The impact of Niacin on PCSK9 levels in vervet monkeys (*Chlorocebus aethiops*)

By

Thobile Ngqaneka

3880262

A full thesis submitted in fulfilment of the requirements for the degree of Masters Pharmaceutical Sciences in the School of Pharmacy, University of the Western Cape.

Submitted October 2020

Supervisor: Dr. Kenechukwu Obikeze

Co-supervisors: Dr. Chesa Gift Chauke

Dr. Zandisiwe Emilia Magwebu

KEYWORDS

Atherosclerosis

Cardiovascular disease (CVD)

High-density lipoprotein (HDL)

Lipids

Lipoproteins

Low-density lipoprotein (LDL)

Mutations

Niacin

Proprotein convertase subtilisin/kexin type 9 (PCSK9)

Vervet monkeys

ABSTRACT

The impact of Niacin on PCSK9 levels in vervet monkeys (*Chlorocebus aethiops*)

Thobile Ngqaneka

MSc thesis, School of Pharmacy, University of the Western Cape

Cardiovascular diseases (CVDs) such as ischaemic heart diseases, heart failure and stroke remain a major cause of death globally. Various deep-rooted factors influence CVD development; these include but are not limited to elevated blood lipids, high blood pressure, obesity and diabetes. A considerable number of proteins are involved directly and indirectly in the transport, maintenance and elimination of plasma lipids, including high and low-density lipoprotein cholesterol (HDL-C and LDL-C). There are several mechanisms involved in the removal of LDL particles from systemic circulation. One such mechanism is associated with the gene that encodes proprotein convertase subtilisin/kexin type 9 (*PCSK9*), which has become an exciting therapeutic target for the reduction of residual risk of CVDs. Currently, statins are the mainstay treatment to reduce LDL-C, and a need exists to further develop more effective LDL-C-lowering drugs that might supplement statins. This study was aimed at contributing to the generation of knowledge regarding the effect of niacin in reducing LDL levels through PCSK9 interaction.

The aims/objectives of this study were achieved by utilizing two approaches, which included animal intervention with niacin followed by genetic screening of five prioritized genes involved in cholesterol synthesis and regulation. For animal intervention, 16 vervet monkeys were divided into two groups of eight animals consisting of a control and an experimental (niacin) group. The control group was given a normal standard diet of pre-cooked maize meal throughout the study, while the experimental group received the same diet supplemented with 100 mg/kg of niacin (SR) for 12 weeks. During the niacin intervention, blood was collected at baseline, every four weeks during the treatment period and the end of the washout period. The collected blood was used for biochemical analysis (total cholesterol, triglycerides, LDL-C, and HDL-C) and downstream genetic applications. The second phase included the screening of *PCSK9, LDLR, SREBP-2, CETP* and *APOB-100* using genotyping and gene expression.

Niacin administration produced statistically significant increases in plasma HDL-C at four-time points (T_1, T_2, T_3 and T_4), which resulted in an overall increase in plasma HDL-C. Additionally, niacin administration resulted in a slight reduction in LDL-C and total cholesterol

levels. Furthermore, the genotyping analysis revealed 13 sequence variants identified in *PCSK9, LDLR, SREBP-2, CETP* and *APOB-100* genes. Five of these variants were predicted to be disease-causing and correlated with gene expression patterns. Three identified *PCSK9* variants (H177N, R148S, G635G) were categorized as LOF mutations, and this was supported by a decline in gene expression in animals harbouring these variants. The *LDLR* also had LOF variants that were the reason for its decreased mRNA expression. Additionally, *SREBP-2* proved to be a key mediator of cholesterol pathways. Therefore, the findings of the study conclusively suggest that niacin does increase HDL-C and decrease LDL-C and total cholesterol. Moreover, an interaction between niacin administration and *PCSK9* was observed which resulted in decreased gene expression.

DECLARATION

I declare that "**The impact of Niacin on PCSK9 levels in vervet monkeys (*Chlorocebus aethiops*)"** is my own work, that it has not been submitted before for any degree or examination in any other University, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Thobile Ngqaneka

Signature…  Date…20/10/2020

ACKNOWLEDGEMENTS

Firstly, I thankfully wish to acknowledge my supervisors: Dr. Chesa Gift Chauke, Primate Unit and Delft Animal Centre (PUDAC) of the South African Medical Research Council (PUDAC/SAMRC), for her guidance, supervision, support, encouragement and for controlling the budget for the project. Dr. Kenechukwu Obikeze, University of the Western Cape (UWC), for his supervision, guidance, and his ability to constructively critique the writing-up process of this thesis. Dr. Zandisiwe E. Magwebu (PUDAC/SAMRC), for her support, encouragement, guidance and supervision of all aspects of this study. Then, I would like to thank the PUDAC/SAMRC technical team: Joritha van Heerden, Timothy Collop, Abraham Davids, Philida Beukes and Mbuyiseli Billy for their excellent technical assistance and expertise in primate management. The SAMRC for funding the MSc degree.

My family, for their love, emotional support and encouragement. My daughter, Yamihle imisebenzi yamaMpondomise, whom was born in the middle of this project. Lastly and definitely not the least, the vervet monkeys that were used for this study, without them, this project wouldn't have been possible.

TABLE OF CONTENT

		Page
	KEYWORDS	**I**
	ABSTRACT	**II**
	DECLARATION	**IV**
	ACKNOWLEDGEMENTS	**V**
	TABLE OF CONTENTS	**VI**
	LIST OF FIGURES	**VIII**
	LIST OF TABLES	**XI**
	LIST OF ABBREVIATIONS	**XII**
	CHAPTER 1: Introduction and Literature Review	
1	Introduction	1
1.1	Background on atherosclerosis	4
1.2	Lipid pathway/mechanisms	7
1.2.1	Role of Lipoproteins in CVD/ atherosclerosis	9
1.2.1.1	LDL levels and atherosclerosis	9
1.2.1.2	HDL levels and atherosclerosis	10
1.2.1.3	Triglycerides and atherosclerosis	12
1.2.1.4	Total cholesterol and atherosclerosis	12
1.3	Cholesterol-lowering drug intervention	13
1.3.1	Statins	13
1.3.2	Niacin	15
1.3.2.1	Side effects of niacin	16
1.3.2.2	Mechanisms of action of niacin	16
1.3.2.2.1	Modulation of triglyceride lipolysis in adipose tissue	16
1.3.2.2.2	Modulation of triglyceride synthesis and secretion of VLDL particles by liver	17
1.3.2.2.3	Role of niacin in HDL metabolism	18
1.4	Gene activities in atherosclerosis /CVDs	20
1.4.1	Involvement of PCSK9 in CVD	20
1.4.1.1	PCSK9 inhibition – evidence from animal studies	22
1.4.1.2	Specific antibodies directly against PCSK9	23
1.4.1.3	Inhibition of PCSK9 by using ASO	24
1.4.1.4	Inhibition of PCSK9 by using SiRNA	26
1.4.1.5	Inhibition of PCSK9 by using an anti-PCSK9 antigen binding fragment	26
1.4.2	Interaction of PCSK9 with niacin	27
1.4.3	Interaction between LDLR and PCSK9 leading to atherosclerosis development	27
1.4.4	Involvement of SREBP 2 in the lipid metabolism leading to atherosclerosis formation	30
1.4.5	The regulation of Lipoproteins by CETP	31
1.4.5.1	CETP and atherogenesis	32
1.4.6	Involvement of APOB-100 in the lipid metabolism leading to atherosclerosis formation	35
1.4.6.1	The mechanism of APOB-100 Synthesis	36
1.4.7	The use of non-human primates (NHPs) as research models	38
1.4.7.1	Vervet monkey as an animal model of atherosclerosis	40
1.5	Research Objectives	41
1.5.1	Aims and specific objectives	41
1.5.2	Hypothesis	41
1.5.3	Overview	41

2	**CHAPTER 2: The effects of short-term niacin treatment on plasma lipoproteins in the vervet monkey model.**	42
2.1	**INTRODUCTION**	42
2.2	**MATERIALS AND METHODS**	43
2.2.1	Animal ethics	43
2.2.2	Animal housing, diet and group selection	43
2.2.3	Compound administration and food consumption	44
2.2.4	Clinical observations and body weights	44
2.2.5	Blood collection	45
2.2.6	Biochemistry analysis	45
2.3	Statistical analysis	45
2.4	**RESULTS**	45
2.4.1	Total cholesterol	46
2.4.2	LDL levels	47
2.4.3	HDL levels	48
2.4.4	Triglycerides	48
2.5	**DISCUSSION**	49
2.6	**CONCLUSION**	52
3	**CHAPTER 3: The impact of PCSK9 in lipid metabolism and its relationship to new therapies for lowering cholesterol in a non-human primate model of atherosclerosis.**	53
3.1	**INTRODUCTION**	53
3.2	**MATERIALS AND METHODS**	55
3.2.1	DNA extraction and quantification	55
3.2.2	Bioinformatics and candidate gene selection	55
3.2.3	PCR amplification, purification and sequencing analysis	56
3.2.4	Gene expression	57
3.2.4.1	RNA extraction, purification and integrity	57
3.2.4.2	cDNA generation	58
3.2.4.3	Quantitative real-time PCR (qRT-PCR)	59
3.2.4.4	Statistical analysis	60
3.3	**RESULTS**	61
3.3.1	Genotyping analysis	61
3.4	Gene expression analysis	67
3.5	**DISCUSSION**	71
3.6	**CONCLUSION**	78
4	**CHAPTER 4: OVERALL DISCUSSION/ CONCLUSION**	79
5	**CHAPTER 5: REFERENCES**	85
6	**CHAPTER 6: APPENDICES**	106

LIST OF FIGURES

Figure 1.1: Endogenous Lipoprotein Pathway. 7

Figure 1.2: HDL metabolism and reverse cholesterol transport. 11

Figure 1.3: Depicts different stages of the statin mechanism of action. (**A**) The catalytic nature of HMG-CoA reductase is mitigated by statins. (**B**) Cholesterol production is reduced Intracellularly. (**C**) SREBP is split and transferred to different parts. (**D**) The *LDLR* gene expression is stimulated by SREBP. (**E**) The transcription and translation of the *LDLR* gene starts. (**F**) LDLR concentration is increased on cell surface. (**G**) LDL binding, endocytosis, and subsequent degradation. (**H**) Intracellular cholesterol increase toward cellular baseline. (**I**) Plasma LDL levels decrease. 14

Figure 1.4: Outline of modern theories on niacin modus operandi against the metabolism of lipid and lipoprotein. Niacin, is able to inhibit fatty acid recruitment from adipose tissue by using lipase-facilitated hormone-sensitive actions. 19

Figure 1.5: The *PCSK9* gene is located on the short arm of chromosome 1p32 and has 12 exons. The mature form of PCSK9 consists of an SP, a PRO, and a catalytic domain and a CHRD in the C-terminal domain. CHRD, cysteine and histidine-rich domain; PRO, pro-segment; SP, signal peptide. 21

Figure 1.6: The illustration of the functions of PCSK9 and treatment approaches focusing on PCSK9. (**A**) SREBP-2 has the ability to induce simultaneous expression of *PCSK9* and *LDLR*. (**B**) Drug development tactics directed at PCSK9 biosynthesis or role are being developed. 25

Figure 1.7: Schematic representation of the *LDLR* gene and LDLR protein structures. OLS, O-linked sugar domain; TM, transmembrane. 29

Figure 1.8: The role of PCSK9 in LDLR metabolism. 29

Figure 1.9: Low intracellular cholesterol levels activate SREBP-2 mediated *LDLR* expression, thereby increasing hepatic LDL-C uptake. Concurrently, SREBP-2 induces expression of *PCSK9*, which enhances hepatic LDLR degradation, thus preventing excessive cholesterol uptake in order to preserve cholesterol homeostasis. 31

Figure 1.10: Depicts the reverse cholesterol transport (RCT) pathway. This figure details all the key mechanisms of RCT including apolipoprotein A-I (APOA-I), high-density lipoprotein (HDL), lecithin: cholesterol acyltransferase (LCAT), ATP-binding cassette transporter (ABCA1/ABCG1) and cholesterol ester transfer protein (CETP). 34

Figure 1.11: Structure of APOB-100, is made up of interchanging α helices and β strands that possesses polar and non-polar ends. Regions of the MTP 36

and LDLR binding domains are also depicted. The length of APOB-48 is indicated by the arrow.

