miR-33a significantly decreased the levels of VLDL-associated triglycerides by upregulating the expression of miR-33a target genes involved in fatty acid oxidation and down-regulating the expression of genes involved in fatty acid production. The combined impact of these regulatory changes resulted in a significant

decrease in plasma triglyceride levels. (184)

MicroRNA-33 as a drug target

Given the pivotal role of ABCA1 in the production of plasma HDL, when miR-33a is suppressed, HDL levels might increase, through the regulation of ABCA1 expression. (38) In an attempt to identify miRNAs that might play a role in cholesterol homeostasis Marquart *et al.* (2010) (187) performed an *in silico* study of human gene analysis encoding for nuclear receptors and transcription factors, known to affect lipid homeostasis. The researchers identified sequences corresponding to miR-33a located within intron 16 of the human SREBP-2. To confirm the hypothesis that SREBP-2 and miR-33a are co-expressed, the researchers incubated both mouse and human primary macrophages in media containing low and high cholesterol levels. This resulted in increased expression of the Liver X Receptor (LXR) gene, ABCA1, with repression of the SREBP-2 gene, LDL-R, in media containing a high level of cholesterol, compared to the opposite expression pattern in media containing low levels of cholesterol. The same expression pattern was observed with miR-33a as with SREBP-2. This resulted in a 29% reduction in HDL. (187)

Consistent with the findings of Marquart *et al.* (2010) (187), Najafi-Shoushtari *et al.* (2010) (186) showed that members of the SREBP family of transcription factors are hosts to miR-33a/b that function in combination with the SERBP host gene products to modulate intracellular cholesterol levels and cholesterol homeostasis. The researchers also showed that miR-33a exerts a posttranscriptional control of ABCA1 transporter. Thus, supporting the suggestion by Marquart *et al.* (2010) (187), to treat patients with cardiometabolic diseases with miR-33a/b antisense drugs in combination with statins to elevate HDL levels (186, 187)

Evidence shows significant upregulation of miR-33a after statin (mostly simvastatin) treatment leading to down-regulation of cholesterol efflux transporters and reduced HDL levels. (38, 39) Given the important role ABCA1 plays in cholesterol homeostasis and the generation of HDL, inhibition of miR-33a may lead to increased expression of ABCA1 and plasma HDL. Thus, targeting miR-33a may lead to an increase in plasma HDL and might be an attractive drug target to improve the HDL/LDL ratio. Recently it has been shown that whilst mi-33 deficiency leads to obesity and hyperlipidemia, the

loss of mi-33 in macrophages decreases lipid accumulation and inflammation, leading to a decrease in the plaque burden, which may be its primary mechanism to reduce atherosclerosis. (38, 39) To corroborate this theory, Rayner *et al.* (2011)(192) inhibited miR-33a/b in green monkeys using anti-mRNA oligonucleotides which caused an increase in plasma HDL and expression of ABCA1 over 12 weeks. (40, 183, 192, 193)

The inhibition of miR-33a has been investigated as a drug target in numerous studies (38, 39) and prescribing miR-33a antisense drugs in combination with statin therapy may offer a promising approach to raising HDL levels and treating atherosclerosis. Although targeting miR-33a as a potential therapeutic target to modulate lipid metabolism, increase HDL levels and mitigate atherosclerosis is a promising avenue, research on the exact mechanism and therapeutic implications of miR-33a variations are still underway. (40, 183, 192) As apoA-1 is a major protein component of HDL, this study aimed to quantify miR-33a using Quantitative Polymerase Chain Reaction and Apolipoprotein A – 1 using Enzyme-linked immunosorbent assay, and their relationship, in control, statin naïve and statin intolerant groups.

b. MicroRNA-133

MicroRNA-133 (miR-133a) belongs to a family of miRs that are involved in the development and function of muscle cells, namely the myogenic family. (194) miR-133a is expressed in a variety of tissues, but it is predominantly expressed in cardiac- and skeletal muscle cells. (195-197)

MicroRNA-133 and its role in muscle cells

Skeletal muscle cells develop from embryonic and lateral mesoderm (198) during development, and transcription factors such as, for example myoblast determination protein 1 (MyoD) (199), myogenin (200), myocyte Enhancer Factor 2 (MEF2) (201), paired box protein (Pax) 7 (202) and nuclear factor of activated T-cells (NFAT) (203), and cellular signaling molecules for example insulin-like growth factor (ICFs), Transforming growth factor (TGF) β, Hedgehog signaling pathway, Wnt signaling pathway and notch signaling pathway, play a crucial role in controlling their proliferation and differentiation. (198, 204)

In cardiac muscle cells MEF2, the MADS (MCM1, Agamous, Deficiens, Serum response factor) box transcription factor and serum response factor (SRF), play an important role in the activation of cardiac gene expression by associating with GATA, T-box, Nkx2.5 transcription factors as well as the myocardin family of transcriptional co-activators. Contrary to this, in skeletal muscle MEF2, the basic-helix-loop-helix (bHLH) and MyoD and myogenin activate myogenic differentiation. (205, 206)

Evolving evidence shows that miRs also play a crucial role in the differentiation of skeletal muscle cells. (198) MicroRNAs regulate myoblast proliferation and differentiation, thereby influencing the overall process of myogenesis. MicroRNA-133 is a family of miRNAs most widely studied for their prominent expression in skeletal and cardiac muscle cells. MicroRNA-133 belongs to a family of miRNAs that are involved in the development and function of muscle cells, namely the myogenic family. (194) MicroRNA-133 is expressed in a variety of tissues, but it is predominantly expressed in cardiac- and skeletal muscle cells. (195-197) MicroRNA-133a-1 (Figure 10) and miR-133a-2 (Figure 11) are identical in structure, however a slight difference of 2 nucleotides is noted in miR-133b. (Figure 12) (structures generated from miRBase). MicroRNA-133a-1 and miR-133a-2 are muscle-specific miRNAs that are regulated by the SRF transcription factor. Mature sequences of miR-133a-1, miR-133a-2 and miR-133b are shown in Table 6. (207)

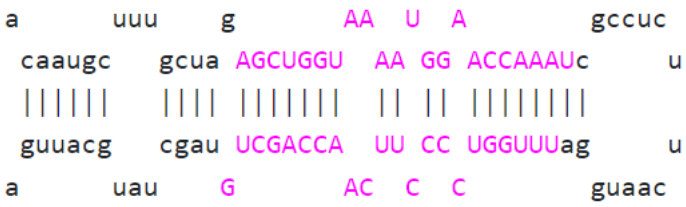

Figure 10: miR-133-a-1 structure.
MicroRNA-133a-1 is transcribed from the intron of the MID1 gene. It is initially synthesised as a precursor hairpin loop structure, pre-miR-133a-1, before it is cleaved into its mature form of 22 nucleotides.

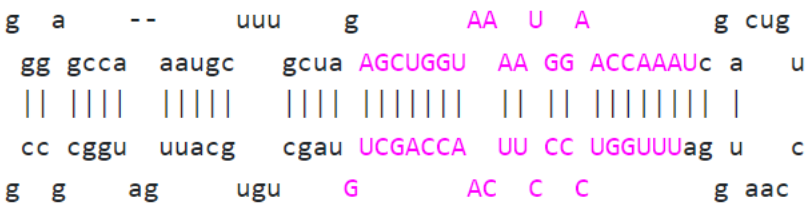

Figure 11: miR-133a-2 structure.
MicroRNA-133a-1 and miR-133a-2 are closely related miRNAs which share similar sequences and functions. It is initially synthesised from a precursor hairpin loop structure, pre-miR-133a-2, before it is processed into a mature form of 22 nucleotides.

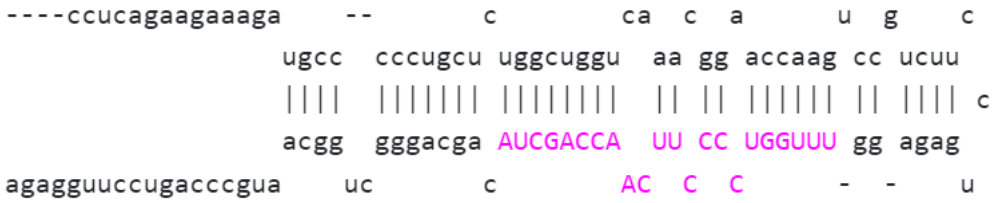

Figure 12: miR-133b structure.
MicroRNA-133b is initially synthesised from a precursor hairpin loop structure, pre-miR-133a-2, before it is processed into a mature form of 22 nucleotides. It shares functional similarities with miR-133a.

Table 6: Mature sequences of miR-133a-1, miR-133b-1, miR-133b obtained from miRbase.

microRNA	Sequence
hsa-miR-133a-1	53 - UUUGGUCCCCUUCAACCAGCUG - 74
hsa-miR-133a-2	59 - UUUGGUCCCCUUCAACCAGCUG - 80
hsa-miR-133b	66 - UUUGGUCCCCUUCAACCAGCUA - 87

MicroRNA-133a has been shown to regulate the expression of several genes involved in muscle development and function. Serum Response Factor, MEF2 and MyoD are responsible for regulating the expression of miR-133a in both cardiac and skeletal muscle. In the miR-1-2/133a locus, SRF controls cardiac specific expression, while MEF2 directs ventricular expression. Research shows that miR-133a inhibits myoblast differentiation and promotes myoblast proliferation by inhibiting SRF. In addition, miR-133a is involved in the regulation of muscle hypertrophy and atrophy, as well as respond to muscle injury. (208-211)

To understand the mechanism of miRNAs involvement in the proliferation and differentiation of skeletal muscle cells, Chen et al. (2006) (212) studied the expression

of miRNAs during skeletal muscle cell differentiation using an already established microarray analysis. The researchers made use of the C2C12 cell line as these myoblasts mimics skeletal muscle differentiation *in vitro.* The researchers showed an upregulation of miR-133a and miR-1 in differentiated C2C12 myoblasts using RNA blot analysis. To assess the function of miR-1 and miR-133a in skeletal muscle, the researchers performed multiple experiments, which included inhibiting the function of miRNAs by transfecting C2C12 myoblasts with 2'-*O*-methyl antisense inhibitor oligoribonucleotides. Consistent with the conclusion made by Callis *et al.* (2008) (208), inhibition of miR-133a led to myoblast differentiation being enhanced and proliferation was inhibited, confirming miR-133a's role in promoting myoblast proliferation and inhibiting myoblast differentiation. (212)

Various functions have been attributed to miR-133a, however evolving research suggest that the primary function of miR-133a is to regulate proliferation and differentiation, whether it be in a cell-type or signal-specific manner. (213, 214)

miR-133a has also been shown to have important roles in other tissues, including the brain, where it is involved in neuronal differentiation and synapse formation. Abnormal miR-133a expression has been implicated in various diseases, including cardiovascular disease, cancer and neurological disorders. (195-197)

MicroRNA-133 and statin intolerance

Muscle injuries are a common occurrence, especially in sports and drug-induced injury. (215, 216) Statin is one of the marketed drugs most commonly associated with muscle injury, often leading to the cessation of the drug. (217-221) Statin intolerance is a term that loosely embraces all skeletal muscle related adverse events. (13, 22, 137) Statins are likely to be the cause of the above-mentioned symptoms, if symptoms such as muscle aches and pains, cramps, fatigue, and weakness start within 3 months of treatment initiation they resolve once treatment is terminated. (22, 138)

There is some evidence to suggest that miR-133a could potentially be a biomarker or therapeutic target for muscle damage caused by statins. (209, 222, 223)

In a study conducted by Nakasa *et al*. (2009) (209), the researchers reasoned that based on the mechanism of miRNAs role in muscle development and regeneration, overexpression of miR-1, miR-133a and miR-206 during muscle injury could fast-track muscle regeneration. The aim of the study was to demonstrate the assumption made by injecting rat tibialis anterior muscle laceration model with a single injection of muscle specific miRNAs (the study consisted of two treatment groups, one injected with double-stranded miR-1, miR-133a and miR-206 and the other with small interfering RNA (siRNA) control). The researchers showed that the combination of miR-1, miR-133a and miR-206 can promote myotube development and differentiation by also upregulating the expression of myoD1, myogenin and Pax7. Thus a local injection of double stranded miRNAs could be a therapeutic target for skeletal muscle injury. (209)

According to a study conducted by Mishima *et al.* (2009) (224), the researchers identified 245 target mRNAs that are regulated by myogenic miRNAs. MicroRNA-1 and miR-133a appear to down-regulate these targets in muscle development and play a role in embryonic muscle gene expression. MicroRNA-1 and miR-133a regulate actin-related and actin binding proteins. Down regulation of miR-1 and miR-133a disrupts actin organization during sarcomere assembly thus altering muscle gene expression. The study concluded that miR-1 and miR-133a actively regulate gene expression in muscle tissue. (224)

As per the study carried out by Laterza *et al.* (2009) (222) the researchers generated pools of plasma from rats treated with different molecules, including HMG-CoA reductase inhibitors (statins). Quantitative polymerase chain reaction (qPCR) analysis identified an increase in miR-133a expression in all 3 groups. miRNA expression was plotted in comparison to the respective AST/ALT data, the conservative biomarkers associated with liver and muscle injury. HMG-CoA reductase inhibitor-treated animals presented with the highest increase in ALT and an 800-fold increase in miR-133a. (222) The study identified miR-133a as a plausible biomarker for skeletal muscle injury associated with HMG-CoA reductase inhibitors although the mechanism is not yet fully understood, and the theory has not yet been tested on participants prescribed chronic statin therapy. (222)

Watanabe *et al.* (2019) (223) confirmed the findings of Laterza *et al.* (2009) (222). The main aim of the study was to determine a mouse model of drug-induced rhabdomyolysis by co-administering statins and fibrates. The study showed a significant increase in the plasma levels of creatine phosphokinase (CPK), AST and miR-206-3p and miR-133a-3p with no increase in miR-122-5p and miR-208-3p, confirming the increase in CPK, AST, miR-206-3p and miR-133a-3p is due to skeletal muscle toxicity in the lovastatin treated group. (223)

Overall, these studies suggest that miR-133a is an important regulator of muscle development and regeneration and may be a promising target for therapies aimed at promoting muscle repair and regeneration following injury or disease. While the exact mechanisms underlying the relationship between miR-133a and statins are not yet fully understood, there is some evidence to suggest that miR-133a could potentially be a biomarker for muscle damage caused by statins. However, further research is needed to confirm miRNA-133a as a reliable biomarker for muscle damage caused by statins. As ALT is an enzyme found in various tissues and organs, it is used as a marker for liver and muscle toxicity, thus this study aimed to quantify miR-133a using Quantitative Polymerase Chain Reaction and aspartate aminotransferase levels using ELISA in control, and their relationship, in control, statin naïve and statin intolerant groups.

c. microRNA-499a

MicroRNA-499a, belongs to a group of recently discovered microRNAs encoded by the myosin gene family. (225, 226) It is expressed in the cardiac ventricles and its main functions include controlling cardiac cell proliferation, differentiation, and angiogenesis. (226-230) MicroRNA-499a is expressed in the liver and skeletal muscle, where it contributes to the development of liver diseases like hepatocellular carcinoma and potentially plays a role in muscular diseases such as muscular dystrophy. (228, 231, 232)

Traditional cardiac biomarkers

Acute myocardial infarction (AMI) is a common condition characterised by the death of cardiomyocytes due to prolonged ischemia. (233) Atherosclerosis is thought to play

a pivotal role as the underlying cause for AMI. (234) Several tests have been marketed for the diagnosis of AMI. However, due to the numerous limitations experienced, e.g. prolonged waiting times for electrocardiograms, prolonged waiting time from troponin levels administering thrombolytic agents, and most notably and the infrequent use of emergency angiography as a standard procedure, AMI still remains one of the leading causes of mortality worldwide and thus alternative diagnostic markers are needed. (233, 235-237)

Myocardial infarction (MI) is a leading cause of death worldwide, making early diagnosis crucial for appropriate medical intervention. While electrocardiograms (ECGs) are still the recommended test to determine MI in emergency patients, they lack sensitivity. (238-240)

The detection of MI has been studied since the 1950s, with various biomarkers being used as a method of diagnosis. (241) Biomarkers are proteins found in blood samples that can indicate the presence of damage to the heart. The most commonly used biomarkers for MI are myoglobin, creatine-kinase myocardial band (CK-MB), and cardiac troponin. (242)