Figure 1.12: The diagram depicts the interaction of APOB and VLDL. Emerging APOB particles associate themselves with lipid-free MTP, leading to a detachment from lumen of the ER into the cytosol (**A**) and thereafter degraded. On the other hand, the interaction of naïve APOB particles with MTP-lipid complexes, results in the formation of VLDL precursor (**B**). A complete VLDL molecule is formed when the VLDL precursor is merged with a triglyceride (*TG*) rich lipid droplet (**C**). 37

Figure 2.1: Animal body weight (Kg). Mean weight of the animals in the niacin and control group over the study period. 46

Figure 2.2: Total cholesterol (mmol/L). The total cholesterol expressed as mean ± SD of the niacin and control groups. * indicates statistical significance ($p < 0.05$). 47

Figure 2.3: LDL-cholesterol (mmol/L). Mean plasma LDL concentration of the animals in the niacin and control groups over the study period. 47

Figure 2.4: HDL-cholesterol (mmol/L). Mean plasma HDL concentration of the animals in the niacin and control groups over the study period. * indicates statistical significance ($p < 0, 05$). ** indicates statistical significance ($p < 0, 005$). **** indicates statistical significance ($p < 0, 0001$). 48

Figure 2.5: Triglycerides (mmol/L). Mean plasma triglycerides concentration expressed as mean ± SD of the animals in the niacin and control groups over the study period. 49

Figure 3.1: Genetic analysis for *PCSK9* in captive-bred vervet monkeys. Sequence chromatogram are showing a nucleotide change at codon 422 (C442>A) located in exon 1. The circles on the chromatographs and the arrow on the protein alignment (R148S) indicate the position of the missense mutation. 62

Figure 3.2: Genetic analysis for *LDLR* in captive-bred vervet monkeys. The chromatographs depict a nucleotide change (G820>A) identified in exon 6. The circles on the chromatographs and the arrow on the protein alignment (D274N) indicate the position of the missense mutation. 63

Figure 3.3: Genetic analysis for CETP in vervet monkeys. Sequence chromatogram Genetic analysis for *CETP* gene in captive-bred vervet monkeys. Sequence chromatogram and protein alignment showing a missense mutation (A274>T) located in exon 2. The circles on the chromatographs and the arrow on the protein alignment (M92L) indicate the position of the mutation. 64

Figure 3.4a:
Genetic analysis for *APOB-100* gene in captive-bred vervet monkeys. The chromatograms are showing a nucleotide change in codon 1903 (T1903>C) identified in exon 14. The circles on the chromatographs and the arrow on the protein alignment (F635L) indicate the position of the missense mutation 65

Figure 3.4b:
The chromatographs are illustrating a nucleotide change (G1939>A) identified in exon 14 of *APOB-100*. The circles on the chromatographs and the arrow on the protein alignment (A647T) indicate the position of the missense mutation. 66

Figure 3.5:
PCSK9 mRNA gene expression in captive-bred vervet monkeys. Gene expression of the control group was compared with the experimental group. The controls were given a maintenance diet while the experimental group received 100 mg/kg of niacin in their food bolus for a period of 12 weeks. 67

Figure 3.6:
LDLR mRNA gene expression in vervet monkeys. The control group (maintenance diet) was compared with the experimental group (100 mg/kg of niacin) at T_0-T_5. The data was expressed as mean ± SD and mRNA expression in a.u. (arbitrary units). 68

Figure 3.7:
SREBP-2 mRNA gene expression in captive-bred vervet monkeys. Gene expression of the control group was compared with the experimental group at T_0-T_5. The data was expressed as mean ± SD and mRNA expression in a.u. (arbitrary units).* indicates a statistical significance difference ($p < 0, 05$). 69

Figure 3.8:
CETP mRNA gene expression in captive-bred vervet monkeys. Gene expression of the control group was compared with the experimental group at T_0-T_5. The data was expressed as mean ± SD and mRNA expression in a.u. (arbitrary units).* indicates statistical significance ($p < 0, 05$). 70

Figure 3.9:
APOB-100 mRNA gene expression in captive-bred vervet monkeys. Gene expression of the control group was compared with the experimental group. The data was expressed as mean ± SD and mRNA expression in a.u. (arbitrary units). 71

LIST OF TABLES

		Page
Table 2.1:	Experimental design.	44
Table 3.1:	The selected candidate genes.	56
Table 3.2:	PCR reaction used for genotyping.	57
Table 3.3:	PCR program used for genotyping.	57
Table 3.4:	cDNA reaction mixture.	59
Table 3.5:	Selected candidate genes for mRNA expression assays.	60
Table 3.6:	Identified sequence variants in the colony of captive-bred vervet monkeys.	61

LIST OF ABBREVIATIONS

ABCA1	ATP binding membrane cassette transport protein A1
ACC	The American college of cardiology
ACC	Acetyl-CoA carboxylase
ADH	Autosomal dominant hypercholesterolemia
AHA	American heart association
APOA-1	Apolipoprotein A-1
APOB	Apolipoprotein B
APOB-100	Apolipoprotein-100
APOCI	Apolipoprotein CI
APOCII	Apolipoprotein CII
APOE	Apolipoprotein E
ATP	Adenosine triphosphate
ASCVD	Atherosclerotic cardiovascular disease
ASOs	Antisense oligonucleotides
BMI	Body mass index
bp	Base pair(s)
°C	Degrees Celsius
CAD	Coronary artery disease
cDNA	Complementary
CE	Cholesteryl esters
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
CVD	Cardiovascular disease
c-AMP	Cyclic adenosine monophosphate
DNA	Deoxyribonucleic acid
ECRA	Ethics Committee for Research on Animals
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
FA	Fatty acid
FASN	Fatty acid synthase
FDA	United States Food and Drug Administration

FH	Familial hypercholesterolemia
GOF	Gain-of-function
HDL-C	High density lipoprotein cholesterol
HL	Hepatic lipase
HMG-CoA	3-Hydroxymethyl-3-methylglutaryl coenzyme A
HMGCR	3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase
HMGCS	HMG-CoA synthase
IMT	Intima-media thickness
I.V	Intravenous injection
Hep G2	Human hepatoblastoma cells
LCAT	Lecithin-cholesterol acyltransferase
LDL-C	Low density lipoprotein cholesterol
LDLR	Low density lipoprotein receptor
LNA	Locked nucleic acid
LNP	Lipidoid nanoparticle
LOF	Loss of function
LPL	Lipoprotein lipase
MI	Myocardial infarction
mRNA	Messenger ribonucleic acid
MTP	Microsomal triglyceride transfer protein
NAD+	Nicotinamide adenine dinucleotide
NADP+	Nicotinamide adenine dinucleotide phosphate
NHPs	Non-human primates
PCSK9	Proprotein convertase subtilisin/kexin type 9
PCR	Polymerase chain reaction
PUDAC	Primate Unit and Delft Animal Centre
RCT	Reverse cholesterol transport
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse transcription-polymerase chain reaction
SAMRC	South African Medical Research Council
SANS	South African Bureau of Standards
SCAP	SREBP cleavage activating protein

siRNAs	Small interfering RNAs
SNPs	Single-nucleotide polymorphisms
SR-B1	Scavenger receptor type B1
SREBP-2	Sterol regulatory element binding protein-2
TGRL	Triglyceride-rich lipoproteins
VLDL	Very low density lipoprotein cholesterol
WHO	World health organization

CHAPTER ONE:
INTRODUCTION AND LITERATURE REVIEW

1. Introduction

Cardiovascular diseases (CVDs) remain the principal reason for loss of life globally and this includes several diseases such as ischaemic heart diseases, venous thromboembolism and stroke (Chauhan and Aeri, 2013). Global annual mortality rate due to CVDs is estimated to be between 17.5 to 17.7 million people (WHO, 2014). These disorders continue to be a public health concern, due to their common occurrence in most populations (Alwan, 2011). Furthermore, a high mortality rate, loss of independence, impaired quality of life and socio-economic costs are associated with these diseases (WHO, 2008). The prevention and epidemiology of these disorders require an understanding of their causes, identification of sustainable preventative means and the monitoring of high-risk populations (Alwan, 2011). There is evidence that indicates CVDs to be prevalent in low, middle and high-income countries (Mozaffarian *et al.*, 2016). CVDs vary widely in manifestations; however, the focus has been primarily on atherosclerosis and ischaemic heart diseases. The progression of these two conditions occurs as a result of longstanding diseases of the walls of arteries, especially in the heart and brain, and as a consequence of persistently high blood pressure (Labarthe, 2010). There are instances where both atherosclerosis and hypertension do coexist in certain individuals. Heart attacks and strokes are two manifestations of these disorders and are the major reasons for the health sector to place much emphasis on CVDs (Labarthe, 2010).

Several factors influence the development of CVDs, including blood lipids, blood pressure, obesity and diabetes (Morrish *et al*., 2001; Steinberg, 2011; Chen *et al*., 2013; Blacher *et al*., 2016). These risk factors are intermediate phenotypes, which are underpinned by inherent and environmental elements, such as nutrition, diet, hormones, smoking, alcohol intake and physical inactivity. Investigations conducted in both mice and human beings have revealed that the nature and the amount of lipids within the blood can predict the likelihood of CVD development (Reichl and Miller, 1989; Fielding and Fielding, 1995; Rader and Maugeais, 2000; Tall *et al*., 2000). High levels of total cholesterol, especially the presence of low-density lipoproteins (LDL) have been

found to have a positive and growing association with the development of ischemic heart disease (Trialists, 2005). A significant number of clinical trials have validated the lipid theory of atherosclerosis, which consistently links decreased total cholesterol to the reduction of coronary heart disease (CHD), stroke and CV mortality (Abifadel *et al*., 2003; Jacobson, 2013; Kassner *et al*., 2015).

There is a possibility that the high numbers of morbidity and mortality due to CVDs could be reduced through approaches that are more geared to spreading awareness. This strategy could prevent the development of CVDs in people that are known to be susceptible and at high risk of developing the disease (WHO, 2003; Manuel *et al*., 2006; Lopez *et al*., 2006). Some of the underlying factors that lead to the development of CVDs are gender and age, with relatively older men more prone than young women (Mendis *et al*., 2011). In some cases, an individual would develop CVDs as a result of many combined factors that coexist and act in a coordinated manner. There is a great need to initiate well-timed and sustainable lifestyle interventions which require drug interventions to reduce events such as heart attacks and strokes in people with a high total risk of CVDs, leading to reduced premature morbidity, mortality and disability (Mendis *et al*., 2011). A lot of people remain unaware of their risk status, due to the lack of information dissemination, however, testing for raised blood pressure, abnormal blood lipids and blood glucose is performed by many health care facilities (Tunstall-Pedoe *et al*., 2003). This information about an individual's risk status is critical in making clinical decisions on the intensity of preventive interventions. The risk assessment approach is particularly suitable in low or middle-class societies with limited resources, where saving the greatest number of lives at the lowest cost becomes imperative (WHO, 2002).