In 1966, van der Veen and Willebrands demonstrated that CK-MB was a specific marker for MI, however CK-MB is also released during, myocarditis and cardiac trauma. (243, 244) During the 1970s, CK-MB became the standard for diagnosing MI, with improved sensitivity through isoenzyme analysis. (244) The development of rapid and accurate laboratory testing for CK-MB revolutionised the treatment of patients with acute cardiac events in the 1970s and 1980s by allowing earlier diagnosis of AMI and detection of reinfarction. (245) The CK-MB assays relies on the measurement of enzyme activity, but improved accuracy and ease of use were established by the use of mass assays. (246, 247) In the mid-1980s, immunologic detection of biomarkers through mass assay was developed, allowing direct analysis of CK-MB levels. (248, 249) CK-MB is not only present in the heart, but also in other tissues making diagnosis complicated. The use of the percent relative index improved the specificity of elevations for cardiac muscle injury, but it was still insensitive when concurrent cardiac injury and skeletal muscle injury were present. (250, 251). Despite improvements in precision and sensitivity over time, CK-MB was still not specific to heart injury. With

the increasing diagnostic sensitivity and specificity of troponins, CK-MB's role has diminished. (252-257)

Cardiac troponin T (cTnT) and cardiac troponin I (cTnI) are considered to be the most sensitive and specific biomarkers for MI and have led to the development of new devices and technologies for detection. (258) These biomarkers are released from necrotic cardiac cells within 2-4 hours and 3-4 hours, respectively, after the onset of MI symptoms. (259-261) They remain in the bloodstream for over 10 days and reach peak levels 1-2 days after the injury. (262) Elevated levels of cTnI can indicate myocardial injury and should be defined as a measurement exceeding the 99th percentile of a reference control group. There is also a biomarker called troponin C (cTnC), however, it is not considered to be a cardiac-specific biomarker and is not favoured for the diagnosis of cardiac injury. (263)

It is widely believed that a rapid and correct diagnosis of CVD, such as AMI, has an important impact on patients' treatment and prognosis. Therefore, biomarkers with a higher sensitivity and specificity than the current diagnostic markers are needed. (225)

MicroRNA-499a as a diagnostic biomarker for cardiovascular disease

In CVD, specifically AMI, miRNAs are proposed as an important biomarker for the diagnosis and prognosis of CVDs. (264, 265) van Rooij *et al*. (2009) showed that quantification of certain miRNAs showed organ- and cell-specific expression (266), Creemers *et al.*, conducted *in-vitro* studies which suggest up- and down regulation of certain miRNAs in CVD (265), and single nucleotide polymorphisms (SNPs) in miRNAs have also been associated with different types of CVDs. (267-269)

Although various miRNAs have been identified to be upregulated in patients with CVD, specifically AMI, miR-499a showed diagnostic promise when compared to standard and conventional biomarkers, including cTnI and cTnT. (265, 270, 271)

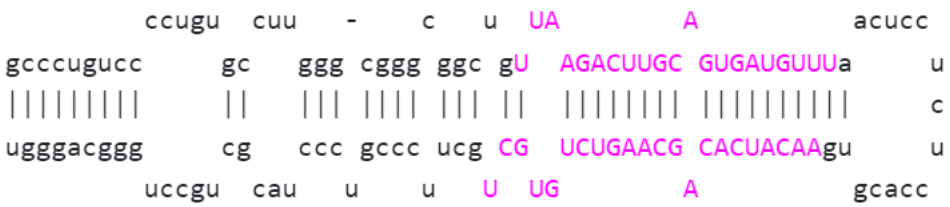

Figure 13: miR-499a structure
MicroRNA-499a is encoded within the intron region of MYH7B. It is initially synthesised as a precursor molecule with a characteristic stem-loop structure, pre-miR-499a, before it is processed into its mature form of 22 nucleotides.

MicroRNA 499 which is encoded by intron 19 within the myosin heavy chain 7B (Myh7b) gene is a recently discovered miRNA encoded by the myosin gene family. (225, 226, 272) MicroRNA 499 is a muscle-specific miRNA and is mainly expressed in the cardiac ventricles, structure presented in Figure 12. The high expression of miR-499a in the heart promotes the idea that miR-499a might participate in the cardiac pathophysiological- and physiological processes. (273) MicroRNA 499 plays an important role in regulating the development of cardiovascular disease, due to their involvement in the regulation of cardiac cell proliferation, differentiation, and angiogenesis. (226-230) However, miR-499a is also expressed in the liver and skeletal muscle. MicroRNA 499 plays a vital role in the development of liver diseases, such as hepatocellular carcinoma, (274, 275) and may also play a role in the development on muscular diseases, such as muscular dystrophy. (228, 231, 232)

Due to the challenges experienced in practice with the detection of cardiac troponin elevation, especially in elderly patients (average age higher than 80 years) who present with symptoms of a non-ST elevation myocardial infarction (NSTEMI) and acute CHF, Olivieri *et al*. (2012) conducted a study to examine miRNA plasma levels in elderly participants. Ninety-two NSTEMI patients, eighty-one patients with acute CHF without AMI and 99 age matched healthy participants were enrolled in the study. Plasma levels of miR-1, miR-21, miR-133a, miR-208a, miR-423-5p and miR-499a-5p were analysed in all participant groups. MicroRNA 208a was undetectable and thus excluded from the analysis. However, it was noted that, the mean fold increase of all analysed miRNAs (except miR-208a) was significantly higher in NSTEMI, and acute CHF participants compared to the control group. Micro-RNA 499-5p was significantly increased in participants diagnosed with NSTEMI compared to acute CHF participants

and the control group. (276) It was noted that miR-499a-5p showed a higher sensitivity and specificity compared to cTnT when differentiating NSTEMI patients from patients with acute CHF. (276)

During an earlier study. Wang *et al*. (2010) showed muscle enriched miRNAs, including miR-499a, is elevated in plasma. However, the researchers also confirmed miR-499a is exclusively expressed in cardiac muscle. Wang *et al*. (2010) conducted microarray and qPCR analysis to detect miRNA levels in plasma of healthy participants and noted that miR-1, miR-499a and miR-208a were undetectable by microarray, with only trace amounts of miR-499a and miR-1 detectable by qPCR. (277) To investigate whether these miRNA levels spiked after an MI, the researchers identified an AMI rat model in which qPCR showed that levels of miR-1, miR-133a and miR-499a reached its peak 3 to 12 hours post coronary ligation and decreased after 12 hours. (277)

Likewise, Agiannitopoulos *et al*. (2018) examined the expression of 2 cardiac specific miRNAs, miR-499a and miR-208, in 2 groups, participants with AMI and healthy subjects using TaqMan® MiRNA assays. The expression of both miRNAs were significantly increased in the patients who presented with AMI, miR-499a was increased by 675-fold, compared to a 256-fold increase of miR-208b. (278)

These studies suggest that similar to cardiac enzymes, cardiac associated miRNAs, specifically miR-499a, is elevated in patients who experience cardiac injury, such as AMI, suggesting miRNAs might be a new and more sensitive biomarker for the diagnosis of CVD. (226, 264-270, 276-278)

A recent study by Sheng *et al.* (2023) (279) showed that miR-499a can directly bind to Sry-box (SOX) 6 and induce an increased expression of proliferating cell nuclear antigen (PCNA), cyclin D1, matrix metalloproteinase (MMP2). This suggests that miR499a may boost the proliferation and migration of vascular smooth muscle cells by directly binding to- and inhibiting the expression of SOX6. These findings support the notion that miR-499a might be a promising target for the prevention and treatment of CVD. (279)

However, despite large number of promising results, it should be noted that detection and quantification of miRNAs is not an easy and affordable process. Several methods are available for the detection and quantification of miRNAs, including, qPCR, microarrays, enzymatic luminescence miRNA assay, deep sequencing and *in situ* hybridisation. (280) Zampetaki and colleagues (2012) highlighted the various obstacles experienced with quantification and detection of miRNAs, such as technical aspects of sample collection, factors such as anticoagulants and cell remnants may lead to contaminated samples, the age of the sample, the tissue type and the storage conditions, laboratory conditions, the lack of standardised protocols (normalised controls), which affect inter-study comparability, reproducibility and difficulty in measuring the absolute levels of miRNAs. (281) In qPCR data, normalization is critical in accurately profiling circulating miRNAs. There are different methods of normalization, including the use of synthetic non-mammalian miRNAs, endogenous miRNAs, or the average of all miRNAs tested. The researchers suggest the use of different normalization methods to ensure robustness of data and inter-study comparability. (281)

Quantitative polymerase chain reaction is the gold standard for gene expression analysis and the most commonly used for miRNA quantification due to its high specificity and sensitivity, However, despite its advantages , this method has various limitations, including miRNAs that are present in low concentrations in samples may degrade during extraction and purification leading to low yield and sensitivity, false results due to interference from other RNA species, RNA degradation and variation in extraction efficiency might impact the quality of the extracted miRNAs. Variations in reagent quality, laboratory conditions, and operator expertise can introduce variability into the results and limit the comparability and reproducibility of results. Reverse transcription quantitative polymerase chain reaction is a highly sensitive technique but is also affected by technical variability, such as variations in PCR efficiency, instrument performance, and reaction conditions which may further impact the reproducibility of the results. (280, 281) These findings were supported by a review of de Planell-Saguer *et al*. (2013) that highlighted the challenges experienced during miRNA detection are due to the intrinsic molecular characteristics such as their small size and large of differences in the number of expressed copies among different miRNAs. (280)

Personalised medicine, also known as precision medicine, an approach to treat and diagnose patients considering the genetic and molecular make up of each individual, also play a vital role in the expression and detection of various miRNAs. MicroRNAs expression can vary greatly between individuals making it challenging to accurately detect and quantify these miRNAs in a given sample. Inter-individual differences such as environment, disease, genetics, lifestyle etc. can influence expression on miRNAs. Researchers have overcome these challenges by using multiple methods/techniques to detect and quantify miRNAs. However, inter-individual variability should still be considered when analysing data. (280-283)

MicroRNA-499 and statin intolerance

MicroRNA-499 is responsible for, cardiomyocyte differentiation (41), mitochondrial function, protection against cardiomyocyte apoptosis (284) and skeletal muscle fibre type switching. (285)

It is notable that statin tolerability may be affected by an array of factors, including genetic variation. (141) Although there is limited research available specifically linking miR-499a to statin intolerance, a study conducted by Min *et al.* (2016)(286) combination of *in vitro* and *in vivo* modeling of pre-, during- and post exercising muscle to evaluate the release of different miRNAs during muscle contraction in both chronic statin users and non-statin users. MicroRNA-499a was upregulated due to statin exposure in athletes and cultured myotubes. (286, 287)

A possible mechanism implicated in statin-associated muscle toxicity is mitochondrial dysfunction. The possible association between statins and their ability to induce mitochondrial-mediated apoptosis in muscle cells has gained more attention over the past few years, (285) supporting the theory that miR-499a may serve as a biomarker associated with statin-induced muscle damage. To date, there is a paucity of *in vitro* studies drawing a correlation between CK levels and upregulation of miR-499a in hypercholesterolemic participants on chronic statin therapy, thus this study aimed to quantify miR-499a in using Quantitative Polymerase Chain Reaction and Creatine Kinase levels using ELISA, and their relationship, in control, statin naïve and statin intolerant groups . (286)

1.5 Study Objectives

Simvastatin and atorvastatin are the first line treatment prescribed for hypercholesterolemia – they are generally effective and affordable, but a large part of the population experiences some form of intolerance. MiR-33a, miR-133a and miR-499a are some of the miRNAs hypothesized to be most frequently implicated in statin intolerance, due to their direct association with cholesterol homeostasis and circulatory statin levels. Intolerance to simvastatin and atorvastatin results in nausea, vomiting, diarrhoea, abdominal pain, skin rash, and muscle-related symptoms. Therefore, although these medications themselves are affordable, the cost associated with treating these adverse effects can be expensive. Although the bigger cost is related to treating CVD complications, it is important that we continue to determine if there are alternative therapies which allows for patients sensitive to statins, to have a better quality of life while reducing cholesterol

In South Africa, the health care system is divided into a disproportionate blend of private and public health care. A small segment of the population, treated in the private health sector, is serviced by a large proportion of available healthcare resources. Due to the imbalance experienced in the current health care system, South Africa lags behind in personalised medicine and targeted therapy, e.g. limited access to genetic testing, underrepresentation in genomic databases, high prevalence of communicable disease which results in resources being diverted to disease such as HIV and TB, cost and resource constraints making advanced diagnostic and targeted therapies inaccessible to most South Africans. (288-290) To bridge this gap, South Africa will need to keep pace with the technological advances and fast changing world of precision medicine. In May 2024, the president signed the National Health Insurance (NHI) Bill which aims to provide universal access to quality health care to all South Africans as per the South African Constitution. The NHI Bill will take many years to be fully implemented and effective as the entire South African health care system will need to be reformed, which includes the integration of the private and public health care sectors. (291) The South African Government has opted to implement the NHI Bill in 3 incremental phases, in an effort to allow public health infrastructure and services to be improved. (292) Thus, the results of this study will help to gain a better

understanding of epigenetics and its effect on statin pharmacokinetics, which will allow a more personalised approach to statin therapy, especially relevant within our diverse South African population.

The aim of this study was therefore to identify potential circulating biomarkers of statin intolerance in a cohort of hypercholesterolaemic patients from Gauteng, South Africa.

The objectives of this study were to:

1. Determine if there is a difference in demographic and clinical variables between control, statin naïve and statin-treated groups.

2. Perform *in silico* screening of miR-33a, miR-133a and miR-499a using miRBase and TargetScan.

3. Quantify miR-33a using Quantitative Polymerase Chain Reaction and Apolipoprotein A – 1 using Enzyme-linked immunosorbent assay, and their relationship, in control, statin naïve and statin-treated groups.

4. Quantify miR-133a using Quantitative Polymerase Chain Reaction and Alanine Aminotransferase levels using ELISA in control, and their relationship, in control, statin naïve and statin-treated groups.

5. Quantify miR-499a in using Quantitative Polymerase Chain Reaction and Creatine Kinase levels using ELISA, and their relationship, in control, statin naïve and statin-treated groups.

Chapter 2: Materials and methods

This cross-sectional observational study aimed to identify potential circulating biomarkers of statin intolerance in a cohort of hypercholesterolaemic patients from Gauteng, South Africa.

The study design, materials, methods, statistical analysis, and ethics are described in detail in this chapter.

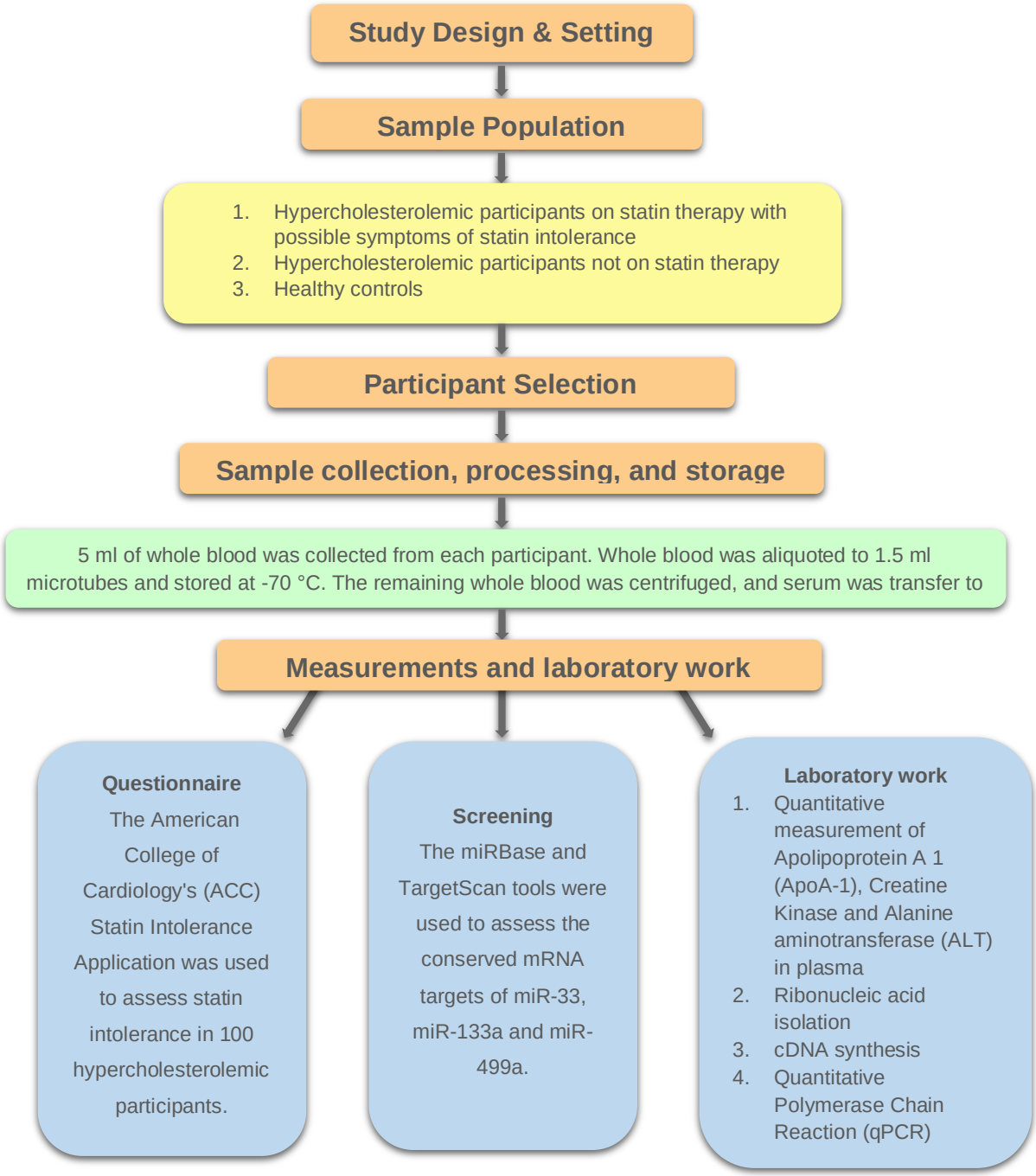

Figure 14: Flow diagram illustrating the study design.