The transport, maintenance and elimination of plasma lipids, which include high and low-density lipoprotein cholesterol (HDL-C and LDL-C) require a well-coordinated effort from a considerable number of protein molecules (Trialists, 2005). Hepatic uptake via the LDL receptor (LDLR) is the first mechanism mainly employed for the removal of LDL particles from blood circulation. However, both environmental and genetic parameters play a role in the regulation of plasma concentrations of LDL-C. Secondly, mutations in the LDLR have been linked to familial hypercholesterolemia (FH), which is caused by mutations in the ligand-binding domain of

apolipoprotein (APOB-100). However, the mechanism driving the development of FH is less frequent as compared to the hepatic uptake (Marduel *et al*., 2010). The third mechanism is associated with the gene that encodes proprotein convertase subtilisin/Kexin type 9 (*PCSK9*) which has become an exciting therapeutic target for the treatment of hypercholesterolemia (Farnier, 2011).

The main function of the human *PCSK9* gene is the internal regulation of LDLR levels and it is associated with some FH events due to the identification of dominant gain-of-function (GOF) mutations in certain individuals (Abifadel *et al*., 2009). This gene is found in some organs, particularly the liver, small intestines and the kidney (Seidah and Prat, 2012). The *PCSK9* gene can either increase or decrease the ability to break down LDLR (Abifadel *et al*., 2003). Mutations that cause the loss of function minimize the proficiency of the transformed PCSK9 to breakdown LDLR, resulting in a greater amount of this molecule in the circulation and lowered levels of LDL-C in plasma (Dubuc *et al*., 2004; Cameron *et al*., 2006; Hochholzer *et al*., 2011). On the contrary, sequence variants that lead to the gene attaining its capacity to degrade LDLR are responsible for high levels of LDL found in circulation. The eventual outcome of these sequence variants is that the plasma levels of LDLR are reduced leading to subsequent visible increases in LDL-C in plasma (Cameron *et al*., 2006; Lagace *et al*., 2006; Hochholzer *et al*., 2011; Song and FitzGerald, 2013; van Capelleveen *et al*., 2016). The findings of these studies and the results obtained from PCSK9 inhibition investigations have encouraged researchers to look at LDLR as a possible drug treatment to lower LDL-C (Alwan *et al*., 2010). Therefore, techniques that are focused on inhibiting the function of PCSK9 are predicted to be harmless and have efficiency in treating CHD (Dubuc *et al*., 2004). Some PCSK9 inhibition strategies that have been investigated with regards to treating atherosclerosis will be dealt with further down in this thesis. The focus of this study was on atherosclerosis pathogenesis since it is the underlying pathology of CVDs, which progresses for years and is generally identified at an advanced stage, usually during the middle age when symptoms manifest (WHO, 2007; WHO, 2008; Alwan, 2011).

1.1 Background on atherosclerosis

Atherosclerosis refers to a disorder in which an artery wall thickens as the result of a build-up of fatty materials such as cholesterol (Fioranelli *et al*., 2018). This disease occurs as a result of inflamed arterial blood vessels, which is the result of the buildup of macrophages (Mota *et al*., 2001). The presence and the subsequent insufficient elimination of LDLs, fats and cholesterol from the macrophages by active HDL, is generally known as the strengthening or furring of the arteries and results in the formation of multiple plaques within the arteries (Tabas *et al.*, 2007; Libby *et al*., 2011). In most cases, this accrual is discovered after 20 years of life and develops further with age. The classification of atherosclerosis as being chronic or acute relies on important factors, ageing and early development. The development of vascular lesions leads to the decline of the flow of blood to the affected tissues (Homeister and Willis, 2010).

These lesions are generally found in many areas in and around the arteries but are predominantly inclined to take place at corners of vessels and intersections where blood flow is normally hindered by blood flow forces, which modify cell movement and replication in the vessel wall (Winkel *et al*., 2015). This hindered blood flow results in reduced blood transfer to coronary, carotid and cerebral arteries and the iliofemoral system, leading to the formation of several disorders such as myocardial ischaemia and infarction, stroke and peripheral vascular disease (Homeister and Willis, 2010). Developed countries are reported to have the highest number of affected individuals, however, the burden is also increasing in developing nations (Herrington *et al*., 2016). The American Heart Association published statistics which revealed that in 2013 more than 17 million fatalities worldwide occurred as a result of cardiovascular diseases (Mozaffarian *et al*., 2016). Cardiovascular diseases claimed 800,937 deaths in the United States, this accounted for about 31 % of all reported deaths in that year (Mozaffarian *et al*., 2016). Therefore, it is forecasted that in 10 years the annual medical budget allocated to deal with cardiovascular diseases would have escalated to $1,044 billion globally (Mozaffarian *et al*., 2016). The major cause of most of these reported deaths is atherosclerosis. The death rate of atherosclerosis-related diseases has declined in cases where better-quality medical care and the modification of known predisposition factors, in addition to other factors, have been applied. However, a substantial increase due to other CVD related diseases (heart failure) has been reported and continues to rise regardless of these interventions (Homeister and Willis, 2010). This might indicate that some individuals who were

at high risk of acute atherosclerosis can live a bit longer, but are incapacitated by the enduring consequences of the disease. The unfortunate scenario is that in developing nations there is an observed increase in atherosclerosis-related diseases, due to the consumption of high-fat foods (Homeister and Willis, 2010). The global death rate associated with ischaemic heart disorder is projected to double between 1990 and 2020 (Yusuf *et al*., 2001a, Yusuf *et al*., 2001b). There are a lot of studies focusing on atherosclerosis, however, this seems to have thus far not fully translated into reducing the number of affected people.

Changing the attitude of those at risk does prove to be effective, especially reducing cigarette smoking and lowering the intake of foods that have a high cholesterol content. However, the genetic background of a particular individual is one of the most powerful risk factors for developing atherosclerosis (Homeister and Willis, 2010). Unfortunately, the exact genetic mechanisms underlying the development of atherosclerosis have not been fully understood. One of the most researched genetic mechanisms is the role played by lipids in CVD development (Homeister and Willis, 2010). Lipids are said to be a pivotal cog in the development of lesions, even though the underlying mechanisms of the association of lipids and atherogenesis is yet to be fully understood. However, several studies have revealed that plasma lipids are associated with the risk of developing CVD (Steinberg, 2011). High LDL levels, in particular, do satisfy a mode of a cause of disease similar to that of Koch's postulates (Goldstein and Brown, 2009). A link can be drawn between high LDL levels and the risk of cardiovascular events in human populations. Consequently, single-gene diseases which are responsible for increasing plasma levels of LDL worsen the cardiovascular risk, and the mechanisms that are focused on reducing LDL levels are able to lower the risk of atherosclerosis (Libby *et al*., 2011). The identification of sequence variants in PCSK9 has been demonstrated to influence atherosclerosis and CHD development, and this has been hailed as a significant breakthrough in the clinical research of CVD (Cariou *et al*., 2011b).

Studies have presented a detailed analysis of the exact involvement of PCSK9 in the breaking down of LDLR. However, for the degradation of LDLR to occur, *PCSK9* gene needs to be first available (Cameron *et al*., 2006; Lagace *et al*., 2006; Holla *et al*., 2007). The binding of PCSK9 and LDLR is facilitated by the epidermal growth factor (EGF), which takes place at the EGF homology domain (Zhang *et al*., 2007). The process of endocytosis is used to transport PCSK9

bound to LDLR inside the cell by making use of clathrin-coated pits (Lagace *et al*., 2006; Nassoury *et al*., 2007; Qian *et al*., 2007). After this process, the PCSK9/LDLR compound moves to the sorting endosomes. The binding affinity of the complex increases from 150-170 folds at the acidic conditions of the endosomes as compared to the basic conditions of the cell surfaces (Cunningham *et al*., 2007a; Fisher *et al*., 2007). As a result of the tight-binding between PCSK9 and LDLR in the endosomes, LDLR is broken down in the lysosomes (Zhang *et al.*, 2007; Nassoury *et al.*, 2007; Qian *et al*., 2007). Therefore, the attachment of PCSK9 to the LDLR prevents the recycling of the endocytosed LDLR. Consequently, LDLR levels on the cell surface are lowered, resulting in the partial removal of LDL from plasma.

Currently, guidelines on CVD prevention and treatment recognize LDL-C lowering treatment as the primary therapy. These guidelines focus on treatment with statins, which are reported to have significant residual cardiovascular risk (Hochholzer *et al*., 2011). Statins are also known to elevate PCSK9 levels thereby resulting in LDLR degradation and high LDL levels (Dubuc *et al*., 2004). Although statins are currently the mainstay treatment to reduce LDL-C, many patients still have atherogenic cholesterol levels greater than the recommended values (Cohen *et al.*, 2012). Therefore, there is an increasing interest in developing more effective LDL-C-lowering drugs that might supplement statins. Niacin (nicotinic acid) is a lipid-altering drug that has been used to lower cholesterol (Song and FitzGerald, 2013). This agent can favourably influence the levels of HDL-C, LDL-C, and triglycerides (van Capelleveen *et al*., 2016). Niacin has been used in combination with other LDL-lowering drugs (statins and fibrates), however, the effect of such combined therapies on PCSK9 was observed to slightly decrease LDL-C (Khera *et al*., 2015a). Despite the positive effect of niacin in lowering LDL-C and increasing HDL-C, there are still limited studies reporting on the interaction of niacin as a primary drug with PCSK9. Even though reduction of LDL-C by inhibition of PCSK9 in NHPs has previously been demonstrated (Rashid *et al.*, 2005a; Graham *et al*., 2007; Palmer-Smith and Basak, 2010), the link between the gene, its sequence variants and niacin administration require further investigation. This study contributes to the knowledge regarding the effect of niacin in reducing LDL levels through PCSK9 interaction.

1.2. Lipid pathway/mechanisms

LDL-C lowering therapies have been a focus area for researchers who are exploring techniques to improve the health of patients at high risk of developing CVDs, and this is considered as an important endpoint for clinical events by the Food and Drug Administration (FDA) of America (Stone *et al*., 2014). Therefore, it is important to look closely at LDL-C metabolism to develop therapeutic approaches to treat diseases such as hyperlipidemia. Lipid metabolism starts with the liver releasing undeveloped very-low-density lipoprotein (VLDL) or budding VLDL (Figure 1.1). The latter is made up of apolipoprotein B-100 (APOB-100), apolipoprotein E (APOE), apolipoprotein C1 (APOC1), CE, cholesterol and triglycerides (Chaudhary *et al*., 2017). The maturation of immature VLDL occurs when HDL that is circulating in the blood offers apolipoprotein C-II (APOC-II). Capillary beds of adipose tissues, cardiac muscle and skeletal muscle cells become a base at which mature VLDL interacts with lipoprotein lipase (LPL) leading to the elimination of triglycerides from VLDL to be stored or to produce energy in these tissues (Chaudhary *et al*., 2017). The interaction of VLDL and HDL occurs once more, and a tradeoff ensues where APOC-Ⅱ is transported back to HDL alongside phospholipids and triglycerides in exchange for CETP.

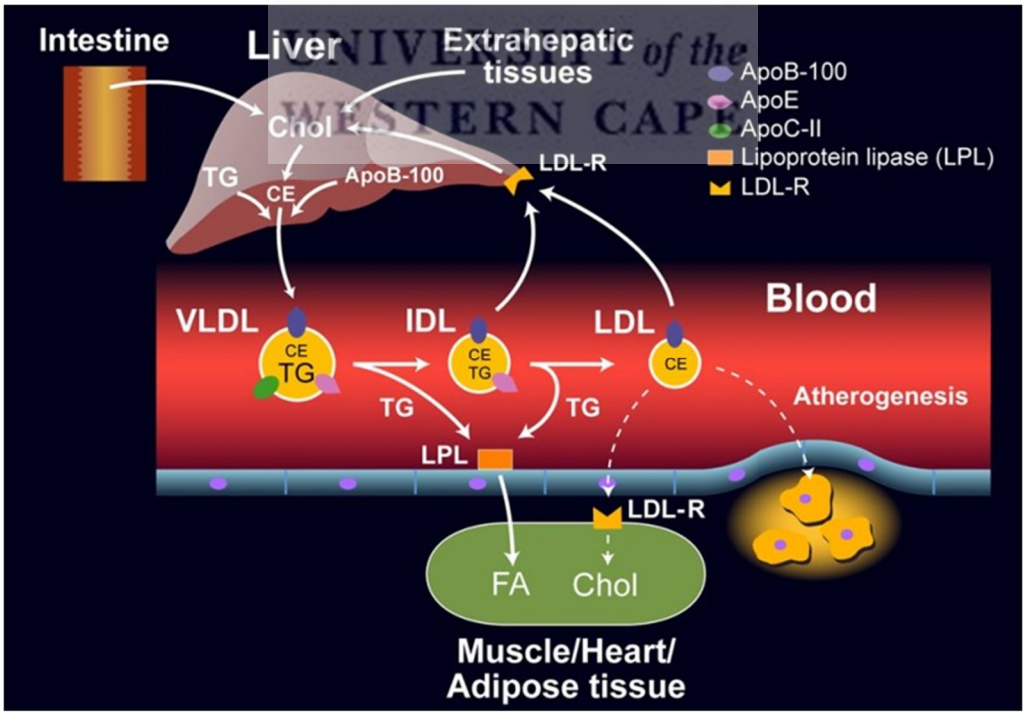


Figure 1.1: Endogenous Lipoprotein Pathway (Feingold and Grunfeld, 2018)

Furthermore, VLDL is converted to intermediate-density lipoprotein (IDL) as a result of the exchange and removal of triglycerides. The presence of APOB-100 and APOE on about half of IDLs makes them vulnerable to attack mounted by liver cells. As a survival mechanism, these IDLs release APOE leading to higher cholesterol concentrations in comparison to triglycerides, after which they change into LDL (Chaudhary *et al*., 2017). APOB-100 is the ligand contained in the formed LDL particles, linking them to LDL receptors (LDLR). The LDL/LDLR compound is kept inside the clathrin-coated vesicles by endocytosis. When this complex gets to the cytosol, it dissociates and LDLR particles are transported again to the cell surface. The process of recycling LDLR is continuous and each receptor has the task of recycling about 150 times following which they are discarded (Chan *et al*., 2009).

The function of statins is to inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGCR), the main compound in the synthesis of cholesterol (Stancu and Sima, 2001). Following this inhibition, intracellular cholesterol levels are decreased leading to the accumulation of LDLR in cell surfaces, which in turn captures LDLs in circulation (Stancu and Sima, 2001). Previous studies conducted after the discovery of PCSK9 demonstrated the importance of sterol regulatory element-binding protein-2 (SREBP-2) in the regulation of cholesterol metabolism. The SREBP-2 molecule is activated by low levels of intracellular cholesterol prompting the expression of *LDLR* (Abifadel *et al*., 2003; Denis *et al*., 2012). The increased levels of LDLR enhance the clearance of LDL from circulation (Abifadel *et al*., 2003; Denis *et al*., 2012). Concurrently, SREBP-2 can also encourage PCSK9 expression, and by so doing promote the breaking down of LDLR (Maxwell *et al*., 2003; Horton *et al*., 2003). Therefore, this harmonized interaction of SREBP-2 induces the production of both LDLR and PCSK9 with the end goal of controlling LDL levels (Maxwell *et al*., 2003; Horton *et al*., 2003). Based on this coordinated interplay, scientists were able to explore and develop substances to counter the actions of PCSK9. There is therefore the need to understand the role played by these four lipoproteins in the development of CVDs/atherosclerosis, which were the focus of this study.

1.2.1 The role of lipoproteins in CVD/ atherosclerosis

1.2.1.1 LDL levels and atherosclerosis

There is an established relationship between small dense LDL and atherosclerosis (Austin and Krauss, 1995). The LDL particle is known for possessing a substantial amount of cholesterol particles, with limited triglycerides. LDL contains a layer of hydrophilic phospholipids on the surface, free cholesterol and APOB-100 which is responsible for the packaging and stability of the particle (Tomkin and Owens, 2012). The main part of the LDL particle is made up of triglycerides coupled with the fatty acid tails of the phospholipid and esterified cholesterol. Similar to other proteins that are in constant movement, the molecule may pick up further molecules that are difficult to dissolve in water such as free fatty acids which might be bound with no strong affinity (Phillips *et al.*, 2005). One of the most important attachments to the LDL particle is that of lipoprotein lipase which enables it's pairing with the endothelial surface (Tomkin and Owens, 2012). The increase of lipoprotein lipase has been reported on diabetic LDL (Phillips *et al*., 2005). Gradient gel electrophoresis is often employed to distinguish between the two sizes of the LDL particle (pattern A and pattern B), the latter pattern is also named small dense LDL (Krauss, 1994). An association between the latter pattern and an increase in atherosclerosis has been reported, even though the exact modifications in the conformation of the LDL that lead to the amplified atherogenicity are not yet well understood (Tomkin and Owens, 2012).

Scientists have been debating about the existing reasons that associate small dense LDL with atherogenic risk for several years and the prominent feature in the discussion is Familial hypercholesterolaemia (FH). Familial hypercholesterolaemia is known to be responsible for the early formation of atherosclerosis, which is associated with bigger LDLs rather than small dense LDLs (P Mikhailidis *et al*., 2011). The increase in the size of LDL particles has been suggested to be responsible for the facilitation of oxidation and glycation, with both processes leading to antibody formation (Tomkin and Owens, 2012). The formed antibodies lead to an escalation in scavenger receptor/fc receptor uptake by the macrophage (Tomkin and Owens, 2012). The oxidation of the LDL particle relies on the oxidation of the protein and/or fatty acids (Tomkin and Owens, 2012). Polyunsaturated fatty acids are more prone to oxidation as opposed to monounsaturated fatty acids, resulting in particles that are rich in linoleic acid which is more vulnerable to oxidation than the ones rich in oleic acid (Tomkin and Owens, 2012). There is an

association between the increased number of free fatty acids in poorly-managed diabetes with a high number of fatty acids accompanying the LDL unit, this potentially results in more oxidation of the particle (Phillips *et al*., 2005; Chung *et al*., 2009).

1.2.1.2 HDL levels and atherosclerosis

Ultracentrifugation was the method first used to isolate HDLs (Rader and Hovingh, 2014). The ability to measure HDL-C was made possible by improvements in reactions that precipitate APOB from lipoproteins (Rader and Hovingh, 2014). This led to the development of major epidemiological studies that focused on the association between HDL-C concentrations and CHD (Rader and Hovingh, 2014). The Framingham Heart Study became the first group to publish convincing findings of the existing inverse relationship between HDL-C and coronary heart disease (Badimon and Vilahur, 2012). These findings paved the way for HDL to be broadly regarded as beneficial cholesterol, leading to the indication that HDL might play a role in preventing the development of CHD. Thus, medical therapies that elevate HDL-C levels could contribute to the reduction of CHD, and this is referred to as the HDL hypothesis (Rader and Hovingh, 2014). The reverse cholesterol transport system was discovered and it was projected that since this pathway is mediated by HDL, this leads to protection against CHD (Badimon and Vilahur, 2012). The notion that the HDL hypothesis is critical in preventing the development of atherosclerosis was reinforced by many *in vivo* investigations in the 1980s and 1990s. A study conducted by Badimon and colleagues injected HDL into rabbits and described the prevention of atherosclerosis (Badimon *et al*., 1990). It was also reported that mice producing more apolipoprotein A1 (APOA-I) which is the major HDL protein, gain protection against atherosclerosis (Rubin *et al.*, 1991). The aforementioned preclinical data correlated with the results from epidemiological studies and strongly supported the HDL hypothesis, thus making HDL one of the main targets for innovative treatment methods to minimize the chances of atherosclerosis development (Rader and Hovingh, 2014).

The synthesis of HDL takes place in the liver and intestine, where APOA-I is produced and stored (Rader and Hovingh, 2014). The APOA-I is secreted as a lipid-poor-protein that forms a connection with the cholesterol–phospholipid transporter ATP Binding Cassette A1 (ABCA1) (Figure 1.2). This transporter is contained by hepatocytes and enterocytes to obtain lipids to create

a nascent HDL particle (Parks *et al*., 2012). HDL further attains other lipids and apolipoproteins, which are retrieved by breaking down triglyceride-rich lipoproteins. The process that leads to the transitioning of nascent HDL to mature HDL particles is catalyzed through lecithin cholesteryl acyl transferase (LCAT) by acting on cholesterol in the naive form to create CE, the main component of the molecule (Rousset *et al*., 2009). Two metabolic pathways are utilized for the removal of CE in HDL. The first pathway occurs in the liver or steroidogenic tissues due to direct uptake by the HDL receptor scavenger receptor B1 (SR-BI) (Rader and Hovingh, 2014). CETP facilitates the second pathway, where cholesteryl ester is transferred to lipoproteins that contain APOB in exchange for triglycerides. The SR-BI mediated mechanism is carefully coordinated and after CE removal, the reduced size APOA-I comprising of HDL unit separates and is recycled (Hoekstra *et al*., 2010). Also, CETP does not only serve the function of removing CE from the HDL molecule; it is also used to add triglycerides to the HDL unit (Barth and Argraves, 2001).