2.1 Ethics

This protocol was submitted to the Faculty of Health Sciences Ethics Committee (protocol number - 709/2021). The study was conducted in accordance with the International Conference on Harmonization Good Clinical Practice (ICH-GCP) guidelines. Written informed consent was obtained from all participants before any sample or data was collected. Confidentiality and anonymity were and will be maintained by using numerical codes as patient identifiers. All participant data

collected for the study was stored on a specifically designed excel database which is stored on a password protected google drive. Relevant blood samples used for analysis were collected by a physician or nurse.

2.2 Study design

This was a cross-sectional and case-controlled study which included a quantitative questionnaire of 100 control participants, 100 hypercholesterolemic participants not taking statins and 100 statin-treated hypercholesterolemic participants.

2.3 Setting

This study was conducted at the University of Pretoria, in the Gauteng Province of South Africa.

2.4 Participant selection

Statin-treated hypercholesterolaemic patients and non-statin treated patients diagnosed with hypercholesterolemia were recruited from medical centers and pharmacies in the Gauteng area as required. Control participants were recruited from the general population in Gauteng, e.g., people recruited from shopping centers, staff members at Clinical Research Unit (CRU) and University of Pretoria and volunteers from public spaces, who have not been diagnosed with hypercholesterolemia. Participants were required to meet all inclusion criteria prior to enrolment. All participants on statin therapy who met the age and hypercholesterolemia criteria were initially approached for possible inclusion in the statin-treated group. Symptoms potentially suggestive of statin intolerance (e.g., muscle aches, muscle weakness, or other adverse effects) were assessed prior to enrolment as a pre-screening measure to ensure compliance with the inclusion criteria. All participants included in the study was age, sex and race matched to limit possible biological and clinical variability. Participant demographic information, specifically pre-existing co-morbidities, concomitant medication and lifestyle factors were collected used for sample selection and analysis.

2.4.1 Inclusion criteria for participants with hypercholesterolemia on statin therapy

A hypercholesterolaemic patient was eligible for participation if all the following

inclusion criteria were met:

1. Written informed consent for study participation prior to the start of any study related procedures.
2. Patients older than 18 years of age.
3. Confirmed diagnosis of hypercholesterolemia. (Confirmed verbally by the participant or the treating physician.)
4. Possible symptoms of statin intolerance (including muscle aches, muscle weakness, tingling or twitching)
5. On continuous and stable minimum 12-week atorvastatin-/ simvastatin dose.

2.4.2 Exclusion Criteria for participants with hypercholesterolemia on statin therapy

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

1. Unwilling to give consent.
2. Patients younger than 18 years of age.
3. Any disruption of atorvastatin or simvastatin therapy within the preceding 12-week period.
4. Any chronic disease, screened by medical history, including but not limited to, Mycobacterium tuberculosis (TB), cancer, Human immunodeficiency virus (HIV), current COVID-19 infection, renal disease, and hepatic disease.

2.4.3 Inclusion criteria for participants with hypercholesterolemia not on statin therapy

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

1. Written informed consent for study participation prior to the start of any study related procedures.
2. Patients older than 18 years of age.
3. Confirmed diagnosis of hypercholesterolemia. (Confirmed verbally by the participant or the treating physician.)
4. Not currently on a statin treatment regimen, including atorvastatin, simvastatin, Pravastatin, Rosuvastatin, Lovastatin, Fluvastatin or Pitavastatin

2.4.4 Exclusion criteria for participants with hypercholesterolemia not on statin therapy

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

1. Unwilling to give consent.
2. Patients younger than 18 years of age.
3. Currently on a treatment regimen that includes Atorvastatin, Simvastatin, Pravastatin, Rosuvastatin or Pitavastatin
4. Any chronic disease, screened by medical history, including but not limited to, TB, cancer, HIV, current covid-19 infection, renal disease, and hepatic disease.

2.4.5 Inclusion criteria for control participants

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

1. Written informed consent for study participation prior to the start of any study related procedures.
2. Participants older than 18 years of age.

2.4.6 Exclusion criteria for control participants

A participant was not eligible for participation if any of the following exclusion criteria were met:

1. Unwilling to give consent.
2. Participants younger than 18 years of age.
3. Participants diagnosed with hypercholesterolemia.
4. Participants on any lipid-lowering therapy.
5. Any chronic disease, screened by medical history, including but not limited to, TB, cancer, HIV, current covid-19 infection, renal disease, and hepatic disease.

2.5 Measurements and laboratory work

2.5.1. Questionnaire

The American College of Cardiology (ACC) Statin Intolerance Application was used to assess statin intolerance in 100 hypercholesterolemic patients currently on statin therapy. The ACC Statin Intolerance Application's calculator was employed to determine statin intolerance risk. (Appendix 6) (293)

2.5.2, Quantitative measurement of Apolipoprotein A 1 in plasma

Apolipoprotein A1 levels were estimated using the ApoA-1 Human SimpleStep ELISA® Kit. (Elabscience. Catalogue number: E-EL-H0125)

Blood samples were collected in 5 ml citrate tubes and centrifuged at 2000 x *g* for 10 min. Plasma samples were transferred to collection tubes to be stored at -80°C. Plasma samples were thawed at room temperature and vortexed for 5 min. Samples were diluted (100 fold dilution factor) into Sample Diluent NS. A total of 50 µl of each sample were added to the appropriate wells in a 96 well plate, after which 50 µl of the Antibody Cocktail was added to each well. The plate was sealed and incubated for 1 hour at room temperature on a plate shaker set to 2000 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. (Complete removal of liquid at each step was essential.) After the last wash, the plate was inverted and blotted with clean paper to ensure removal of all excess liquid. A total of 100 µl of TMB Substrate was added to each well and the plate was incubated for 10 min, in the dark, on a plate shaker set at 2000 x *g*. (Given variability in the laboratory environment, the incubation time varied between 5 – 20 min.) After incubation, 100 µl of Stop Solution was added to each well and the plates were placed back onto the plate shaker for 1 min to ensure thorough mixing. The intensity was recorded at 450 nm. The optical density and standards with known concentrations were used to create standard curves. The equation generated from the standard curve was used to calculate the concentration of Apo-A1 in unknown samples.

2.5.3. Quantitative measurement of plasma Creatine Kinase

Creatine kinase levels were estimated using the CKM Human SimpleStep ELISA® Kit. (Elabscience. Catalogue number: E-EL-H1433)

Blood samples were collected in 5 ml citrate tubes and centrifuged at 2000 x *g* for 10 min. Plasma samples were transferred to collection tubes and stored at -80°C. Plasma samples were thawed at room temperature and diluted with Sample Diluent in a 1:50 ratio. Standards were also reconstituted and prepared in sample diluent. A total of 50 µl of each sample or standard with known concentration was added in duplicate to the appropriate wells in a 96 well plate, after which 50 µl of the antibody cocktail was added to each well. The plate was sealed and incubated for 1 hour at room temperature on a plate shaker set to 2000 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. Complete removal of liquid at each step was essential. After the last wash, the plate was inverted and blotted with clean paper to ensure removal of all excess liquid. A total of 100 µl of TMB substrate was added to each well and the plate was incubated for 5 min on a plate shaker set at 2000 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 optical density was measured using a spectrophotometer (Biotek Synergy HT, Software GEN 5.1) at an absorbance 450 nm. A standard curve was constructed, and the concentration of the unknown samples was calculated using the equation derived from the standard curve (y=mx+c). All samples were diluted for the assay and then multiplied by the dilution factor when final concentrations were calculated.

2.5.4. Quantitative measurement of Alanine aminotransferase (ALT) in plasma

Alanine aminotransferase (ALT) levels were estimated using the ALT Activity Assay Kit (Colorimetric). (Cell Biolabs, Inc. Catalogue number: MET-5123)

Blood samples were collected in 5 ml citrate tubes and centrifuged at 2000 x *g* for 10 min. Plasma samples were transferred to collection tubes and stored at -80°C. Plasma samples were thawed at room temperature and diluted with Sample Diluent in a 1:50 ratio. Pyruvate Standards were also reconstituted and prepared in sample diluent. A total of 50 µl the selected samples or pyruvate standards with known concentrations and the positive control was added in duplicate to the appropriate wells of the 96 well plate. After which a 100 µl of the prepared Reaction Reagent was added to each well. The contents of each well were mixed thoroughly. The plate was covered to protect the contents from light exposure. Absorbance of each well was read immediately using

a spectrophotometric plate reader at an absorbance of 540-570 nm.

2.5.5. Ribonucleic acid (RNA) isolation

Total RNA was extracted from Whole Blood (WB) of hypercholesterolemic patients and healthy volunteers using the QIAzol lysis reagent. Whole blood samples were collected in citrate tubes.

QIAzol lysis reagent (cat. no.: 79306, QIAGEN, USA) was added to the appropriate vessel for disruption and homogenization and subsequent centrifugation. Five hundred µl of QIAzol lysis reagent was be added to each tube per 500 µl WB. The tube containing the homogenised ruptured WB was incubated on the bench top at room temperature for 5 min. Following incubation, 0.1 ml of chloroform per 0.5 ml QIAzol lysis reagent was added to each tube and vortexed for 15 s.

After incubation at room temperature for 2-3 min, the samples were centrifuged at 12 000 xg for 15 min at 4°C. The upper, aqueous phase was transferred into a new 1.5 tube and 0.1 ml isopropanol was added to the tubes and mixed thoroughly. The samples underwent a second centrifugation at 2 000 xg for 10 min at 4°C. The supernatant from each tube was carefully aspirated and discarded.

An aliquot of 0.5 ml 75% ethanol was added to each sample and the samples were centrifuged at 7400 xg for 5 min 4°C. The supernatant was removed completely, and the RNA pellet was air-dried briefly. 12.5 µl of nuclease-free water was used to re-dissolve the RNA. The total isolated RNA was quantified (1 µl) spectrophotometrically (NanoDrop2000c, Thermo-Fischer, USA). The quantified RNA was standardised to 200 ng/µl and used to prepare cDNA for qPCR.

2.5.6. cDNA synthesis and Quantitative Polymerase Chain Reaction (qPCR)

Following RNA isolation, cDNA was synthesised using the TaqMan Advanced miRNA cDNA synthesis kit (Thermo Fisher Scientific, South Africa). A four-step conversion was performed to prepare cDNA of the miRNAs from total RNA.

i. The poly(A) tailing reaction.

   Two µl of sample was added to each well of a 96-well plate. After which, 3 µl of the poly(A) reaction mix which contains 5.5 µl of 10X poly (A) buffer, 5.5 µl

of ATP, 3.3 µl of poly(A) enzyme and 18.7 µl of RNase-free water was added to each well. The plate was sealed, vortexed, centrifuged and placed into the Roche Light Cycler96 (Roche, South Africa).

Cycling conditions:

Polyadenylation: 1 cycle at 37ºC for 45 min

Stop reaction: 1 cycle at 65ºC for 10 min

Hold: 1 cycle at 4ºC

ii. The adaptor ligation reaction.

A total of 33 µl of 5X DNA ligase buffer, 49.5 µl of 50% PEG 800, 6.6 µl of 25X ligation adaptor, 16.5 µl of RNA ligase and 4.4 µl of RNase-free water were mixed together to make up the ligation reaction mix. 10 µl of the ligation reaction mix was added to each well containing the poly(A) tailing reaction product. The plate was vortexed and centrifuged and then placed into the Roche Light Cycler96 (Roche, South Africa).

Cycling conditions:

Ligation: 1 cycle for 16ºC for 60 min

Hold: 1 cycle at 4ºC

iii. Reverse transcription (RT) reaction.

A total of 66 µl of 5X RT buffer, 13.2 µl of dNTP mix (25mM each), 16.5 µl of 20X universal RT primer, 33 µl of 10X RT enzyme mix and 36.3 µl of RNase-free water were mixed to make the RT reaction mix. An amount of 15 µl of the mix was then added to each of the wells that contained the adaptor ligation reaction product. The plate was then vortexed and centrifuged and then placed into the Roche Light Cycler96 (Roche, South Africa).

Cycling conditions:

Reverse transcription: 1 cycle at 42ºC for 15 min

Stop reaction: 1 cycle at 85ºC for 5 min

Hold: 1 cycle at 4ºC

iv. The miRNA-Amp reaction.

A total of 275 µl of 2X miRNA-Amp master mix, 27.5 µl of 20X miRNA-Amp primer mix, 192.5 µl of RNase-free water were mixed together to make the miRNA-Amp reaction mix. An amount of 45 µl of the mix was then transferred into each well of a new plate, then 5 µl of the RT reaction product was added to each well of the plate. The plate was vortexed, centrifuged and placed into the Roche Light Cycler96 (Roche, South Africa).

Cycling conditions:
Enzyme activation: 1 cycle at 95ºC for 5 min
Denaturation: 14 cycles at 95 ºC for 3 sec
Anneal/extension: 14 cycles at 60 ºC for 30 sec
Stop reaction: 1 cycle at 99 ºC for 10 min
Hold: 1 cycle at 4ºC

Following cDNA synthesis, the miR-33a, miR-133a and miR-499a miScript Primer assays (Qiagen SA Biosciences, Frederick, Maryland, USA) were used to profile the levels of all 3 miRNAs together with a housekeeping miRNA. A total of 10 µl of the Taqman Fast Advanced master mix (Thermo Fisher Scientific, South Africa) was added to each well. Then, 1 µl Taqman advanced miRNA assay, 2 µl RNase free water and 2.5 µl cDNA was added into each well, in duplicate for each sample. Parallel to this, the reference miRNA, U6, was prepared on the same plate for each sample. The plates were sealed with optically clear adhesive and inspected from the bottom to ensure that there were no bubbles. Plates were centrifuged at 250 x*g* for 2 min at room temperature to remove any bubbles and then transferred to the Roche Light Cycler96 (Roche, South Africa). The cycling conditions for the amplification of miR-33a, miR-133a and miR-499a and the reference miRNA, U6, are detailed as follows.

PCR cycling conditions:
Enzyme activation: 1 cycle at 95ºC for 20 sec
Denaturation: 40 cycles at 95 ºC for 1 sec
Anneal/Extension: 40 cycles at 60 ºC for 20 sec

2.6 Statistical analysis

The study set out to determine the relationship of protein biomarkers with statin treatment and the levels of associated miRNAs. The levels of circulating miRNAs (miR-33a-5p, miR-133a-3p and miR-499a-5p) in whole blood measured by qPCR and serum levels of protein biomarkers (ApoA-1, ALT and CK) quantified by ELISA was used to determine if a relationship between the respective miRNA and circulating protein biomarker exists in hypercholesterolaemia patients either on statins or statin-naive. Participant demographic information, specifically pre-existing co-morbidities, and any medication use was captured and used for sample selection and analysis. Sample size requirements usually are to enroll at least 10 to 15 patients per predictor/independent variable considered in the multivariable regression analysis. Not more than 10 – 12 variables were anticipated and therefore a sample size of at least 300 patients (100 per group) was the target.

Participant demographics are presented as mean with the range for age, percentage differences for gender and number of patients for other categorical variables related to clinical history. Fold-regulation for all the miRNAs were calculated using the 2 to the minus delta delta cycle threshold method and comparisons were completed compared to the healthy control group. All ELISA data generated is continuous. Thus, for each data set, a normality test was conducted to determine if the data followed a normal distribution. This guided whether or not the t-tests and one-way ANOVA tests which were conducted between each group is considered as parametric or non-parametric. Where data is parametric, the mean and standard deviations were reported. Where the data is non-parametric the median and interquartile ranges were reported. The t-tests were conducted between control vs. statin naïve, control vs. statin users and statin naïve vs. statin users – for miRNAs and ELISA datasets. Relationships between miRNAs and the respective quantified proteins were evaluated using a linear regression and correlation analysis. Testing was done at the 0.05 level of significance. GraphPad prism 9.0 was used for statistical analysis.

Chapter 3: Results

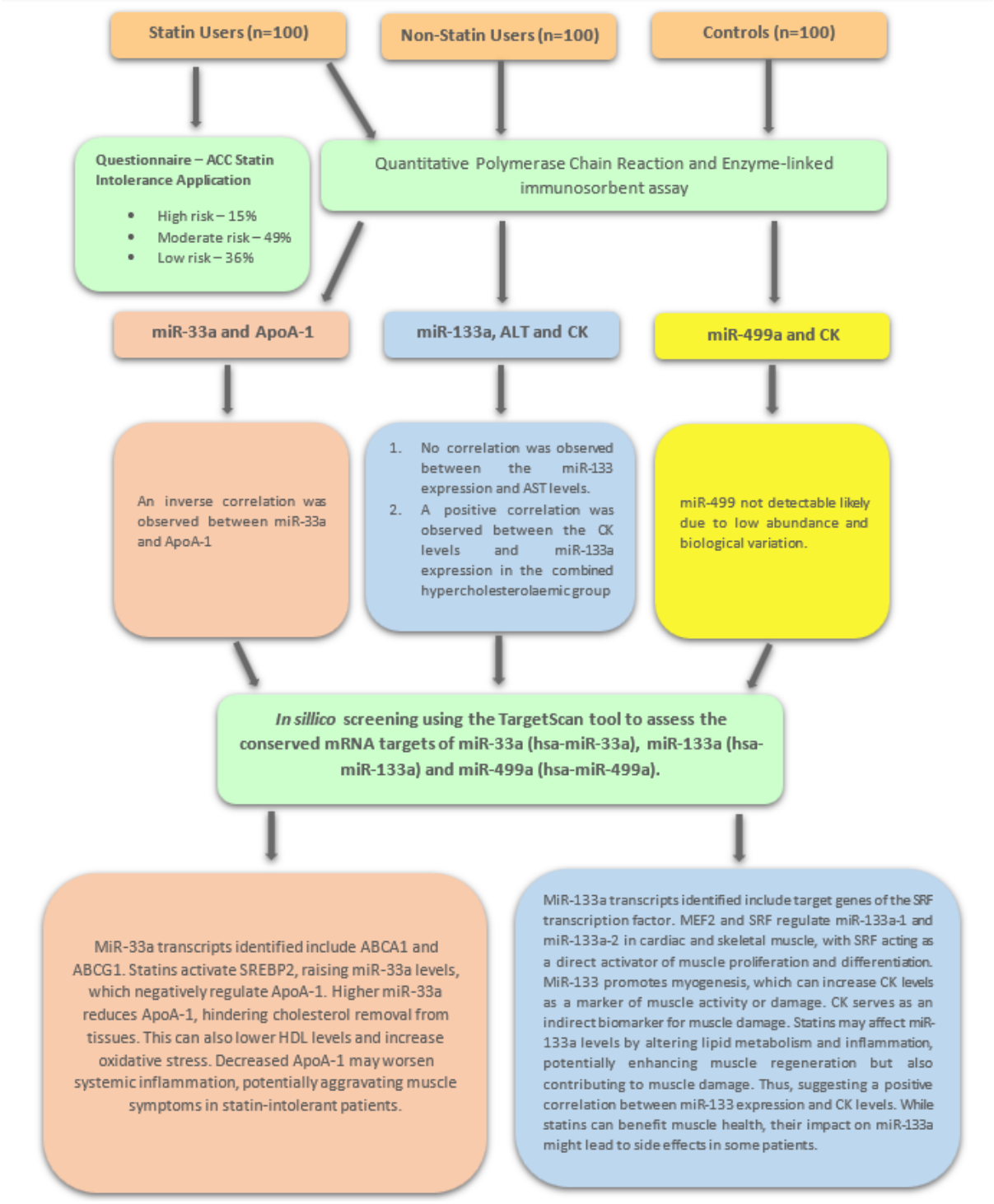

Figure 15: Summary of results

3.1 Cohort Demographics

The cohort demographics recruited for this study included 300 participants between the ages of 19 – 79. Of these, 100 were control participants, 100 were

hypercholesterolemia patients not taking statins and 100 were statin-treated hypercholesterolemia patients. Cohort demographics are shown in Table 7. Muscle related signs and symptoms reported by each of the statin users are shown in Table 8.

Table 7: Participant (cohort) demographics

	Controls	Non-Statin Users	Statin Users
Number of participants	100	100	100
Age Median (range) in years	49 (19 - 78)	47 (22 - 79)	49 (19 - 78)
Average height (m) (range)	-	-	1,72 (1,58 – 1,82)
Average weight (kg) (range)	-	-	79,38 (58 – 131)
Average BMI	-	-	26,87 (18,41 – 40,77)
Sex			
Male (%)	49%	50%	49%
Female (%)	51%	50%	51%
Race			
Black (n)	86	80	85
White (n)	14	20	15
Treatment group			
Atorvastatin	0	0	30
Simvastatin	0	0	70
Co-morbidities			
Cardiovascular Disease	0	13	9
Type 2 Diabetes Mellitus	0	38	30
Hypertension	0	88	65
Obesity *	0	28	21
*Obesity was defined as having a BMI of greater than or equal to 30.			

Sampling was informed by the population ratios in the country, ~80% Black South African, ~20% White South African, however, despite South Africa's diverse multi-racial population, it was decided to only include Black and White South Africans in the study as the majority of the population consist of these two ethnic groups.

Of note is the higher prevalence of comorbidities in the non-statin users compared to the statin-users. This observation is unexpected given that patient with comorbidities, such as diabetes mellitus and cardiovascular disease, have strong indications for stain therapy. However, this observation can be attributed to a number of causes, such as statin intolerance, delayed initiation of statin therapy, differences in socio-economic status and healthcare access and adherence to treatment. It is also important to note

that the group referred to as “non-statin users” are hypercholesterolemic patients on other lipid lowering therapies, besides statins.

Table 8: Muscle related signs and symptoms reported by each of the statin users

	Statin dose				
	Atorvastatin 10 mg	Atorvastatin 20 mg	Simvastatin 10 mg	Simvastatin 20 mg	Simvastatin 40 mg
Aches/Weakness	9	18	27	29	8
Tingling/twitching	1	2	0	6	0
Bilateral	9	19	24	25	7
Unilateral	1	1	3	10	1
Severity of symptoms	4	4	4	3	5
How many times in past 7 days did the patient have symptoms	3	4	4	3	5

3.2 The American College of Cardiology's (ACC) Statin Intolerance Application – Statin Intolerance Risk Score

All participants for the hypercholesterolemia group who are currently taking statins as their primary lipid-lowering therapy were requested to complete a questionnaire adapted from the ACC’s Statin Intolerance Application. (Appendix 6) The ACC’s Statin Intolerance Application was developed as part of the ACCs “LDL: Address the Risk” Initiative, to assist clinicians to manage cholesterol and as well as managing and treating patients who present with or report muscle related symptoms while on statin therapy. (294) The application collects information on CK levels, muscle related symptoms and their associated severity, frequency, effects on daily exercise and activities, and any medical history that may attribute to a participant’s likelihood of presenting with statin intolerance. By using the application, a clinician can answer questions to assess a patient’s possible intolerance to their current statin therapy and employ steps to manage and treat the reported muscle symptoms. (294) For this study, the ACC Statin Intolerance Risk Application was used to assess how likely it was that the symptoms experienced by the participants were related to statins, distribution highlighted in Table 9. Of the 100 participants enrolled, 5 participants presented with risk factors that may increase their risk of statin intolerance, 3 presented with low body mass index (BMI) and T2DM.

Table 9: Statin intolerance risk score cohort distribution.

Statin Intolerance Risk Score	**Statin dose**					
	Atorvastatin 10 mg	Atorvastatin 20 mg	Simvastatin 10 mg	Simvastatin 20 mg	Simvastatin 40 mg	Population distribution
High risk (n)	-	2 (*10%*)	7 (*26%*)	3 (*8,5%*)	3 (37,5%)	15 (15%)
Moderate risk (n)	4 (*40%*)	12 (*60%*)	13 (*48%*)	16 (*45,7%*)	4 (*50%*)	49 (49%)

Low risk (n)	6 (*60%*)	6 (*30%*)	7 (*26%*)	16 (*45,7%*)	1 (*12,5%*)	36 (36%)
Total	10 (*10%*)	20 (*20%*)	27 (*27%*)	35 (*35%*)	8 (*8%*)	100 (100%)

3.3. In silico screening of miR-33a, miR133a and miR-499a using TargetScan

The TargetScan tool was used to assess the conserved mRNA targets of miR-33a (hsa-miR-33a), miR-133a (hsa-miR-133a) and miR-499a (hsa-miR-499a). Conserved sites refer to the specific region within the 3' untranslated region (3' UTR) of the target mRNA where the specific miRNA, in this case miR33a, miR-133a and miR-499a, is expected to bind, these binding sites are conserved across multiple species.

a) miR-33a

A total of 545 transcripts with conserved sites were identified for miR-33a using the TargetScan tool. The aggregate P_{CT} score was used to determine the likelihood of the identified targets to be a true target site of miR-33a. The table generated from TargetScan was sorted according to the aggregate P_{CT} score, and targets were filtered based on their association to cholesterol homeostasis and atherosclerosis. Following a thorough screening of the selected targets, a list was compiled. (Table 10)

Table 10: Target genes on miR-33a associated with cholesterol homeostasis and atherosclerosis.

Target Gene	Representative transcript	Gene name	Aggregate P_{CT}
CROT	ENST00000331536.3	carnitine O-octanoyltransferase	0,58
ABCB11	ENST00000263817.6	ATP-binding cassette, sub-family B (MDR/TAP), member 11	< 0.1
ABHD13	ENST00000375898.3	abhydrolase domain containing 13	< 0.1
ADAMTS3	ENST00000286657.4	ADAM metallopeptidase with thrombospondin type 1 motif, 3	0,21
ADCYAP1	ENST00000579794.1	adenylate cyclase activating polypeptide 1 (pituitary)	< 0.1
ADRA2A	ENST00000280155.2	adrenoceptor alpha 2A	< 0.1
SLC25A25	ENST00000373069.5	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 25	0,63
ABCA1	ENST00000374736.3	ATP-binding cassette, sub-family A (ABC1), member 1	0,9
CPT1A	ENST00000265641.5	carnitine palmitoyltransferase 1A (liver)	0,69
BAG2	ENST00000370693.5	BCL2-associated athanogene 2	< 0.1
ATP8B1	ENST00000283684.4	ATPase, aminophospholipid transporter, class I, type 8B, member 1	0,45

CSNK1D	ENST00000392334.2	casein kinase 1, delta	0,21
ENC1	ENST00000302351.4	ectodermal-neural cortex 1 (with BTB domain)	< 0.1
HADHB	ENST00000317799.5	hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit	0,21
IQSEC2	ENST00000375365.2	IQ motif and Sec7 domain 2	0,21
IRS2	ENST00000375856.3	insulin receptor substrate 2	< 0.1
ITGBL1	ENST00000545560.2	integrin, beta-like 1 (with EGF-like repeat domains)	0,21
MAP3K1	ENST00000399503.3	mitogen-activated protein kinase kinase kinase 1, E3 ubiquitin protein ligase	< 0.1
MAP3K7	ENST00000369325.3	mitogen-activated protein kinase kinase kinase 7	< 0.1
MAP4K4	ENST00000413150.2	mitogen-activated protein kinase kinase kinase kinase 4	0,21
MAPK6	ENST00000261845.5	mitogen-activated protein kinase 6	0,31
MAPK8	ENST00000374182.3	mitogen-activated protein kinase 8	< 0.1
MDM4	ENST00000391947.2	Mdm4 p53 binding protein homolog (mouse)	< 0.1
MMP16	ENST00000286614.6	matrix metallopeptidase 16 (membrane-inserted)	0,21
PDGFRA	ENST00000257290.5	platelet-derived growth factor receptor, alpha polypeptide	0,21
PPL	ENST00000345988.2	periplakin	0,21
SGCD	ENST00000435422.3	sarcoglycan, delta (35kDa dystrophin-associated glycoprotein)	0,21
SIK1	ENST00000270162.6	salt-inducible kinase 1	< 0.1
SNX9	ENST00000392185.3	sorting nexin 9	0,14
UBA2	ENST00000246548.4	ubiquitin-like modifier activating enzyme 2	0,21
CDK6	ENST00000265734.4	cyclin-dependent kinase 6	0,78
CDK8	ENST00000536792.1	cyclin-dependent kinase 8	0,21
CDK16	ENST00000457458.2	cyclin-dependent kinase 16	0,31

b) miR-133a

A total of 703 targets were identified using the TargetScan tool. The aggregate P_{CT} score was used to determine the likelihood of the identified targets to be a true target site of miR-133a (hsa-mir-133a). The table generated from TargetScan was sorted according to the aggregate P_{CT} score, and all targets with an aggregate P_{CT} score of ≤ 0.25 were selected. Each target was then assessed for its association with muscle growth, morphogenesis, differentiation, and contractility of muscle cells and apoptosis.

Following a thorough screening of the selected targets, a list was compiled. (Table 11)

Table 11: Target genes on miR-133a associated with muscle growth, morphogenesis, differentiation, and contractility.

Target Gene	Representative transcript	Gene name	Aggregate P_{CT}
AOX1	ENST00000374700.2	aldehyde oxidase 1	< 0.1
BNIP3L	ENST00000380629.2	BCL2/adenovirus E1B 19kDa interacting protein 3-like	0,24
CCDC30	ENST00000342022.4	coiled-coil domain containing 30	< 0.1
CCNDBP1	ENST00000300213.4	cyclin D-type binding-protein 1	< 0.1
DCLRE1A	ENST00000361384.2	DNA cross-link repair 1A	0,21
ENPP5	ENST00000371383.2	ectonucleotide pyrophosphatase/phosphodiesterase 5 (putative)	0,2
GABRB1	ENST00000295454.3	gamma-aminobutyric acid (GABA) A receptor, beta 1	< 0.1
GARNL3	ENST00000373387.4	GTPase activating Rap/RanGAP domain-like 3	0,2
IFIT2	ENST00000371826.3	interferon-induced protein with tetratricopeptide repeats 2	0,23
IL6ST	ENST00000381287.4	interleukin 6 signal transducer (gp130, oncostatin M receptor)	< 0.1
ISCA2	ENST00000554924.1	iron-sulfur cluster assembly 2	< 0.1
KIAA0141	ENST00000194118.4	KIAA0141	0,24
KIAA1024	ENST00000305428.3	KIAA1024	0,12
LGALS8	ENST00000526589.1	lectin, galactoside-binding, soluble, 8	< 0.1
MAP3K2	ENST00000409947.1	mitogen-activated protein kinase kinase kinase 2	0,24
MED8	ENST00000372457.4	mediator complex subunit 8	< 0.1
MMP14	ENST00000311852.6	matrix metallopeptidase 14 (membrane-inserted)	< 0.1
MRPL44	ENST00000258383.3	mitochondrial ribosomal protein L44	< 0.1
MTMR4	ENST00000579925.1	myotubularin related protein 4	0,25
NEBL	ENST00000377122.4	nebulette	0,23
PDE1C	ENST00000396193.1	phosphodiesterase 1C, calmodulin-dependent 70kDa	< 0.1
PIP4K2B	ENST00000269554.3	phosphatidylinositol-5-phosphate 4-kinase, type II, beta	0,24

POLH	ENST00000372226.1	polymerase (DNA directed), eta	< 0.1
SFMBT2	ENST00000361972.4	Scm-like with four mbt domains 2	0,1
SFT2D3	ENST00000310981.4	SFT2 domain containing 3	< 0.1
SOCS2	ENST00000548537.1	suppressor of cytokine signaling 2	< 0.1
SPRN	ENST00000414069.2	shadow of prion protein homolog (zebrafish)	< 0.1
SUMO1	ENST00000392246.2	small ubiquitin-like modifier 1	0,25
THNSL1	ENST00000524413.1	threonine synthase-like 1 (S. cerevisiae)	< 0.1
TUBB1	ENST00000217133.1	tubulin, beta 1 class VI	< 0.1
ZNF354A	ENST00000335815.2	zinc finger protein 354A	0,25
ZNF362	ENST00000539719.1	zinc finger protein 362	0,19

c) miR-499a

The TargetScan tool was used to assess the conserved mRNA targets of miR-499a (hsa-miR-499a) and their associated pathways. A total of 452 transcripts with conserved and 189 with poorly conserved sites were identified using the TargetScan tool. The table generated from TargetScan was sorted according to the aggregate P_{CT} score (The likelihood of the identified targets to be a true target site of miR-499a.), and targets were filtered based on their association to cardiac- and skeletal muscle cell differentiation, mitochondrial functions and cardiac- and skeletal muscle cell apoptosis. Following a thorough screening of the selected targets, a list was compiled. (Table 12)

Table 12: Target genes for miR-499a associated with cardiac- and skeletal muscle cell differentiation, mitochondrial functions and cardiac- and skeletal muscle cell apoptosis.