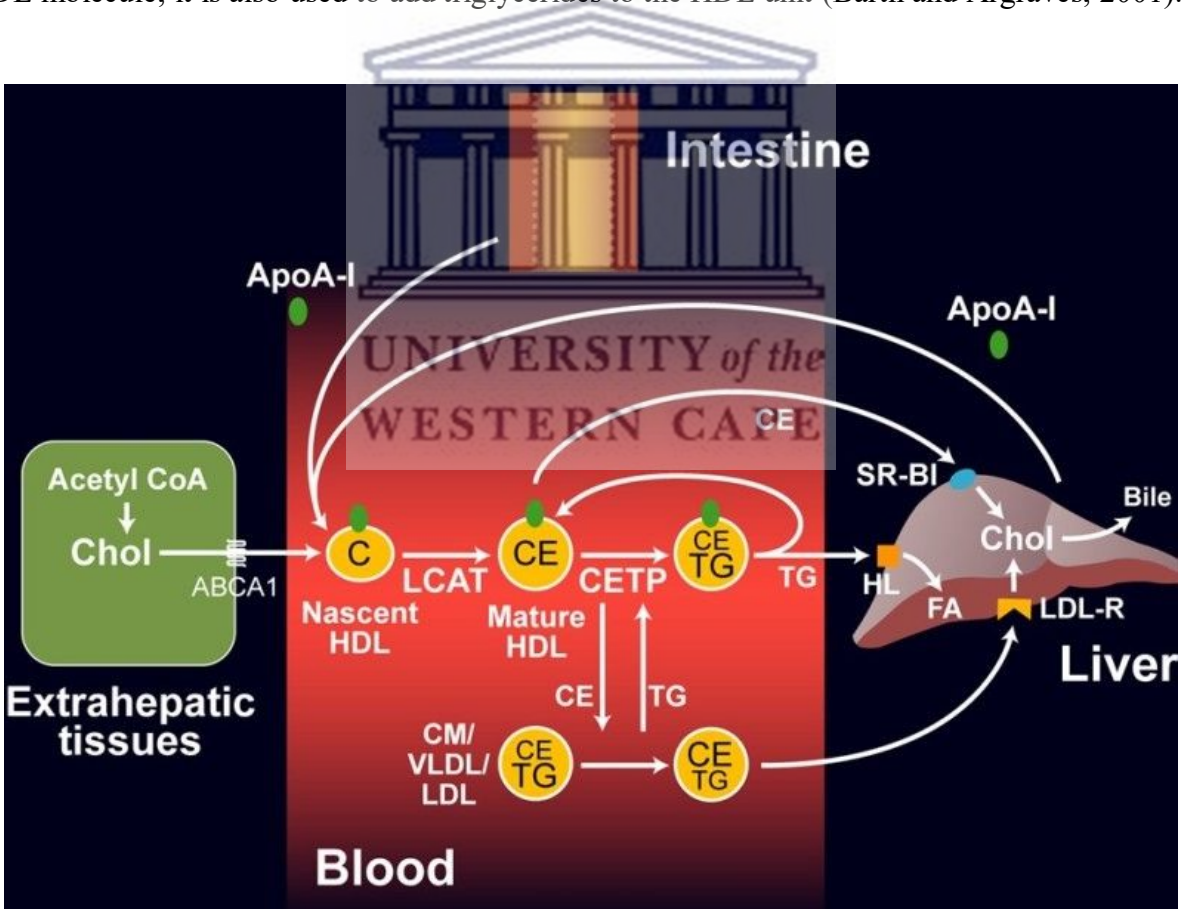


Figure 1.2: HDL metabolism and reverse cholesterol transport (Feingold and Grunfeld, 2018).

1.2.1.3 Triglycerides and atherosclerosis

The transportation of triglycerides to the liver and intestines is carried out by VLDL and chylo-microns, to be distributed to the peripheral tissues for energy requirements (Khetarpal and Rader, 2015). The hydrolysis of the triglyceride molecule results in the core containing triglyceride-rich lipoproteins, covered by the remaining VLDL and chylomicrons becoming moderately cholesterol-enriched (Handelsman and Shapiro, 2016). Triglycerides are not known to collect in the foam cells, therefore, their association with plasma triglycerides and atherosclerotic cardiovascular disease (ASCVD) may result from the remnants of lipoproteins (Handelsman and Shapiro, 2016). The residues are capable of collecting in the arterial endothelium, resulting in their uptake by macrophages. Once taken up they contribute to foam cell formation and eventually, fatty streak formation resulting in plaque progression (Talayero and Sacks, 2011; Khetarpal and Rader, 2015). These fragments exhibit distinctive features in comparison to LDL particles, especially since they do not require oxidative modification for their uptake by arterial macrophages and are also linked to significant inflammation (Varbo *et al*., 2013b). Hypertriglyceridemia is also characterized by greater concentrations of small dense LDL particles, minor HDL particle, APOA-I concentrations, and larger concentrations of APOC-III–containing particles (Miller, 2015). When changes are made to the structural makeup of these lipoprotein particle subclasses, there is the likelihood that atherosclerotic processes could be fast-tracked. Consequently, lipoprotein particles that possess a larger triglyceride content may be oxidized more freely, thus increasing their atherogenic potential (Handelsman and Shapiro, 2016). Also, lipoprotein lipase facilitates the hydrolysis of traces of triglycerides at the endothelial cell surface and within the sub-endothelial space and this produces pro-inflammatory mediators, including free fatty acids (Saraswathi and Hasty, 2006; Varbo *et al*., 2013b).

1.2.1.4 Total cholesterol and atherosclerosis

Remnant cholesterol refers to cholesterol comprised mainly of triglyceride-rich lipoproteins (Nordestgaard and Varbo, 2014). Numerous approaches that measure residues in general and cholesterol residues specifically do exist. Since lipoprotein remnants are diverse both in the conformation of lipids and apolipoproteins as a result of different stages of metabolism, an assay that measures all remnants at the same time remains elusive (Scriver, 2001; Chapman *et al.*, 2011). Remnant cholesterol can, however, be collated as non-fasting total cholesterol without HDL-C and

LDL-C (Nordestgaard *et al*., 2007; Jørgensen *et al*., 2012; Varbo *et al*., 2013a; Varbo *et al*., 2013b). This is beneficial since cholesterol can be calculated from a standard non-fasting lipid profile at no additional cost. Increased levels of the calculated remnant cholesterol have been associated with increased risks of cardiovascular disease after being confirmed by ultracentrifugation methods (Jørgensen *et al*., 2012; Varbo *et al*., 2013a; Varbo *et al*., 2013b; Varbo *et al*., 2014).

1.3 Cholesterol-lowering drug intervention

1.3.1 Statins

The development of coronary atherosclerosis results from a multifaceted interaction between metabolic and inflammatory processes (Libby *et al*., 2014). Studies that focus on mechanistic and genetic evidence reveal that APOB containing lipoprotein, especially LDL-C, is the underlying factor for atherogenesis (Ference *et al*., 2015). Therefore, the main function of statins is to decrease the synthesis of cholesterol and reduce plasma LDL-C and triglycerides levels (Istvan and Deisenhofer, 2001). Statins obstruct the function of HMGCR in hepatocytes (Figure 1.3). HMGCR is a critical biocatalyst required in the liver for the synthesis of cholesterol and converts HMG-CoA to mevalonic acid, the first substance made in the cholesterol pathway (Davies *et al.*, 2016). The enzyme, HMG-CoA, competes with statins for the binding site within the reductase. After the binding of statins with the active site of the enzyme, the shape and function of the enzyme are changed. Statins act as competitive antagonists that have a greater affinity for the binding position of the enzyme, this prevents the lower affinity endogenous substrate HMG-CoA from binding (Davies *et al.*, 2016). Lovastatin was the first commercially available statin medication approved by the United States Food and Drug Administration (FDA) in 1987 (Endo, 2010). Since then, statins usage has been able to primarily prevent CVDs by reducing the risk and inhibiting the onset of the disease (Hobbs, 2004).

Furthermore, the ability of statins to effectively slow disease progression and lessen cardiovascular associated illnesses and deaths has made it to be regularly used as a secondary drug. The American College of Cardiology (ACC) and the American Heart Association (AHA) in 2013 made amendments to the existing guidelines to reduce the risk of CVDs (Stone *et al*., 2014). Currently, there are numerous types of statins prescribed worldwide which are available in the market

including atorvastatin (Lipitor and Torvast), simvastatin (Zocor and Lipex), lovastatin (Mevacor, Altocor and Altoprev), pitavastatin (Livalo and Pitava), rosuvastatin (Crestor), fluvastatin (Lescol), and pravastatin (Pravachol, Lipostat and Selektine). However, each of these statins has a different effect on lipid profile (Herman, 1999; Harper and Jacobson, 2007; Sewright *et al*., 2007; Di Stasi *et al*., 2010). As much as statin treatment has been effective in lowering LDL-C levels in the majority of individuals, there are reported cases of drug resistance or proneness to develop intolerance (Reiner, 2014). Therefore, this situation warrants the development of new scientific methods aimed at discovering efficient treatment alternatives to statins in individuals where statin therapy is ineffective.

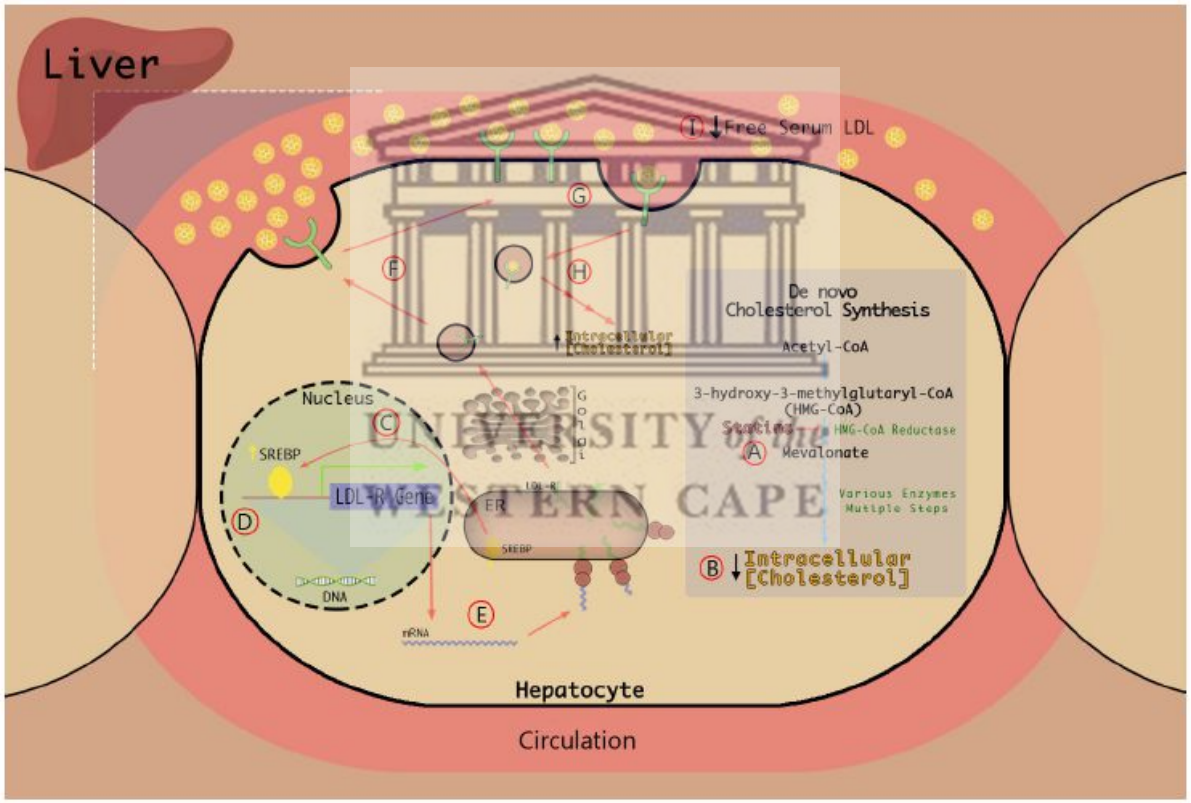


Figure 1.3: Depicts different stages of the statin mechanism of action. (**A**) The catalytic nature of HMG-CoA reductase is mitigated by statins. (**B**) Cholesterol production is reduced Intracellularly. (**C**) SREBP is split and transferred to different parts. (**D**) The *LDLR* gene expression is stimulated by SREBP. (**E**) The transcription and translation of the *LDLR* gene start. (**F**) LDLR concentration is increased on the cell surface. (**G**) LDL binding, endocytosis, and subsequent degradation. (**H**) Intracellular cholesterol increase toward cellular baseline. (**I**) Plasma LDL levels decrease (Davies *et al* 2016).

1.3.2 Niacin

Nicotinic acid and nicotinamide (collectively termed niacin) are precursors of co-enzymes nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate (NAD+ and NADP+) and are water-soluble vitamins of the vitamin B complex (Trapani *et al*., 2013). Niacin is the first drug to lower cholesterol levels in normal subjects as well as in patients with hypercholesterolemia (Sakai *et al.*, 2001). Niacin has proven to be an efficient, distinctive treatment reducing total plasma cholesterol, APOB, triglycerides, VLDL, and LDL while elevating HDL levels (Carlson, 2005; Kamanna, 2008). Several clinical studies have shown that niacin treatment meaningfully lowered the total death rate, coronary events, and prompted the regression of coronary atherosclerosis (Trapani *et al*., 2013). The discovery of human hepatoblastoma cells (Hep G2), enabled researchers to gain advanced theoretical knowledge about the modus operandi of niacin in the modulation of lipoprotein metabolic processes (Kamanna and Kashyap, 2000).

The inhibition of triglyceride biosynthesis is mediated by niacin, this is achieved by hindering the movement of APOB through the endoplasmic reticulum (ER) membrane, resulting in decreased APOB secretion (Kamanna, 2008). These findings supported existing knowledge about the niacin-induced reduction of VLDL-triglyceride transport and VLDL levels, resulting in the production of additional circulating LDL molecules in individuals that have high cholesterol (Kamanna and Kashyap, 2000). Further investigations relating to APOAI and HDL metabolic reactions revealed that APOA-I is increased by niacin action, leading to the reduced hepatocellular upward movement of HDL-APOAI; these events, however, do not inhibit HDL-cholesteryl ester movement (Kamanna and Kashyap, 2000). Studies conducted in the past decade reported that niacin mediated events hinder the hepatic movement of HDL compounds comprising only APOA-I (LPA-I) more than HDL particles made-up of APOA-I and APOA-II (LPA-I+AII) particles. These novel mechanisms result in increased APOA-I concentrations thus, improving the effectiveness of reverse cholesterol transport. Research more geared at uncovering underlying mechanisms may not only lead to new drug discovery but could be a blueprint for combined treatment utilizing compounds with varying mechanisms of action to decrease VLDL/LDL and increase HDL levels (Kamanna and Kashyap, 2000; Kamanna, 2008).

1.3.2.1 Side effects of niacin

Niacin is known to have some unwanted effects. These effects are usually non-life-threatening, but they can affect patient compliance. The most common adverse effect in persons treated with niacin seems to be skin flushing since approximately 70 % of persons receiving niacin suffer from this symptom (Birjmohun *et al*., 2005). Hepatotoxicity has also been reported, this condition usually leads to increased levels of hepatic enzymes called transaminase (Segatto et al., 2011) Other patients do report nausea and abdominal pain when treated with nicotinic acid (Carlson, 2006). Some of them can be alleviated and there is continuing interest in niacin mimetics that could circumvent metabolic pathways causing these effects (Goel and Dunbar, 2016). However, between 50 mg and100 mg has been reported not to elicit such severe side effects (Gille et al., 2008, Chauke et al., 2014). It was therefore not anticipated that any animals would suffer these side effects during the study.

1.3.2.2 Mechanisms of action of niacin

Two mechanisms are largely utilized by niacin that can influence plasma lipids and the storing of lipoproteins such as VLDL particles that contain APOB in the liver. These mechanisms are involved in the modulation of triglyceride lipolysis in adipose tissue, and triglyceride synthesis resulting in increased intracellular APOB degradation (Figure. 1.4) (Ganji *et al.*, 2003).

1.3.2.2.1 Modulation of triglyceride lipolysis in adipose tissue

One of the approaches that are used to minimize high levels of lipids such as triglycerides in blood could be the prevention of lipolysis in adipose tissue (Ganji *et al.*, 2003). Triglycerides are synthesized and stored in a specifically conducive environment, which is adipose tissues and for their subsequent shipping to the liver as energy-carrying compounds in the form of free fatty acids and glycerol (Ganji *et al*., 2003). Triglycerides are specifically constructed to be able to store metabolic energy in adipose tissue (Ganji *et al*., 2003). As depicted in Figure 1.4, niacin can minimize the movement of fatty acids from adipose tissue by stopping triglycerides lipolysis (Wu and Zhao, 2009). This process is essentially managed by cyclic adenosine monophosphate (c-AMP) mediated activation of hormone-sensitive lipase. Niacin blocks adenylate cyclase activity in adipocytes leading to reduced concentrations of c-AMP, thereby inhibiting triglycerides lipolysis (Ganji *et al*., 2004; Bodor and Offermanns, 2008). Furthermore, niacin also inhibits

forskolin-induced c-AMP production in rat adipocytes (Kang *et al*., 2011). The mechanisms that are used for the synthesis of c-AMP are usually controlled by G-protein receptor-dependent or independent mechanisms, niacin is adept at employing a number of these mechanisms in stopping the breakdown of triglycerides in adipose tissue (Ganji *et al*., 2003). Even though the binding of niacin to some G-protein coupled receptors in adipocytes and spleen has been described, the depiction of the exact receptor for niacin or the involvement of this receptor in niacin-facilitated adipocyte triglyceride lipolysis is still vague (Ganji *et al*., 2003).

1.3.2.2.2 Modulation of triglyceride synthesis and secretion of VLDL particles by the liver

APO and its accompanying lipids such as VLDL and Lp (a) particles are primarily synthesized and stored in the liver (Ganji *et al*., 2003). Though the intestine also produces lipoproteins of comparable size and lipid conformation, these, however, contain shortened APOB with lower molecular weight (Kozyraki *et al*., 1999). In studies where niacin treatment was administered to hyperlipidemic patients, a significant decrease in triglycerides by 52% and in VLDL by 36% was achieved (Ganji *et al*., 2003). It has also been suggested that a decrease in triglyceride content of VLDL results in lowered triglyceride content (Ganji *et al*., 2003). The decline of the circulating cholesterol did not result in modifications in faecal excretion of cholesterol or bile acids (Jin *et al*., 1999). However, it was noticed that niacin may have induced a minimal upturn in the hepatic secretion of biliary cholesterol that may be the reason the body losses cholesterol, possibly characterizing improved reverse cholesterol transport (Natarajan *et al.*, 2010). Niacin reduces plasma cholesterol by mainly lowering LDL, and this is an important action in the eventual niacin-mediated plasma cholesterol reduction (Ganji *et al*., 2003). The shortcomings of these investigations conducted on living organisms are that they were unable to reveal the cellular and molecular targets of niacin's effect. These drawbacks necessitated the use of human hepatocyte cell line (Hep G2 cells), as an experimental tool to uncover the specific mechanisms of action of niacin on VLDL/LDL metabolism (Ganji *et al*., 2003). Subsequent studies have used these cells at length in studying hepatocellular lipoprotein metabolism. The results were that the breaking down of intracellular APOB was amplified by niacin and the secretion of APOB particles was also decreased (Jin *et al*., 1999).

1.3.2.2.3 Role of niacin in HDL metabolism

Even though niacin has been able to increase HDLs for decades, the mechanisms of action that control this reported increase are only beginning to come to the fore (Ganji *et al.*, 2003). Early plasma turnover studies in humans indicated that niacin primarily decreased the fractional catabolic rate of APOAI without altering APOAI synthetic rates (Kamanna and Kashyap, 2000; Martinez *et al.*, 2003). The Hep G2 cells were also used in various studies to determine the involvement of niacin in the modulation of several phases included in APOAI metabolism, which involves APOAI biosynthesis and storage, upward movement of HDL particles, and the characteristics of the stored molecules to effectively release cholesterol particles within the cells (Kamanna and Kashyap, 2000).

These studies discovered that niacin treatment was able to increase APOAI buildup in Hep G2 cell culture media. Nevertheless, the treatment did not affect the production of APOAI and the mRNA expression of APOAI (Kamanna and Kashyap, 2000). Therefore, it is thought that this drug might play a role in the elimination or re-uptake of HDL by liver cells. The use of radiolabeling was able to reveal that niacin specifically prevented the accumulation of HDL-APOAI whereas HDL-cholesterol ester buildup was allowed (Kamanna, 2008). Based on these findings, niacin therapy is postulated to cause an increase in the ability of the remaining APOAI molecules to release cholesterol and amplify reverse cholesterol transport pathway (Figure 1.4). As a result of these experiments, it was suggested that the elimination of HDL-APOAI is inhibited by niacin using mechanisms that not fully understood, however, SR-B1-facilitated actions, which are selective to HDL-cholesterol esters were not hindered (Kamanna and Kashyap, 2000).