Target gene	Representative transcript	Gene name	Aggregate P_{CT}
MYEF2	ENST00000324324.7	myelin expression factor 2	< 0.1
PURB	ENST00000395699.2	purine-rich element binding protein B	0,14
SOX6	ENST00000316399.6	SRY (sex determining region Y)-box 6	0,52
PDCD4	ENST00000280154.7	programmed cell death 4 (neoplastic transformation inhibitor)	0,64
MYLK	ENST00000360772.3	myosin light chain kinase	< 0.1
ITGBL1	ENST00000545560.2	integrin, beta-like 1 (with EGF-like repeat domains)	0,33
PRKAA2	ENST00000371244.4	protein kinase, AMP-activated, alpha 2 catalytic subunit	< 0.1

MAP3K2	ENST00000409947.1	mitogen-activated protein kinase kinase kinase 2	0,4
HSPA4	ENST00000304858.2	heat shock 70kDa protein 4	< 0.1
MYH10	ENST00000360416.3	myosin, heavy chain 10, non-muscle	0,4
CAMTA2	ENST00000358183.4	calmodulin binding transcription activator 2	< 0.1
TANC1	ENST00000263635.6	tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 1	< 0.1
MMP16	ENST00000286614.6	matrix metallopeptidase 16 (membrane-inserted)	0,4
CFL2	ENST00000341223.3	cofilin 2 (muscle)	< 0.1
ADCYAP1	ENST00000579794.1	adenylate cyclase activating polypeptide 1 (pituitary)	< 0.1
CELF1	ENST00000395290.2	CUGBP, Elav-like family member 1	< 0.1
FOXO4	ENST00000374259.3	forkhead box O4	< 0.1
TSPAN6	ENST00000373020.4	tetraspanin 6	< 0.1
ABCB7	ENST00000253577.3	ATP-binding cassette, sub-family B (MDR/TAP), member 7	< 0.1

3.4. a) Quantification of Apolipoprotein A–1 and miR-33a

Apolipoprotein A-1 is a major protein component of HDL, which is generally used by clinicians to assess a participant's risk for CVD. Apolipoprotein A-1 plays an important role in cholesterol metabolism, specifically reverse cholesterol transport, which is the process by which excess cholesterol is removed from peripheral tissues and transported back to the liver for metabolism and excretion. (185) To determine the ApoA-1 levels of the participants included in the study, 40 participants were selected from each group based on predefined selection criteria, which included race, age and gender. The ratio of simvastatin/atorvastatin users was kept consistent when the samples were selected for ApoA-1 analysis.

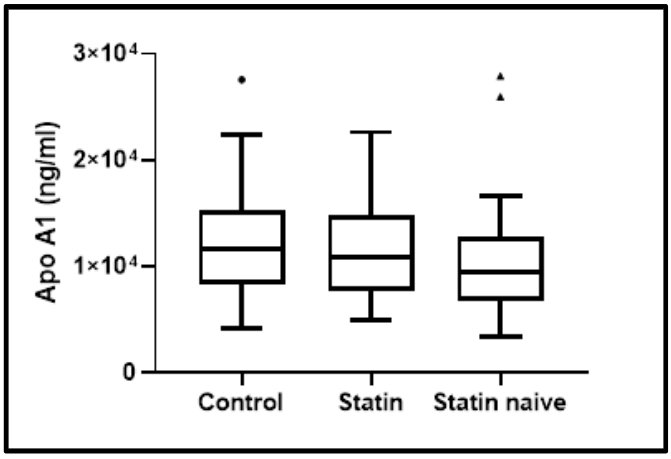

Figure 16: Serum ApoA-1 levels of participants in the control, statin-users and statin naive groups. The data is presented in a box-whisker plot with the median and 25th and 75th percentiles indicated.
p= 0,1781, Indicates no statistical significance with *p*> 0,05

The statistical analysis showed no significant difference in the ApoA-1 levels between participants in the control, statin-users, or statin naïve groups, p= 0,1781, presented in Figure 16. With the average ApoA-1 levels of the control, statin-users, and statin naïve groups being, 12037 ng/ml, 11441 ng/ml and 10322 ng/ml, respectively.

MicroRNA-33 levels was determined as per section 2.5.6 in chapter 2. A significant difference (p= 0,0011) in the relative fold change of miR-33a was noted between statin-users and statin naïve patients compared to the control group, shown in Figure 17. A significant difference in the relative fold change of miR33a was noted between control and statin naïve patients (p= 0,0134) and between statin-users and statin naïve patients (p= 0,0063).

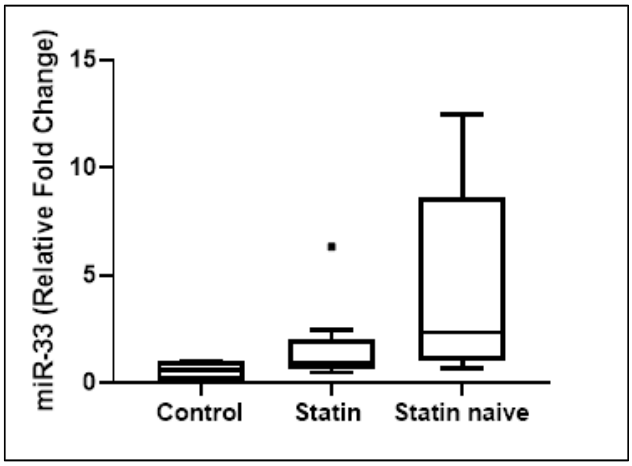

Figure 17: Relative fold change of miR-33a expression between the statin-users and

statin naive patients compared to the control group. The data is presented in a box-whisker plot with the median and 25th and 75th percentiles indicated.
*p= 0,0011, Indicates statistical significance with *p*<0,05

b) Determining the possible relationship between miR-33a and ApoA-1

Following the quantification of ApoA1 and the expression of miR-33a and assessing the differences between the different groups, a linear regression and Spearman R correlation analysis was conducted on matched samples between miR-33a and ApoA-1 levels.

MicroRNA-33 is involved in cholesterol regulation and lipid metabolism by inhibiting genes involved in cholesterol efflux, fatty acid oxidation, and insulin signaling. Interestingly, miR-33a typically suppresses these genes, however ApoA-1 is an activator of these genes, suggesting an inverse relationship between miR-33a and ApoA-1.

The Spearman's rank correlation coefficient was used to assess a possible correlation between the miR-33a expression and ApoA-1 levels. The Spearman R correlation coefficient is a nonparametric measure of rank correlation or statistical measure of the strength and direction of association or correlation between two variables.

An inverse correlation, though weak, between miR-33a and ApoA-1, was observed, with the r = - 0,3496 (95% confidence interval -0,6052 to -0,02868) and p= 0,0291, presented in Figure 18.

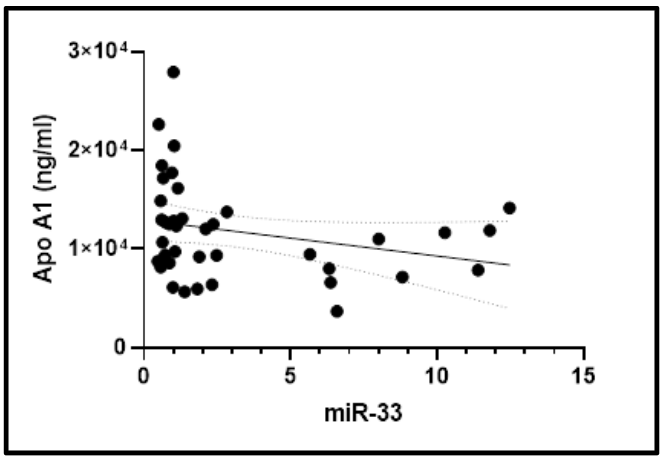

Figure 18: Spearman's correlation analysis of the inverse correlation between miR-33a

and ApoA1.
p= 0,0291, Indicates statistical significance with *p*<0,05

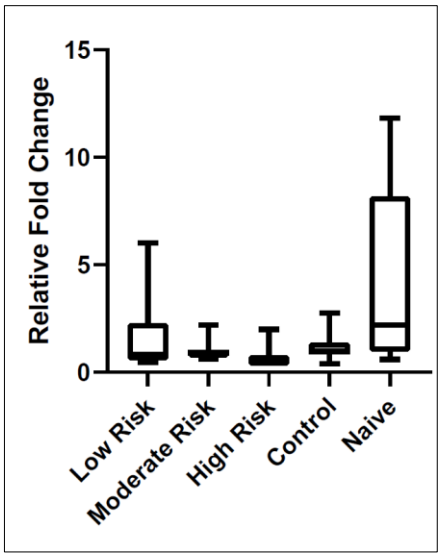

Figure 19: Relative fold change of miR-33a expression within the different Statin Intolerance Risk Score sub groups compared to the control and Statin Naive participants. The data is presented in a box-whisker plot with the median and 25th and 75th percentiles indicated.

Tukey's multiple comparisons test also known as Tukey's Honest Significant Difference test was used to assess the mean difference in expression of miR-33a between low risk -, moderate risk -, and high risk statin users compared to the control and statin naïve patients. A significant difference in the relative fold change of miR-33a expression was noted between low risk statin-users compared to the statin naïve patients (p= 0,0392), moderate risk statin-users compared to the statin naïve patients (p= 0,0244) and the control participants compared to the statin naïve patients. (p= 0,0012) (Figure 19)

3.5. a) Quantification of Creatine Kinase and miR-499a

Creatine Kinase in serum is of great importance in the enzymology of cardiac- and skeletal muscle due to its high sensitivity and specificity during quantification. Elevated circulating CK in plasma is used as an indirect biomarker for cardiac- and skeletal muscle damage as only cardiac- and skeletal muscle contains high concentrations of CK. Elevated CK levels are thus used as a surrogate endpoint to establish possible statin intolerance is patients on statin therapy. (295)

To determine the CK levels of participants in the study, 40 participants were selected based on predefined selection criteria, which mainly included age, race and gender. The ratio of simvastatin to atorvastatin users was kept consistent in the selected sample of statin-users.

Upon completion of the ELISA, CK levels/values were calculated as defined in section 2.5.3 in chapter 2. Numerical values of CK are reported in pg/ml units.

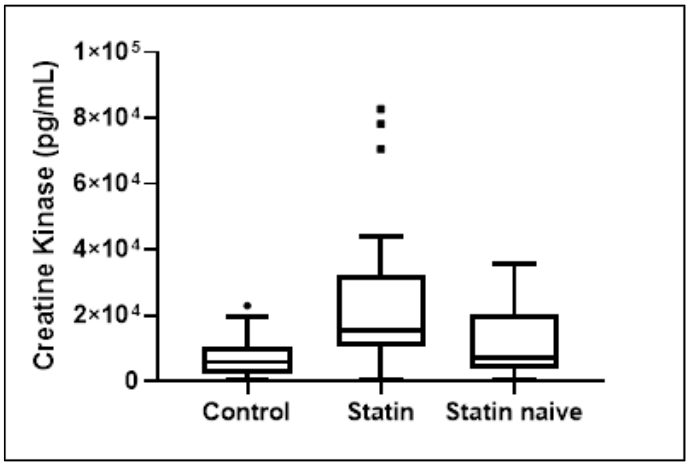

Figure 20: Serum CK levels of participants in the Control, Statin-treated and Statin naive groups. The data is presented in a box-whisker plot with the median and 25th and 75th percentiles indicated.
*p= <0,0001, Indicates statistical significance with *p*<0,05

Statistical analysis showed a significant difference between the CK levels between the different patient groups, p= <0,0001, presented in Figure 20, with the average CK level for the statin-users being significantly higher compared to the other 2 groups. The mean CK level of the statin-users, Statin naïve and Control groups were, 22256 pg/ml, 11788 pg/ml and 6938 pg/ml, respectively. The average CK values based on likelihood of statin intolerance were 15428 pg/ml for the high-risk group, 24436 pg/ml for the moderate risk group and 23092 pg/ml for the low-risk group.

MicroRNA-499 expression was quantified based on the method outlined in section 2.5.6 in chapter 2. MicroRNA-499 was however not detectable in any of the samples used for analysis. There are several reasons why miR-499a may not have been detected in serum, including: low abundance, degradation, technical issues, method sensitivity and biological variation.

Upon troubleshooting and further analysis, it was noted that the house keeping miRNA was detected in both experiments, ruling out technical issues and method sensitivity. The most likely causes being low abundance and biological variation as miR-33a levels were detectable in all three groups ruling out miRNA degradation by RNases present in the serum.

b) Determining the possible relationship between miR-499a and CK levels

MicroRNA-499 has been found to regulate muscle development, function, and regeneration. MicroRNA-499 plays an important role in cardiac muscle function, specifically regulating processes such as hypertrophy, apoptosis, and contractility. Creatine Kinase is an enzyme specifically used as a marker for muscle damage or pathology. Studies suggest that the ability of statins to induce mitochondrial-mediated apoptosis in muscle cells might support the theory that miR-499a may serve as a biomarker for statin-induced muscle damage. Suggesting a direct correlation between CK levels and upregulation of miR-499a.

After quantifying CK levels, measuring the expression of miR-499a, and comparing these values among different groups, the aim was to perform a linear regression and Spearman R correlation analysis on matched samples to assess the relationship between miR-499a and CK levels.

However, miR-499a was not detectable in any of the samples, likely due to low abundance and biological variation, no correlation could be made between the elevated CK levels noted in the different groups and the expression of miR-499a.

3.6. a) Quantification of Alanine aminotransferase (ALT) and miR-133a

To determine the ALT levels of the participants included in the study, 40 participants were selected from each group based on predefined selection criteria, which included race, age and gender. The ratio of simvastatin/atorvastatin users were kept consistent when the samples were selected.

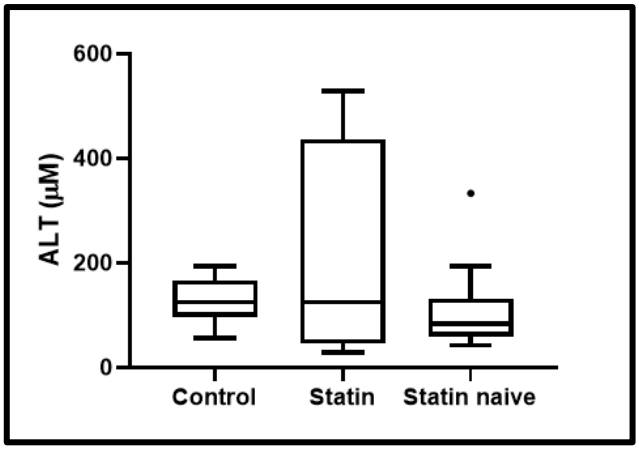

Figure 21: Serum ALT levels of participants in the control, statin-users and statin naive groups. The data is presented in a box-whisker plot with the median and 25th and 75th percentiles indicated.
p= 0,3222, Indicates no statistical significance with p>0,05

The statistical analysis showed no significant difference (p= 0,3222) in the ALT levels between participants in the control, statin-users, or statin naïve groups, presented in Figure 21. With the average ALT levels of the control, statin-users, and statin naïve groups being, 243 µM ± 308,728 µM ± 435,782 µM, 331 µM and 169 µM ± 226,226 µM, respectively.