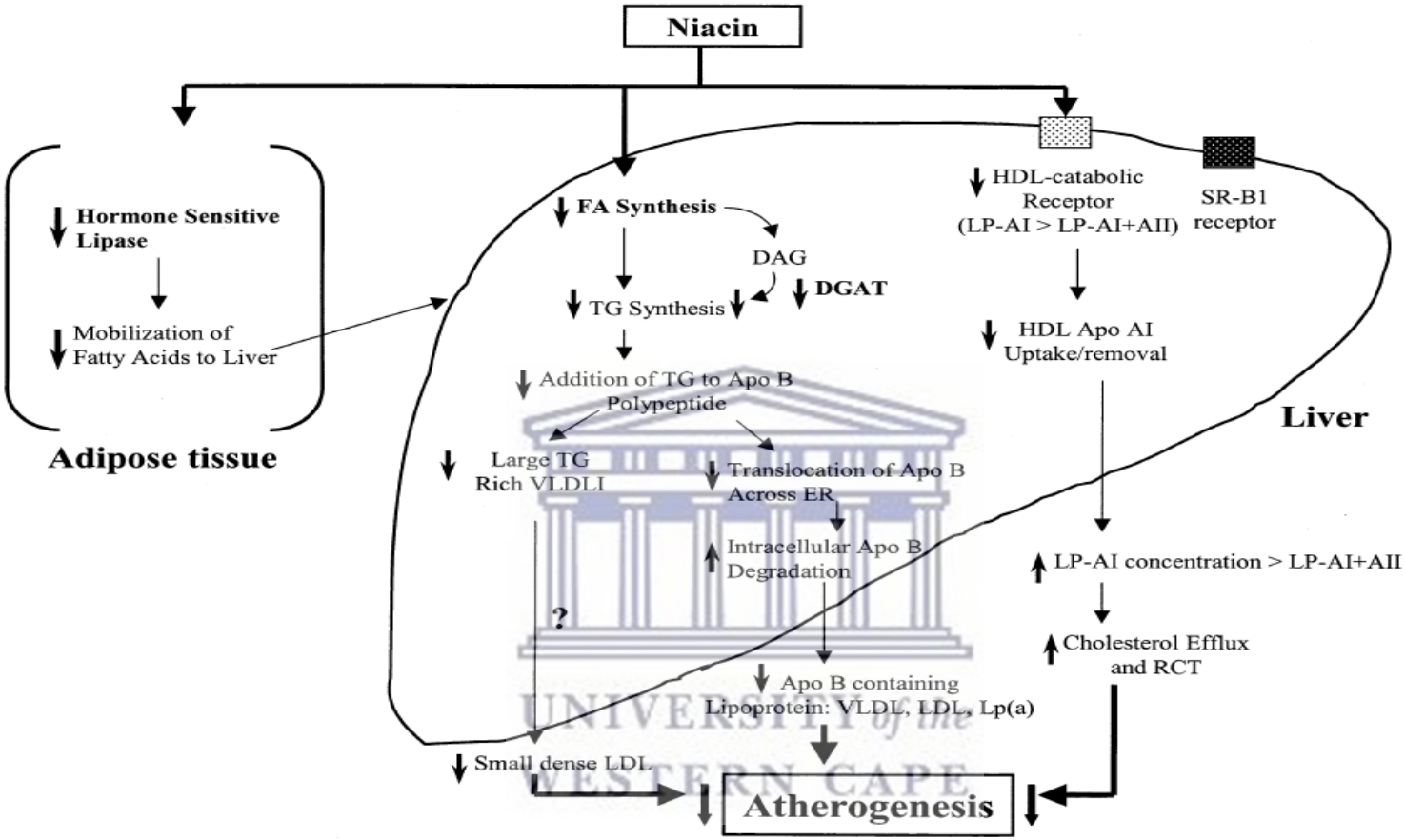


Figure 1.4: Outline of modern theories on niacin modus operandi against the metabolism of lipid and lipoprotein. Niacin can inhibit fatty acid recruitment from adipose tissue by using lipase-facilitated hormone-sensitive actions (Ganji *et al* 2003).

1.4 Gene activities in atherosclerosis /CVDs

1.4.1 Involvement of PCSK9 in CVD

The human *PCSK9* gene is found in chromosome 1p32.3 and it consists of 692 amino acids (Figure 1. 5). This gene is found in several organs, particularly the liver, small intestines and the kidney (Seidah and Prat, 2012). PCSK9 is biosynthesized as a soluble zymogen molecule which is autocatalytically cleaved in the endoplasmic reticulum (ER) (Seidah *et al*., 2003; Maxwell *et al*., 2003; Naureckiene *et al*., 2003). After it is cleaved it becomes two molecules, the prodomain which remains non-covalently bound to the complete PCSK9 molecule and therefore functions as a shuttle to help with the packaging of the complete PCSK9 molecule. To enable the exit of the PCSK9 molecule from the ER its folding is necessary (Seidah *et al*., 2003). The prodomain is also known for blocking the active spot of the mature PCSK9 (Seidah *et al*., 2003). The core role of PCSK9 in humans is suggested to be post-transcriptionally regulate the number of cell surface LDL receptors thereby regulating the levels of plasma LDL-C (Maxwell and Breslow, 2004; Maxwell *et al.*, 2005). The PCSK9 molecule is recognized as the main controller for LDLR activity and a viable target for the treatment of hypercholesterolemia (Seidah, 2009; Farnier, 2011; Rhainds *et al*., 2012; Seidah and Prat, 2012).

Recent studies have given a detailed analysis of the exact modus operandi used by PCSK9 in the breaking down of the LDLR. However, PCSK9 has to be secreted first for it to be able to degrade LDLR (Cameron *et al*., 2006; Lagace *et al*., 2006; Holla *et al*., 2007). In human plasma, the circulating PCSK9 must bind to the epidermal growth factor (EGF) like repeat A in the EGF homoly domain of the cell surface LDLR (Zhang *et al*., 2007). The process of endocytosis is used to transport PCSK9 bound to LDLR inside the cell by making use of clathrin-coated pits (Lagace *et al*., 2006; Nassoury *et al*., 2007; Qian *et al*., 2007). After endocytosis, the PCSK9/LDLR complex moves to the sorting endosomes. The binding affinity of the complex increases from 150-170 folds at the acidic conditions of the endosomes as compared to the basic conditions of the cell surfaces. The tight binding between PCSK9 and LDLR in the endosomes leads to the LDLR to be broken down in the lysosomes (Zhang *et al*., 2007; Nassoury *et al*., 2007; Qian *et al.*, 2007). Therefore, the tight binding between PCSK9 and LDLR leads to the shortage of LDLR molecules in circulation. Consequently, the rate at which LDL particles are removed from the liver cells and in circulation is significantly hampered.

Furthermore, PCSK9 mutations are reported to either increase or decrease the proficiency to break down LDLR. However, sequence variants that cause the loss of function to minimize the ability of the modified PCSK9 to break down the LDLR, resulting in a greater quantity of this molecule in the cell surface and lowered LDL-Cs in plasma (Zhao *et al*., 2006; Cameron *et al*., 2006; Fisher *et al*., 2007). On the other hand, mutations that enhance the function of PCSK9 are responsible for the degradation of LDLR. This results in reduced levels of LDLR on the cell surfaces and a higher numbers of LDL-C in plasma (Cameron *et al*., 2006; Lagace *et al*., 2006; Fisher *et al*., 2007; McNutt *et al*., 2007; Cunningham *et al*., 2007a). Additionally, two gain of function (GOF) mutations were identified in two French families that presented clinical signs of ADH, however, there were no detectable mutations in *LDLR* and *APOB-100* genes (Seidah *et al*., 2003). The results of the above-mentioned studies have led to researchers believing that the exploitation of *PCSK9* gene could lead to the discovery of drug compounds specifically aimed at reducing plasma levels of LDL-C (Benjannet *et al*., 2004; Rashid *et al*., 2005a). Therefore, inhibition of *PCSK9* should be safe and utilized as an effective strategy for the primary prevention of CHD (Zhao *et al*., 2006).

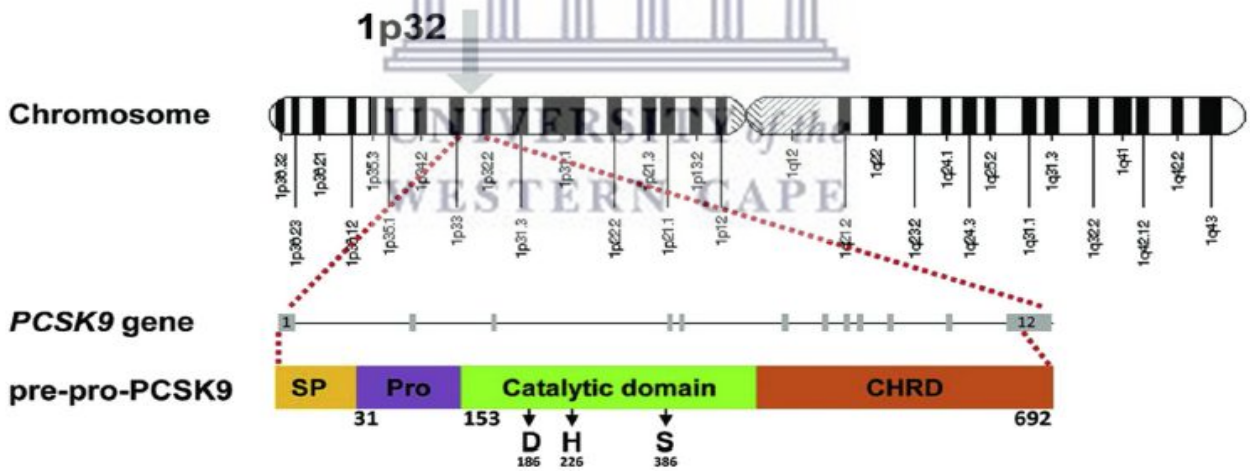


Figure 1.5: The *PCSK9* gene is located on the short arm of chromosome 1p32 and has 12 exons. The mature form of PCSK9 consists of an SP, a PRO, and a catalytic domain and a CHRD in the C-terminal domain. CHRD, cysteine and histidine-rich domain; PRO, pro-segment; SP, signal peptide (Awan *et al*, 2014)

Several inhibition techniques are currently being either used or tested on a variety of models. Hepatic uptake through LDLR is the first mechanism that is mainly used for the removal of LDL particles from blood circulation. However, both environmental and genetic parameters play a role in the regulation of plasma concentration of LDL-C. Secondly, mutations in the *LDLR* have been linked with FH, which is caused by sequence variants in the binding region of APOB-100. However, the formation of the mutations that drive the development of FH is less frequent as compared to the hepatic uptake (Marduel *et al.*, 2010). The third mechanism is associated with *PCSK9* gene which has become an attractive therapeutic target for the treatment of hypercholesterolemia (Farnier, 2011). Currently, guidelines on LDL-C lowering treatment are recognized as the primary therapy of CVDs. The focus of these guidelines is on the treatment with statins, which are reported to have a significant residual cardiovascular risk (Trialists, 2005). Statins are also known to elevate PCSK9 levels thereby resulting in LDLR degradation and elevated LDL levels (Dubuc *et al.*, 2004). Although statins are currently the mainstay treatment to reduce LDL-C, many patients still have atherogenic cholesterol levels greater than the recommended values (Cohen *et al.*, 2012). Therefore, there is an increasing interest in developing more effective LDL-C-lowering drugs that might supplement statins. These approaches are further discussed below.

1.4.1.1 *PCSK9* inhibition – evidence from animal studies

Animal and *in vitro* investigations that are aimed at discovering techniques to block PCSK9 functions, revealed that the use of drugs that act against lipids in addition to the inhibition may provide encouraging results (Cariou *et al.*, 2011a). The inhibition of the expression of *PCSK9* using antisense oligonucleotides (ASOs) (Mayer *et al.*, 2008), locked nucleic acids (Gupta *et al.*, 2010), and RNA interference (RNAi) (Frank-Kamenetsky et al., 2008), or inhibition of *PCSK9* activity on LDLR using neutralizing antibodies (Chan *et al.*, 2009) was tested successfully in *in vivo* models, such as rodents and NHPs. Positive findings regarding LDL-C lowering have also been reported from a study that tested PCSK9 monoclonal antibody (mAb) in humans (Stein *et al.*, 2012). The encouraging animal study results showed a decrease in plasma PCSK9 and LDL-C levels, leading to the manufacture of two antisense oligonucleotides (SPC5001 and BMS-844421, which were tested in phase I clinical trials (Burke *et al.*, 2017). However, these compounds were abruptly discontinued under a cloud of secrecy (Burke *et al.*, 2017).