MicroRNA-133a expression was determined as per section 2.5.6 in chapter 2. Dunn's multiple comparisons test was used to assess the mean difference in expression of miR-133a between statin-users, statin-naïve patients, and control group. A significant difference in the relative fold change of miR-133a expression was noted between the control group and statin-users (p= 0,0006), and the statin-users and the statin naïve patients (p= 0,0239). (Figure 22)

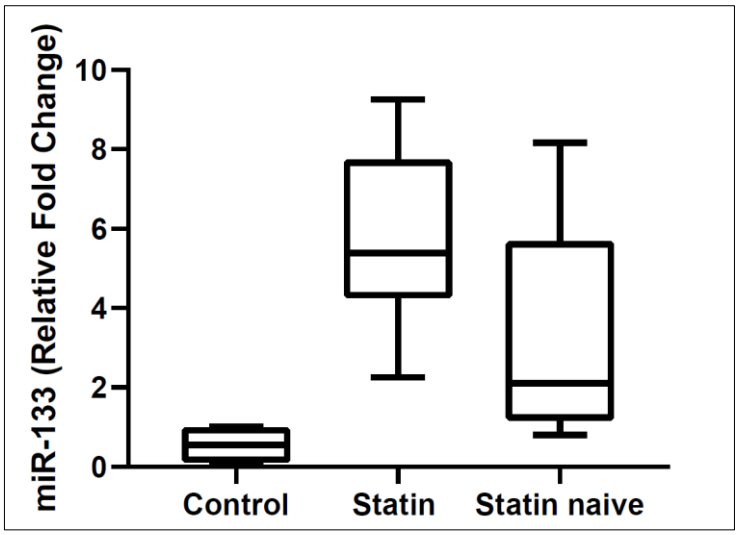

Figure 22: Relative fold change of miR-133a expression between the statin-users, statin-naïve patients, and control group. The data is presented in a box-whisker plot with the median and 25th and 75th percentiles indicated.

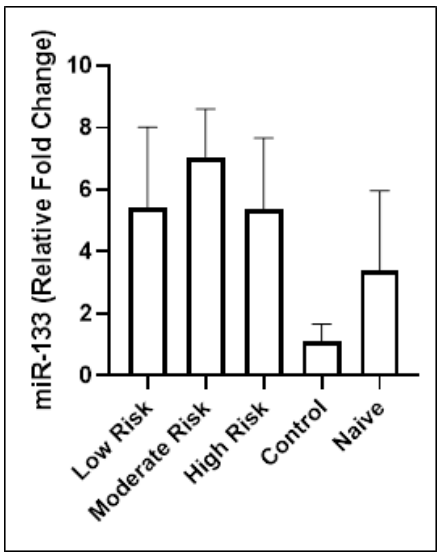

Figure 23: Relative fold change of miR-133a expression within the different Statin Intolerance Risk Score subgroups compared to the control and Statin Naive participants. The data is presented in a box-whisker plot with the median and 25th and 75th percentiles indicated.

Holm-Sidak's multiple comparisons test was used to assess the mean difference in expression of miR-133a between low risk -, moderate risk -, and high risk statin users compared to the control and statin naïve patients. A significant difference in the relative fold change of miR-133a expression was noted between low risk statin-users compared to the control group (p= 0,0036), moderate risk statin-users compared to the control group (p= 0,0003), moderate risk statin-users compared to the statin naïve group (0,0029) and the control participants compared to the statin naïve patients. (p=

0,0052) (Figure 23)

b) Determining the possible relationship between miR-133a and ALT

Following the quantification of ALT and the expression of miR-133a, a linear regression and Spearman R correlation analysis was conducted on matched samples between miR-133a and ALT levels.

MicroRNA-133, predominantly expressed in cardiac- and skeletal muscle cells, plays a pivotal role in muscle development and regeneration, similarly, elevated ALT levels are often associated with cardiac- and skeletal muscle damage. As changes have been observed in miR-133a levels and AST levels during cardiac or skeletal muscle injury, this suggests a positive correlation between miR-133a levels and ALT levels.

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The Spearman's rank correlation coefficient was used to assess a possible correlation between the miR-133a expression and ALT levels in statin-users and subsequently in a combined hypercholesterolaemic group (statin-users and statin naïve patients).

No correlation was observed between the miR-133a expression and ALT levels for either the hypercholesterolaemic participants on statin therapy or the combined hypercholesterolaemic group (hypercholesterolaemic patients on statin therapy and statin naïve hypercholesterolaemic patients), with r = -0,1088 (95% confidence interval -0,6336 to 0,4845) and p= 0,72 and r= 0,07492 (95% confidence interval -0,3326 to 0,4588) and p= 0,7161, respectively.

c) Determining the possible relationship between miR-133a and CK

Although the exact mechanism detailing the relationship between miR-133a and statins are not yet understood, there is evidence to suggest that statins induce muscle damage and miR-133a levels are influenced by muscle damage, thus suggesting that miR-133a may be a possible biomarker for statin induced myopathy. Various biomarkers are used to clinically assess muscle damage is patients, however, CK levels in serum is the gold standard when assessing cardiac- and skeletal muscle damage.

Following the quantification of CK and the expression of miR-133a, a linear regression

and Spearman R correlation analysis was conducted on matched samples between miR-133a and CK levels.

The Spearman's rank correlation coefficient was used to assess a possible correlation between the miR-133a expression and CK levels in statin-users and subsequently in a combined hypercholesterolaemic group (statin-users and statin naïve patients).

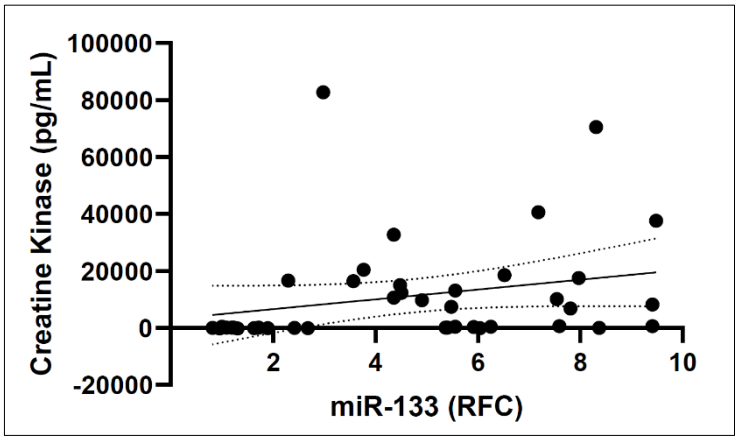

Figure 24: Spearman's correlation analysis of the positive correlation between miR-133a and CK levels in the combined hypercholesterolaemic group.
p= 0,0031, Indicates statistical significance with *p*<0,05

A positive correlation was observed between the CK levels and miR-133a expression in the combined hypercholesterolaemic group, with r= 0,4567 (95% confidence interval 0,1601 to 0,6777) and p= 0,0031, presented in Figure 22. No correlation was observed between the CK levels and miR-133a expression for the statin-users, with r= -0,2197 (95% confidence interval -0,6124 to 0,2600) and p= 0,352.

Chapter 4: Discussion

The World Health Organization (WHO) estimates that in 2019, 17.9 million people died from CVDs globally with 80% of these being due to heart attack or stroke. (296) More than 75% of these deaths were in low- and middle socio-economic countries. (296) The prevalence of CVD increases progressively with age, from 6% at the age of 20, to 77% at the age of 70. (297) Elevated cholesterol levels are a major risk factor for atherosclerotic disease accounting for approximately 4.5 million deaths globally in 2020 making it the eighth leading cause of death worldwide. (298-301)

Statins, also known as HMG-CoA reductase inhibitors, are the gold-standard lipid-lowering therapy. Public health care institutions prescribe a low- or moderate dose statin (usually 10- to 40mg simvastatin or 10- to 20mg atorvastatin) in combination with lifestyle changes as the first-line therapy for hypercholesterolemia. (9) Statins are administered orally, undergo extensive first-pass metabolism (11-13) and are then metabolised by the CYP450 enzymes in the liver. (14) Although statins are the most promising and effective therapy for reducing elevated LDL levels, and are normally regarded as safe and well-tolerated, they may be associated with moderate to severe side effects. (10, 15-22)

Statin intolerance is the leading reason for high discontinuation rates in patients on statin therapy, even in patients with CVD. Around 20% of patients present with statin intolerance. (17-22) Currently, there is no standardised diagnostic criterion to assess statin intolerance, however associated symptoms typically start within 3 months of treatment and resolve upon treatment cessation. (22, 138-140)

The increasing number of reported AEs associated with statin intolerance has prompted the need to investigate potential epigenetic factors that might contribute to the high prevalence of these side effects. Epigenetics is defined as the study of changes in gene function that are mitotically or meiotically heritable and does not include changes in the DNA sequence. (302) Epigenetic changes are mainly influenced by environmental factors, e.g., age, temperature, stress, diet, exposure to different toxins. (32)

Pharmaceuticals, including statins, can cause changes in gene expression that in some cases persist after cessation of the drug. (37) Currently there is a paucity in research on how statins affect miRNA expression or whether miRNAs can be used as potential biomarkers to assess statin-induced AEs, as they leak into circulation from tissues where injury occurs. Recent studies have shown that miRNAs can serve as biomarkers for various diseases and statin's effect on a single miRNA can potentially lead to enormous consequences as a single miRNA has the potential to affect hundreds of mRNAs. (32, 34, 36, 38-41)

The aim of this study was therefore to identify potential circulating biomarkers of statin intolerance in a cohort of hypercholesterolaemic patients from Gauteng, South Africa. MicroRNAs such as miR-33a, miR-133a and miR-499a are hypothesised to be linked to statin intolerance due to their direct involvement or association in cholesterol homeostasis and effect on circulatory statin levels. (38-41, 183, 222, 284, 286)

A total of 300 participants were recruited for the study, 100 control participants, 100 hypercholesterolaemic patients using statin treatment and 100 patients with hypercholesterolemia not on statin therapy. The median age was kept consistent between the control, statin-users and statin-naïve group, 49-, 47-, and 49 years, respectively.

Of the identified miRNAs, miR-33a is one of the most extensively studied miRNAs, especially for its role in hypercholesterolemia and has been identified as the major regulator of cholesterol homeostasis. (180) This notion is supported by the word-cloud compiled for hsa-miR-33a and hsa-miR-33b, obtained from miRBase, which shows the prominent association found between miR-33a and cholesterol. (Figure 25 and Figure 26)

Figure 25: Word-cloud generated from miRBase compiled for 157 open access papers (hsa-miR-33a).

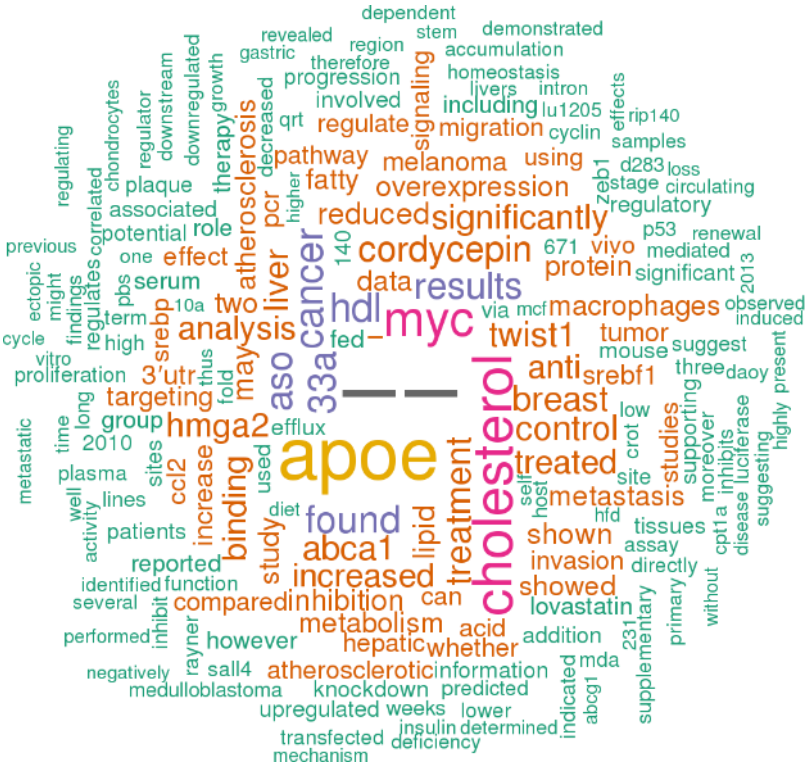

Figure 26: Word-cloud generated from miRBase compiled for 121 open access papers (hsa-miR-33b).

The ABCA1 transporter plays an important role in the reverse transport process of cholesterol by transferring cholesterol extracellularly to HDL molecules which in its turn transports cholesterol to the liver to be exported. (303, 304) Chen *et al.* (2013)(38) highlighted that miR-33a plays an important role in the pathophysiology of atherosclerosis. (38) Studies suggest that miR-33a and Srebp-2 are co-transcribed, aiding to increase cellular cholesterol levels by reducing cholesterol export by inhibiting transporters involved in cholesterol transport, when intracellular cholesterol levels decrease. This suggests that miR-33a might affect cholesterol homeostasis by down-regulating transmembrane transport of cholesterol via specialised transporters, such as ABCA1. The transmembrane transporter, ABCA1, plays an important role in cholesterol transport by mediating the transport of cholesterol from the peripheral tissue to the liver to be metabolised and excreted as well as the export of cholesterol to ApoA-1. (190, 305)

Apolipoproteins are responsible for the formation and function of lipoproteins. Apolipoprotein A1, is a major structural protein accounting for most of HDL. (305) The associated risk of CVD is inversely correlated by HDL and ApoA-1 levels, however pharmacological increase of HDL in statin-treated participants have in certain cases failed to lower the risk of CVD, suggesting the association might be indirect. (306, 307)

An *in silico* screening of miR-33a/b was performed using TargetScan and miRBase. The list of transcripts with conserved sites are presented in Table 10 in Section 3.3 a. A total of 545 transcripts were identified. The identified targets were filtered and sorted according to a pre-defined criteria. Interestingly, the list of target genes with conserved binding sites for miR-33a/b identified, includes genes involved in regulating cholesterol homeostasis, specifically ABCA1 and ABCG1 which play a pivotal role in cholesterol homeostasis by their mediation of cholesterol export from cells to ApoA-1 in circulation (308, 309), and fatty acid metabolism (CPT1A, CROT and HADHB), cyclin dependent kinases (CDK6, CDK8 AND CDK16) and mitogen-activate protein kinases (MAP3K1, MAP3K7, MAP4K4, MAPK6 and MAPK8). This supports the notion that inhibition of miR-33a using miR-33a/b antisense drugs might increase plasma HDL and lead to regression of atherosclerosis, making miR-33a a promising drug target. (183, 308, 310)

Given the important role ABCA1 plays in cholesterol homeostasis and in the production of HDL, researchers theorised that when miR-33a is suppressed, HDL levels may increase. (38) In this study, one of the research objectives was to establish a possible association between ApoA-1 levels and miR-33a expression in 3 groups: control, statin users and statin-naïve participants. As miR-33a mediates cholesterol regulation and lipid metabolism by inhibiting genes associated with these processes, and ApoA1 serves as an activator for these genes, an inverse correlation is suggested. (38, 39)

Although there was no significant difference in the ApoA-1 levels between the different groups (p= 0,1781) a significant difference in the relative fold change of miR-33a expression was noted between the statin-users and statin-naïve group compared to the control group (p=0,0011). We were also able to determine an inverse correlation between miR-33a and ApoA1, r= -0,3496 (95% confidence interval -0,6052 to -0,02868) and p= 0,0291.

The findings of this study are consistent with studies performed by Marquart *et al.* (2010) (187) and Najafi-Shoushtari *et al.* (2010) (186) who showed that miR-33a/b functions in combination with the SREBP host gene products to modulate cholesterol homeostasis and intracellular cholesterol levels. The researchers also showed that miR-33a/b exerts a posttranscriptional control of ABCA1 transporter suggesting that miR-33a/b antisense drugs might be a promising treatment for patients with cardiometabolic diseases as the reduced expression of miR-33a might lead to increased levels of ApoA1 and HDL. (186, 187)

Oladosu *et al*. (2024)(97) demonstrated that lentiviral vector-mediated knockdown of miR-33a-5p effectively reduced the expression of miR-33a-5p in cultured MLCs. This reduction resulted in de-repression of ABCA1, resulting in increased ABCA1 protein levels. An increase in ABCA1 protein expression enhances ABCA1-dependent cholesterol efflux by increasing apoAI-mediated cholesterol efflux. (97)

Hennessy *et al.* (311) identified a primate specific lncRNA, which they named CHROME, which is elevated in plasma and atherosclerotic plaques, of patients with

coronary artery disease. In this study the researchers showed that miR-33a is regulated by CHROME, which plays an important role in cholesterol efflux and HDL biogenesis. When CHROME is inhibited, miR-33a expression increases, resulting in a reduced expression of ABCA1, which reduces cholesterol efflux and HDL particle formation, resulting in reduced ApoA1 levels. (311) Suggesting an inverse relationship between miR-33a and ApoA1.

MicroRNA-133 is also a well-studied miRNA, particularly for its crucial role in regulating gene expression in cardiac and skeletal muscle cells. (195-197) The wordcloud compiled from 384 open access papers that mentioned hsa-mir-133a, obtained from miRBase, shows the prominent association found between miR-133a and muscle. (Figure 27)

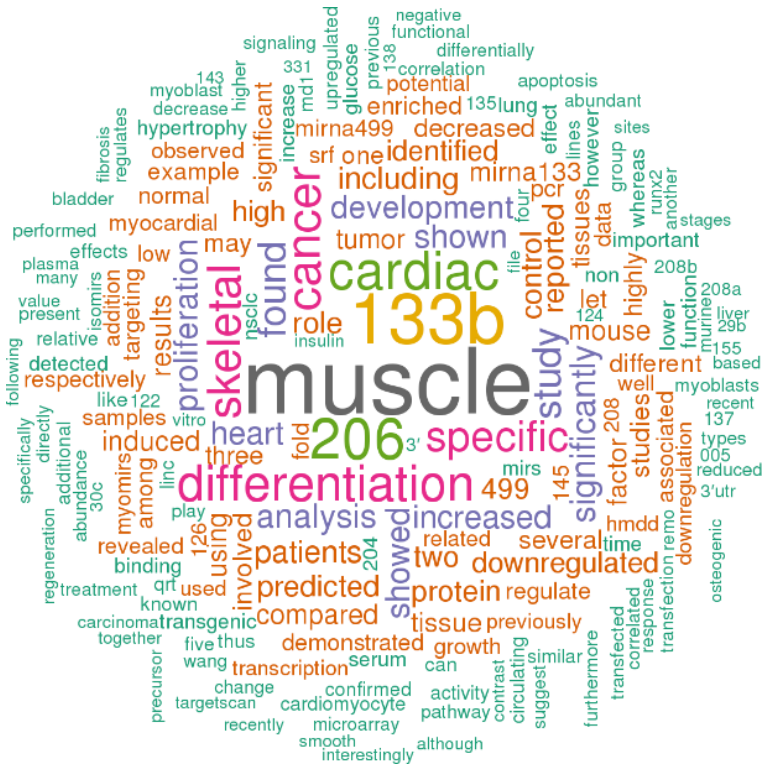

Figure 27: Wordcloud from miRBase compiled for 384 open access papers.

Various studies have been conducted to understand the mechanism in which miR-133a's involvement in proliferation and differentiation of skeletal muscle cells. MicroRNA-133 affects muscle development by targeting specific genes, e.g., SRF, cyclin D2, Histone Deacetylase 4 (HDAC4). (175) Research show that by inhibiting SRF, miR-133a inhibits myoblast differentiation and promotes myoblast proliferation

thus helping to regulate the balance between muscle differentiation and proliferation. (208-211)

The *in silico* screening performed to identify possible target genes for miR-133a using TargetScan generated 703 possible targets, presented in Table 11 in section 3.3 b. Interestingly, the list of target genes with conserved binding sites for miR-133a identified, includes target genes of the SRF transcription factor, such as AOX1, CCNDBP1, ISCA2, LGALS8, MRPL44 and POLH.

To understand the involvement of miRNAs in skeletal muscle proliferation and differentiation, Chen *et al.* (2005)(212) analysed the expression of miRNAs during skeletal muscle differentiation using and established microarray analysis. The researchers found that overexpression of miR-133a, in C2C12 myoblasts, repressed the expression of myogenic markers such as myogenin and MCH and promoted myoblast proliferation. In an effort to identify target genes that might moderate the observed effects of miR-133a, two conserved miR-133a-binding sites were identified on the 3' UTR of the mammalian gene encoding for SRF. (212)

Myocyte Enhancer Factor 2 and SRF regulate the expression of miR-133a-1 and miR-133-a-2 in cardiac and skeletal muscle. Genetic deletion of miR-133a-1 and miR-133-a-2 shows that SRF is a direct target of miR-133a. (207) Serum Response factor is shown to be, depending on its association with co-factors, a direct activator of muscle proliferation and differentiation. (207)

To determine the possible association between miR-133a and statin intolerance, or statin-associate muscle damage, Laterza *et al.* (2009) (222) used qPCR to measure specific miRNAs in the plasma samples of rats treated with liver and muscle toxins. The toxins used included HMG-CoA reductase inhibitor. In the study, miR-133a showed specificity to muscle and liver damage as this miRNA was not detectable in samples with toxicity to other organs. The researchers also plotted the miRNA expression in comparison to the AST and ALT data. Although the HMG-CoA reductase inhibitor groups showed the highest increase in ALT, a lesser increase was seen in AST level (30-fold increase), however an 800-fold increase was seen in miR-133a

levels, indicating a possible correlation between ALT levels and miR-133a expression. (222)

In this study, statistical analysis showed no significant difference in the ALT level between the different study groups. There was also no correlation observed between miR-133a expression and ALT levels for either the hypercholesterolaemic participants on statin therapy, or the combined hypercholesterolaemic group.

The study did, however, show a significant difference in the relative fold change of miR-133a expression between the control group and statin users (p= 0,0006), and the statin-users and the statin naïve patients (p= 0,0239), supported by the data generated by Laterza *et al.* (2009) (222) and Watanabe *et al.* (2019) (223) who both showed a significant increase in miR-133a expression in rat/mouse models when exposed to HMG-CoA reductase inhibitors or statins.

As there is evidence which supports the notion that miR-levels are influenced by muscle damage an alternative biomarker was used in this study to assess a possible relationship between miR-133a and statin induced muscle damage. Creatine Kinase acts as an indirect biomarker for cardiac-and skeletal muscle damage, due to the high concentrations of CK levels in cardiac and skeletal muscle, and is thus the gold standard biomarker for assessing cardiac- and skeletal muscle damage. (295)

Using ELISA, the CK levels of 40 participants were determined. Analysis showed a significant difference between the CK levels of the different groups, control, statin-users, and statin naïve, with, p= <0,0001. Analysis showed that the average CK level of statin-users were significantly higher compared to the other 2 groups.

Spearman R correlation coefficient showed no significant difference or correlation between the CK levels and miR-133a expression for the statin-users, with r= -0,2197 (95% confidence interval -0,6124 to 0,2600) and p= 0,352, however, a positive correlation was observed between the CK levels and miR-133a expression in the combined hypercholesterolaemic group, with r= 0,4567 (95% confidence interval 0,1601 to 0,6777) and p= 0,0031.

Contrary to the findings of this study, Watanabe *et al.* (2019) (223) showed a significant increase in plasma CPK, AST and miR-133a-3p levels in mice treated with an HMG-CoA reductase inhibitor (30 mg/kg/day atorvastatin or 300 mg/kg/day rosuvastatin, lovastatin, simvastatin, pitavastatin or fluvastatin, p.o.), suggesting a positive correlation between CPK, AST and miR133 levels. (223) The increase in miR-133a-3p specifically also supported the notion that miR-133a levels are influenced by skeletal muscle damage. (223)

These contradictory findings can be attributed to various factors, such as varying experimental designs, physiological and genetic variation is the participant population and environmental factors such as stimuli, pathogens and concomitant medication use.

The third miRNA identified in this study, possibly associated with statin intolerance, is miR-449. MicroRNA-499 belongs to a group of miRNAs encoded by the myosin family. (225, 226) MicroRNA-499 is a muscle specific miRNA, mainly expressed in cardiac cells. (273)

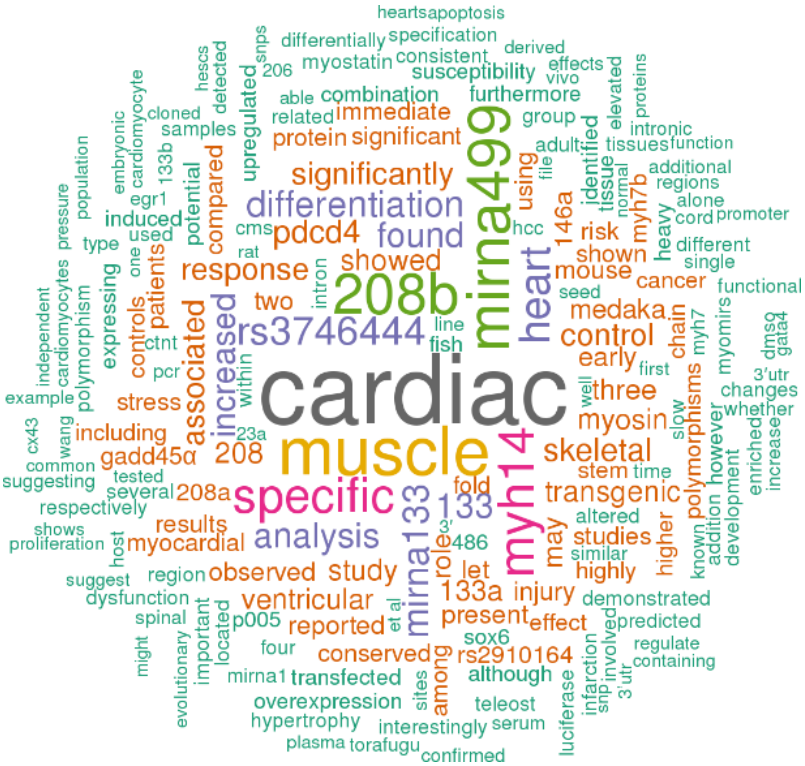

Figure 28: Wordcloud from miRBase compiled for 136 open access papers.

Of importance to note is the wordcloud compiled from 136 open access papers that

mentioned has-miR-499a, obtained from miRBase, that shows the prominent association found between miR-499a and cardiac- and skeletal muscle. (Figure 28).

MicroRNA-499 plays a significant role in the production of myosin heavy chains (MHC), ventricular specification and drives muscle cell specification and slows the MHC upregulation by targeting the β-MHC and SOX6 genes. (228) MicroRNA-499 is also expressed in the liver and skeletal muscle cells and plays a role in the development of various liver- and muscular diseases. (228, 231, 232, 274, 275) MicroRNA-499 plays an integral role in cardiomyocyte differentiation (41), mitochondrial function, skeletal muscle fiber type switching (285) and provides protection against cardiomyocyte apoptosis. (284)

A TargetScan analysis was performed to identify the conserved mRNA target for miR-499a. Of note from the table of conserved targets for miR-499a, presented in Table 12 in section 3.3 c, is FOXO4, PDCD4 and SOX6, of which all are genes associated in pathways related to regulation of smooth and skeletal muscle cell differentiation. (273, 279, 312)

Numerous studies have confirmed that SOX6 is a target of miR-499a (273, 313), however the role of the miR-499a/SOX6 pathway in vascular smooth muscle is unclear. A study performed by Sheng *et al.* (2023) (279) found that miR-499a binds directly to SOX6, decreasing the expression of SOX6 in vascular smooth muscle suggesting that SOX6 is negatively regulated by miR-499a, supporting the notion that miR-499a is implicated in cardiovascular pathophysiological processes. (279)

Forkhead Homeobox 04 (FOXO4), belongs to the forkhead box class O family member proteins (FOXOs) and is expressed quite ubiquitously, however, it is abundantly expressed in skeletal muscle. (314) Forkhead Homeobox04 also plays a role in muscle regeneration and cell proliferation and differentiation by interacting with forkhead box protein K1 (Foxk1) promoting muscle progenitor cell proliferation by repressing FOXO4 transcriptional activity. This results in a decreased expressions of FOXO4 target genes, and increased proliferation of muscle progenitor cells. (314)

A possible mechanism to explain statin-associated muscle toxicity is mitochondrial dysfunction. Statin's potential to trigger mitochondrial-mediated apoptosis in muscle cells has gained interest in research recently. (285) This supports the notion that miR-499a could be a biomarker for statin-induced muscle damage.

Currently, there is no standardised biomarker used to measure statin intolerance. The presence of elevated CK levels in plasma serves as an indirect biomarker for damage to cardiac and skeletal muscle. Creatine kinase plays a crucial role in the enzymology of cardiac and skeletal muscle, owing to its high sensitivity and specificity in quantification. (295)

Despite the current lack of research drawing a correlation between elevated CK levels and upregulation of miR-499a in hypercholesterolemic patients undergoing long-term statin therapy, Min *et al*. (2016)(286) demonstrated that miR-499a release/expression is upregulated due to statin exposure in athletes and cultured myotubes, by using a combination of in vitro and in vivo modeling of pre-, during- and post exercising muscle. (286, 287)

Contrary to the findings of Min *et al*. (2106)(286), miR-499a was not detectable in any of the samples used for analysis. During troubleshooting it was noted that the house keeping miRNA was detected, suggesting the most likely reason for not detecting miR-499a in any of the test samples might be due low abundance or biological variation as both miR-133a and miR-33a was detected.

When accessing the methodology employed for the detection of miR-499, it raises questions about the sensitivity and specificity of the detection techniques used even though both miR-133a and miR-33 was detected. This possibly suggests a need for more advanced technologies like digital droplet PCR or next-generation sequencing. However, lack of detection of miR-499 could also be due to the fact that the samples used were frozen and underwent multiple freeze thaw cycles prior to processing.

Despite results generated from this study and previous studies, research on miR-499a in the context of statin intolerance remains limited. A more comprehensive

investigation is needed to better understand its potential as a possible biomarker for statin intolerance. Suggesting the need for studies with larger, more diverse sample sizes to fill the gaps in the current research, while using more sensitive detection methods to improve the precision and reliability of miR-499a data generated.

Chapter 5: Conclusion

The global statistics on CVD emphasizes its impact on morbidity and mortality, particularly in low- and middle-income countries, like South Africa. Elevated LDL levels, a major risk factor for atherosclerotic disease, supports the need for effective cholesterol-lowering therapies, such as statins. The widespread use of statins is in some instances complicated by statin intolerance, affecting approximately 20% of patients.

The study analysed of the role of statin intolerance, its relationship with cholesterol homeostasis, and the involvement of microRNAs (miRNAs) such as miR-33a, miR-133a, and miR-499a in understanding and in the future potentially managing the adverse effects of statins.

Three hundred participants were included in the study of which 100 were hypercholesterolemia patients not taking statins, 100 were statin-treated hypercholesterolemia patients and 100 were healthy volunteers.

Of the three miRNAs assessed in this study, only miR-33a and miR-133a was detectable. During troubleshooting, to identify possible reasons why miR-499a was not detectable, it was noted that the most plausible reason is due to low abundance or biological variation.

A significant difference in the relative fold change of miR-33a expression was noted between the statin-users and statin-naïve group compared to the control group. The study showed an inverse correlation between miR-33a and ApoA1 levels. The study showed a significant difference in the relative fold change of miR-133a expression between the control group and statin-users, and the statin-users and the statin naïve patients. Although, no correlation was observed between the CK levels and miR-133a

expression for the statin-users, a positive correlation was observed between the CK levels and miR-133a expression in the combined hypercholesterolaemic group.

The analyses of the miRNAs included in this study provides a novel angle, focusing on their possible potential as biomarkers for statin intolerance. MicroRNA-33a, involved in cholesterol homeostasis, modulates cholesterol efflux by regulating the ABCA1 transporter. Its inverse relationship with ApoA1 levels suggests its potential as a therapeutic target for improving cholesterol export and reducing statin intolerance. MicroRNA-133a, is a key role player in muscle-specific pathways, specifically muscle damage and statin-induced side effects. The elevated expression of miR-133a, as shown in this study, aligns with previous research of the upregulation of miR-133a in response to statin-induced toxicity. Although miR-499 was not detected in this study, it has been implicated in muscle and mitochondrial function in previous research. Despite results from this and previous studies, there is a paucity of research on miR-499a in the context of statin intolerance, despite its theoretical relevance, addressing this gap through larger sample sizes or more sensitive detection methods could clarify its role and support its relevance to statin-associated muscle damage.

Unfortunately, the lack of standardisation in diagnostic criteria for statin intolerance limits the generalisability of findings. Developing standardised definitions and incorporating miRNA profiling into routine diagnostic protocols could enhance its clinical application.

This study contributes to the ongoing research on statin intolerance by identifying miRNAs (miR-33a and miR-133a) that could, in conjunction with future research, serve as biomarkers and therapeutic targets. The insights gained from this study, contributes to our understanding of the epigenetic and possible molecular mechanisms underlying statin-induced side effects. The integration of miRNA research with current clinical practices could pave the way for better patient outcomes in the future. The findings highlight the need to investigate larger sample sizes, focusing on the potential correlation between miR-499a and CK levels and evaluating whether varying statin doses produce differential effects in patients.

Limitations

1. The protocol specified that participants should exhibit “possible symptoms of statin intolerance” as part of the study inclusion criteria. However, it did not define a criteria of what constitutes such symptoms or whether they must be directly linked to statin use. This lack of clarity may have led to variability in how inclusion criteria were interpreted during recruitment. While the protocol intended to include only participants with symptoms suggestive of statin intolerance, the recruitment process involved an initial approach to all eligible individuals on statin therapy with any muscle related symptoms, followed by a post-hoc assessment of symptoms using a questionnaire.
2. Due to the heterogenicity of our South African population, significant genetic differences among ethnic groups can affect the susceptibility to statin-induced myopathy and this may complicate identifying a universal biomarker.
3. A questionnaire relies on self-reported symptom like muscle pain which ultimately is a subjective characteristic. Although the use of a questionnaire provided a standardized method to assess the symptoms reported by each participant, the retrospective nature of symptom evaluation introduces the possibility of bias or misclassification, particularly if symptoms were unrelated to statin use but perceived as such by participants during the pre-screening process.
4. The recruitment of participants from a specific population and the potential variability in symptom assessment, as described in limitation 1, may limit the generalizability of the study’s findings to broader populations or to those on statins other than atorvastatin or simvastatin.
5. Lifestyle factors such as diet and physical exercise were not investigated, as they too can influence risk of myopathy.
6. Socioeconomic factors in our unequal South African society were not assessed.
7. The absence of known control specimens, such as those from a hospital chemistry laboratory, for validation of the ELISA assays. While internal quality procedures were implemented to ensure the reliability of experimental results, the inclusion of externally validated control specimens would have provided validity and reproducibility to the data. This limitation limits the ability to directly compare the assays outcomes with clinically established standards.

8. The potential discrepancy between the reported levels of ApoA1 and expected physiological ranges for HDL cholesterol. While ApoA1 was measured as a marker of HDL, the assay was designed to assess ApoA1 levels and not direct HDL-C levels. This may contribute to the observed values, which may not align with typical physiological ranges for HDL-C. Although procedures were applied to ensure data validity and reproducibility, there remains the possibility of dilution errors or assay calibration issues that could have influenced the results.
9. The absence of lipid data for the non-statin and statin users, hinders our ability to fully assess the relationship between biomarkers, cholesterol levels, and statin use. The lack of lipid profiles makes it difficult to differentiate whether observed changes in biomarker levels were due to statin treatment or might reflect underlying metabolic changes.
10. While age and ethnicity were matched, the lack of complete data in controls made it impossible to fully compare demographic and clinical variables across all groups.

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Appendix 1

ETHICS APPROVAL LETTER

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 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

Faculty of Health Sciences **Research Ethics Committee**

11 February 2022

Approval Certificate
New Application

Dear Ms R de Beer

Ethics Reference No.: 709/2021
Title: Identifying circulating biomarkers in a cohort of hypercholesterolaemic patients with statin intolerance from Gauteng, South Africa

The **New Application** as supported by documents received between 2022-01-03 and 2022-02-09 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2022-02-09 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 2023-02-11.
- Please remember to use your protocol number (709/2021) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

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

We wish you the best with your research.

Yours sincerely

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

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

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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

Appendix 2

ANNUAL RENEWAL – 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. 0990-0278 Approved for use through August 31, 2023.

Faculty of Health Sciences **Research Ethics Committee**

16 February 2023

Approval Certificate
Annual Renewal

Dear Ms R de Beer,

Ethics Reference No.: 709/2021 – Line 1
Title: Identifying circulating biomarkers in a cohort of hypercholesterolaemic patients with statin intolerance from Gauteng, South Africa

The **Annual Renewal** as supported by documents received between 2023-02-01 and 2023-02-15 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2023-02-15 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2024-02-16.
- Please remember to use your protocol number (709/2021) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

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

We wish you the best with your research.

Yours sincerely

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

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2016 (Department of Health)

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Fakulteit Gesondheidswetenskappe
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Appendix 3

ANNUAL RENEWAL – 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**

1 March 2024

Approval Certificate
Annual Renewal

Dear Ms R de Beer,

Ethics Reference No.: 709/2021 – Line 2
Title: Identifying circulating biomarkers in a cohort of hypercholesterolaemic patients with statin intolerance from Gauteng, South Africa

The **Annual Renewal** as supported by documents received between 2024-01-30 and 2024-02-28 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2024-02-28 as resolved by its quorate meeting.

Please note the following about your ethics approval:

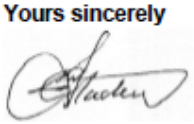
- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2025-03-01.
- The Research Ethics Committee (REC) must monitor your research continuously. To this end, you must submit as may be applicable for your kind of research:
  a) annual reports;
  b) reports requested *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 (709/2021) 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).

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Appendix 4

PhD APPROVAL LETTER

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

23 November 2021

Prof A Phulukdaree
Department of Physiology
Faculty of Health Sciences

Dear Prof Phulukdaree

Student: R de Beer (PhD Physiology)
Title: Identifying circulating biomarkers in a cohort of hypercholesterolaemic patients with statin intolerance from Gauteng, South Africa

The above-mentioned student's protocol has been approved by the PhD committee.
We wish the student all the best with their studies.

Kindly note that the PhD committee requires 6-monthly progress reports.

Kind regards,

Prof. Martin Brand
Chair: PhD Committee

Appendix 5

LETTER OF CLEARANCE FROM THE BIOSTATISTICIAN

Date: 28 / 10 / 2021

LETTER OF CLEARANCE FROM THE BIOSTATISTICIAN

This letter is to confirm that, Ms Rene de Beer from UP

discussed with me the study titled: Identifying circulating biomarkers in a cohort of hypercholesterolaemic patients with statin intolerance from Gauteng, South Africa

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

The analytical tool(s) that will be used is(are): Data summary by treatment group will report, but may not be limited to, descriptive statistics mean, standard deviation, median, range and 95% confidence intervals (CI) for continuous data and frequency, percentage and 95% CI for categorical data. Of principal interest is the comparison of treatment groups with respect to protein biomarker levels, adjusted for associated miRNAs and covariates (demographic and clinical). Fold-regulation for all 4 miR's will be calculated using the 2 to the minus delta delta cycle threshold method. All ELISA data and LC/MS-MS data generated will be continuous. Thus, for each data set, a normality test will be conducted to determine if the data follows a normal distribution. This will guide whether or not the t-tests and one-way ANOVA tests which will be conducted between each group are considered as parametric or non-parametric. Where data is parametric, the mean and standard deviations will be reported. Where the data is nonparametric the median and interquartile ranges will be reported. The t-tests will be conducted between control vs. statin naïve, control vs. statin intolerant and statin naïve vs. statin intolerant – for miR's, ELISA and LC/MS-MS datasets. Relationships between miR's and the respective quantified proteins will be evaluated using a multiple regression analysis which will take all confounding variables into consideration. Testing will be done at the 0.05 level of significance. The relationship of protein biomarkers (Apolipoprotein a, GAA, AST, ALT and CKM) with hypercholesterolemia statin treatment (yes/no), associated miRNA (miR-33 a/b, miR-124a, miR-133a, miR-499) levels, demographic and clinical variables will be assessed using multivariable regression. Sample size requirements usually are to enroll at least 10 to 15 patients per predictor/independent variable considered in the multivariable regression analysis. Not more than 10 – 12 variables are anticipated and therefore a sample size of at least 300 patients (100 per group) will be the target.

Signature

PJ Becker (Tel: 012-319-2203)

Research Office, Faculty of Health Sciences

Appendix 6

QUESTIONNAIRE: AMERICAN COLLEGE OF CARDIOLOGY'S (ACC) STATIN INTOLERANCE APPLICATION

Demographics

Sex:

☐ Male

☐ Female

Age: _____ years

Race: _____

Weight: _____ kg

Height: _____ cm

Smoking:

☐ Yes If yes, number of cigarettes per day: _____

☐ No

Have you been diagnosed with covid-19 infection?

☐ Yes

☐ No

Have you been vaccinated against covid-19?

☐ Yes If yes, comment: _____

☐ No

Rhabdomyolysis Assessment

1. Is the patient's CK above 5x the upper normal limit (UNL)?

☐ Yes

☐ No

☐ Unknown

Muscle symptoms

2. Select the group that best describes the symptoms

☐ Muscle ache, weakness, soreness, stiffness, cramping, tenderness, or general fatigue.

☐ Tingling, twitching, shooting pain, nocturnal cramps, or joint pain

3. Select symptom area

☐ Bilateral (Muscle symptoms are generalized, e.g. neck and shoulder pain or lower back)

☐ Unilateral (Muscle symptoms are isolated, e.g. only on knee of shoulder)

4. Severity of symptoms

a. How severe is the pain?

(0 = no pain to 10 = worse pain)

□ 0 – 2 mild

□ 3 – 5 moderate

□ 6 – 10 severe

b. How many of the last seven days has the patient had the symptoms?

□ 1 – 2 mild

□ 3 – 4 moderate

□ 5 – 7 severe

c. How much have the muscle symptoms impacted everyday activities?

□ Only limits exercise

□ Slightly reduces everyday activity (trouble working, sleeping, performing household chores, climbing stairs etc.)

□ Greatly restricts everyday activities (cannot work, sleep, perform household chores or climb stairs)

5. 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

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

Medical conditions

Primary muscle diseases

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

Rheumatological disorders

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

Additional disorders

- Diabetes
- Adrenal insufficiency/ Cushing Syndrome
- Addison’s disease

- ☐ Anemia
- ☐ Hypoparathyroidism
- ☐ Viral illness
- ☐ Anemia
- ☐ Peripheral arterial disease

8. 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

Concomitant medication

_____

_____

Appendix 7

INFORMED CONSENT FORM: HYPERCHOLESTEROLAEMIC GROUP

PARTICIPANT'S INFORMATION & INFORMED CONSENT DOCUMENT

STUDY TITLE:

Identifying circulating biomarkers related to statin intolerance in a cohort of hypercholesterolaemic patients from Gauteng, South Africa

Protocol no: …… Researcher: René de Beer

Dear Mr. / Mrs. .....

1) INTRODUCTION

You are invited to volunteer for a research study involving the medication called statins, that you are currently using as treatment for your high cholesterol. I am doing research for a doctoral 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 our bodies where it is broken down so it can lower our cholesterol levels and be excreted as waste. However, in some cases this up 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.

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

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

A 5ml (about a teaspoon full) tube of blood will be collected to determine whether you do have any of the biomarkers and to determine your Creatine Kinase (CK) levels, Apolipoprotein A – 1 levels, Guanidino acetoacetate (GAA) levels, Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels.

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

1. Are you older than 18 years?
2. Have you been diagnosed with high cholesterol?
3. Do you experience severe adverse events/ side effects?
4. Have you been on a stable and continuous atorvastatin or simvastatin dose12-weeks prior to this date?

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

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

4) POSSIBLE RISKS AND DISCOMFORTS INVOLVED

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

5) 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 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.

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, René de Beer, will be able to identify you as participant. Results will be published or presented in such a fashion that patients remain unidentifiable. The hard copies of the anonymous data and the samples we collected will be kept in a locked facility at the Department of Physiology, the University of Pretoria.

_____

Participant's name (Please print)

_____ _____

Participant's signature Date

_____

Researcher's name (Please print)

_____ _____

Researcher's signature Date

Appendix 8

INFORMED CONSENT FORM: NON-STATIN USERS

PARTICIPANT'S INFORMATION & INFORMED CONSENT DOCUMENT

STUDY TITLE:

Identifying circulating biomarkers related to statin intolerance in a cohort of hypercholesterolaemic patients from Gauteng, South Africa

Protocol no: …… Researcher: René de Beer

Dear Mr. / Mrs. .....

1) INTRODUCTION

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

2) THE NATURE AND PURPOSE OF THIS STUDY

Statins are normally taken up by our bodies where it is broken down so it can lower our cholesterol levels and be excreted as waste. However, in some cases this up 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 and to determine your Creatine Kinase (CK) levels, Apolipoprotein A – 1 levels, Guanidino acetoacetate (GAA) levels, Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels.

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

5. Are you older than 18 years?
6. Have you been diagnosed with high cholesterol?
7. Not currently on a statin treatment regimen, including Atorvastatin, Simvastatin, Pravastatin, Rosuvastatin or Pitavastatin?

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

3. Are you younger than 18 years?

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) 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 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.

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, René de Beer, will be able to identify you as participant. Results will be published or presented in such a fashion that patients remain unidentifiable. The hard copies of the anonymous data and the samples we collected will be kept in a locked facility at the Department of Physiology, the University of Pretoria.

_____

Participant's name (Please print)

_____ _____

Participant's signature Date

_____

Researcher's name (Please print)

_____ _____

Researcher's signature Date

Appendix 9

INFORMED CONSENT FORM: CONTROL GROUP

PARTICIPANT'S INFORMATION & INFORMED CONSENT DOCUMENT

STUDY TITLE:

Identifying circulating biomarkers related to statin intolerance in a cohort of hypercholesterolaemic patients from Gauteng, South Africa

Protocol no: 709/2021 Researcher: René de Beer

Dear Mr. / Mrs. .....

1) INTRODUCTION

You are invited to volunteer for a research study involving the medication that is commonly prescribed for the treatment of high cholesterol called statins. I am doing research for a doctoral 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 up 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 and to determine your Creatine Kinase (CK) levels,

Apolipoprotein A – 1 levels, Guanidino acetoacetate (GAA) levels, Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels.

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

8. 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:

4. Are you younger than 18 years?
5. Have you been diagnosed with abnormally high levels of cholesterol?
6. 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, René de Beer, will be able to identify you as participant. Results will be published or presented in such a fashion that patients remain unidentifiable. The hard copies of the anonymous date and the sample we collected will be kept in a locked facility at the Department of Physiology, the University of Pretoria.

_____	_____	_____
Participant's name	Participant's signature	Date

_____	_____	_____
Researcher's name	Researcher's signature	Date

Appendix 10

ARTICLE SUBMISSIONS

Gmail Rene Pienaar <renedbr1@gmail.com>

Molecular and Cellular Biochemistry - Receipt of Manuscript 'Evaluation of miR-133,...'

1 message

Molecular and Cellular Biochemistry <ashika.amreen@springernature.com> Wed, Oct 9, 2024 at 6:54 PM

To: renedbr1@gmail.com

Ref: Submission ID 64b1edc5-db7f-4cc5-b2e4-4ce85fac2964

Dear Dr Beer,

Please note that you are listed as a co-author on the manuscript "Evaluation of miR-133, creatine kinase and aspartate aminotransferase in hypercholesterolaemic patients in South Africa – A pilot study", which was submitted to Molecular and Cellular Biochemistry on 09 October 2024 UTC.

If you have any queries related to this manuscript please contact the corresponding author, who is solely responsible for communicating with the journal.

Kind regards,

Editorial Assistant
Molecular and Cellular Biochemistry