1.4.1.2 Specific antibodies directly against PCSK9

An anti-PCSK9 antibody was created by a group of researchers, named mAb1;this antibody was able to bind and neutralize the epitope on PCSK9 neighbouring the area essential for LDLR contact (Chan *et al*., 2009). Laboratory experiments testing the efficiency of mAb1 revealed that PSCK9 interaction with LDLR was obstructed and PCSK9-facilitated decline in LDLRs was weakened, leading to an improved LDL degradation (Chan *et al*., 2009). Additionally, LDL-C was lowered in cynomolgus monkeys when the humanized murine mAb was used to bind to PCSK9 and this blocked its ability to bind to the LDLR (Chan *et al*., 2009). When one dose of mAb1 was administered via intravenous (i.v.) injection, a reported decrease of about 80% of LDLs was observed; however, this lasted for 14 days (Chan *et al*., 2009). A different group manufactured another antibody that also attaches itself to the binding area of PCSK9, thereby preventing the binding with LDLR and has the structure that resembles the LDLR EGF-A (Ni *et al*., 2010; Cariou *et al*., 2011b). The J16 humanized monoclonal antibody was specially synthesized to block the interaction between PCSK9 and LDLR (Liang *et al*., 2012). This antibody and its mouse prototype J10 are specifically constructed to block the binding of the extracellular domain of LDLR to PCSK9 and also prevent the *in vitro* down-regulation of LDLR which is PCSK9 controlled (Liang *et al*., 2012). Serum triglycerides were effectively reduced in C57BL/6 mice after J10 administration (Liang *et al*., 2012). Additionally, when 10 mg/kg of J10 intraperitoneal injection was administered to *C57BL/6* mice who are on a normal diet, cholesterol levels were decreased by 46% at day 7 post-treatment compared to the controls (Liang *et al*., 2012). A dose of 10 mg/kg of J16 administered to healthy and hypercholesterolemic cynomolgus monkeys resulted in approximately 39% LDL-C reduction in both groups without having a significant effect on HDL-C levels (Liang *et al*., 2012). Therefore, these reported antibody findings were aligned to the notion that antibodies acting against PCSK9 can be considered for the treatment of hypercholesterolemia.

1.4.1.3 Inhibition of PCSK9 by using ASO

The use of antisense oligonucleotides as therapeutic drugs against PCSK9 action has a potential to be successful based on its modus operandi which is associated with base-pair hybridization (Figure 1. 6 (1)) (Gouni-Berthold and K Berthold, 2011). The use of non-coding DNA and small interfering RNA initiate a process that encourages the breaking down of the mRNA of PCSK9. It is for this reason that the two approaches result in silencing the *PCSK9* gene (Gouni-Berthold and K Berthold, 2011). In mice, PCSK9 shortage results in a 40 to 50% decline in plasma total cholesterol and a 3-fold rise in hepatic LDLR (Park *et al*., 2004; Rashid *et al*., 2005; Zaid *et al*., 2008; Davignon *et al*., 2010). It has been reported that the administration of ASOs via subcutaneous injections in a mice model produces a decline of 90% in PCSK9 mRNA, 38% in LDL-C and 53% in total cholesterol (Graham *et al*., 2007). The study was significant since it was the initial one where injectable ASOs were reported to induce a decrease in PCSK9 levels (Davignon *et al*., 2010). Additionally, ASOs with reduced size due to the use of locked nucleic acid (LNA) technology are reported to be safe and effective in mice and NHPs and pose a nullifying outcome which persists for three weeks with two injections per week (Davignon *et al*., 2010; Gupta *et al*., 2010). To confirm the efficiency of using the LNA mechanism, a group of researchers made use of two LNA antisense oligonucleotides targeting PCSK9. These two LNA compounds comprised of a once-off loading dose (20 mg/kg) and a maintenance dose (5 mg/kg) that was administered weekly. The dosages managed to result in a persistent decline of LDL-C in NHP with mRNA and plasma PCSK9 protein being reduced by 85%, which caused a 50% decrease in circulating LDL-C (Lindholm *et al*., 2012). There was also a reduction in total cholesterol and no decrease in HDLs. Furthermore, the NHPs were able to tolerate the drugs and did not show any signs of illness (Lindholm *et al*., 2012). Antisense inhibition of PCSK9 is proven to be effective in lowering PCSK9 appearance and cholesterol levels in mice fed a normal diet and hyperlipidemic mice, and reduced liver triglycerides in hyperlipidemic mice (Graham *et al*., 2007; Gupta *et al*., 2010; Cariou *et al*., 2011).

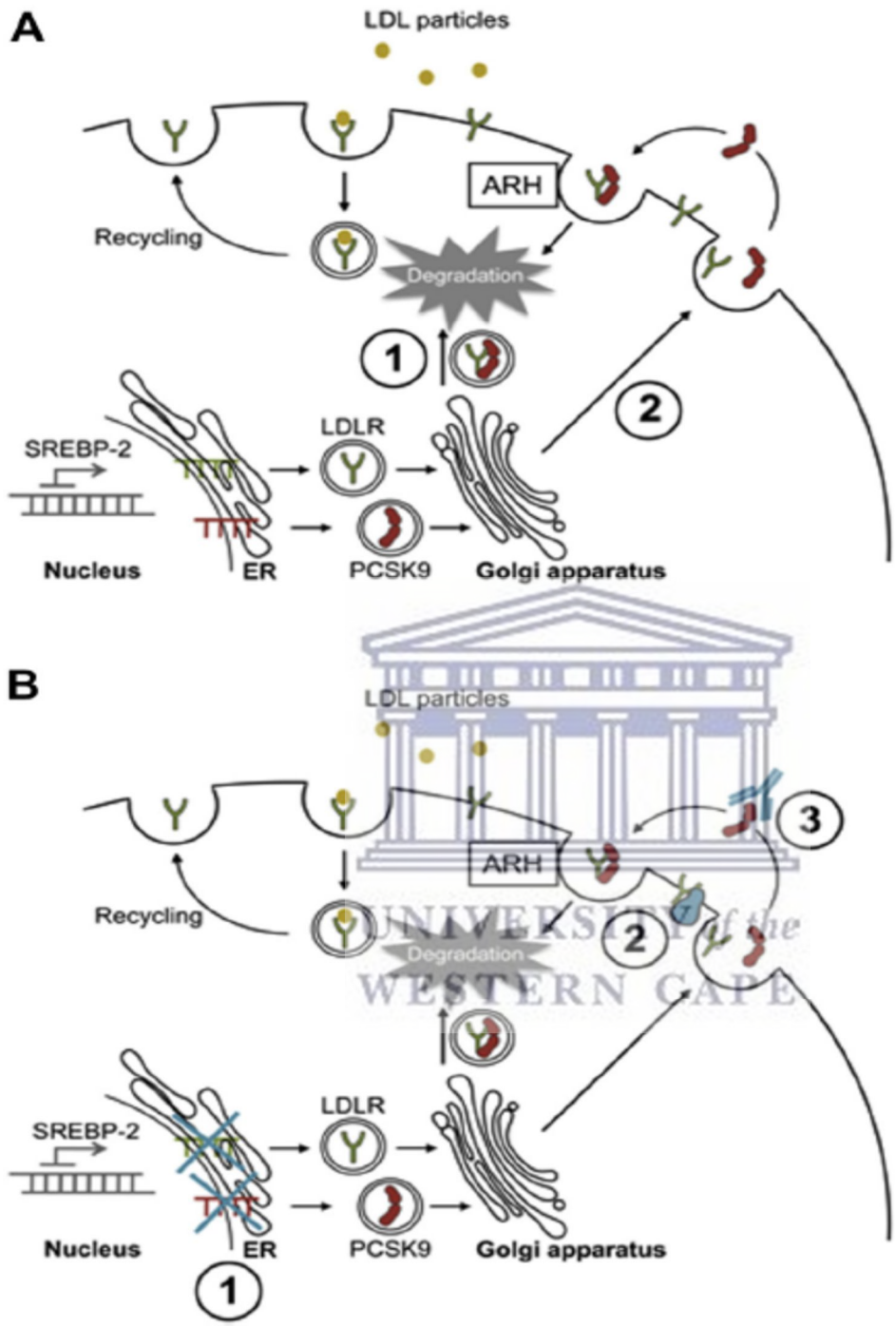


Figure 1.6: The illustration of the functions of PCSK9 and treatment approaches focusing on PCSK9. (**A**) SREBP-2 can induce simultaneous expression of *PCSK9* and *LDLR*. (**B**) Drug development tactics directed at PCSK9 biosynthesis or role are being developed (Urban *et al* 2013).

1.4.1.4 Inhibition of PCSK9 by using siRNA

The inhibition of PCSK9 by making use of small interfering RNAs (siRNAs) was carried out in some species such as the murine, rat and NHP (Frank-Kamenetsky *et al*., 2008). Both PCSK9 and control siRNAs were conveyed in a lipidoid nanoparticle (LNP) for *in vivo* studies. The siRNA induction in mice and rats resulted in the silencing of PCSK9 mRNA levels by 50-70%, which is associated with up to 60% reduction in plasma total cholesterol concentrations (Frank-Kamenetsky *et al*., 2008). In the NHP study, one dose of 5 mg/kg of LNP-PCS-A2 or LNP-PCS-B2 accounted for a substantial decline in LDL-C. Additionally, this investigation discovered that a decrease in LDL-C in the experimental group was aligned with the tendency to lower the quantity of plasma APOB (Frank-Kamenetsky *et al.*, 2008). The targeting of PCSK9 mediated by siRNA accounts for the prompt, sturdy and rescindable dropping of plasma PCSK9, APOB and LDL-C, with no significant changes in both HDL-C and triglycerides (Xu *et al*., 2010). Moreover, another siRNA facilitated experiment revealed a decrease in circulating total cholesterol levels in both transgenic rodent models, with no effect on the liver triglyceride content (Cariou et al., 2011a; Frank-Kamenetsky *et al*., 2008). In monkeys, an average of 60% decrease in LDL-C was observed that lasted for 2–3 weeks after a single I.V. injection (Cariou *et al*., 2011b; Frank-Kamenetsky *et al*., 2008). The above results confirmed that PCSK9 targeting with RNAi can specifically lower LDL-C, paving the way for the development of PCSK9-lowering agents.

1.4.1.5 Inhibition of PCSK9 by using an anti-PCSK9 antigen-binding fragment

A protein molecule with the ability to imitate the binding of LDLR with PCSK9 is capable of inhibiting PCSK9 and LDLR binding, thereby preventing LDLR break-down (Shan *et al*., 2008; Akram *et al*., 2010). Likewise, this arrangement between PCSK9 and LDLR can be disrupted by an antigen, which restores the ability of cells to take-up LDL (Ni *et al*., 2010; Akram *et al*., 2010). A study was able to identify a fragment antigen-binding (Fab), 1D05, that has a nano-molar binding attraction to PCSK9 (Ni *et al*., 2011). A dosage of 3 mg/kg per body weight of 1D05-IgG2 was administered via I.V injection to rhesus monkeys and genetically modified mouse model (*CETP/LDLR*-hemi) that resulted in significant decreases in LDL-C (40%), total cholesterol by about 30% and elevating LDLR particles just about 5-fold for seven days (Ni *et al*., 2011). In summary, these findings revealed that the LDL-lowering effect of the neutralizing anti-PCSK9

1D05-IgG2 antibody is mediated by reducing the amount of PCSK9 that can bind to the LDLR (Ni *et al*., 2011).

1.4.2 Interaction of PCSK9 with niacin

As mentioned, statins are the current therapeutic drugs for the treatment of CVDs; however, some patients are reported not to achieve the targeted LDL-C levels. This results in these patients being offered higher dosages of statins, which are associated with diseases such as myopathy (Armitage *et al*., 2010; Baigent *et al*., 2010). These reasons therefore compel researchers to develop a combination of LDL-C-reducing treatments that are capable of supplementing current therapies and reducing the adverse outcomes of statins (Hochholzer *et al.*, 2011). The focus has been largely on lowering LDL-C, however increasing HDL-C has also been a focus area due to secondary therapeutic features (Expert Panel on Detection, 2001). For these reasons, niacin was chosen as the drug of choice for the current study, due to its reported efficacy in both these aspects (Chauke *et al.*, 2014). Even though niacin has a well-documented success as a drug that lowers lipids, there is inadequate knowledge concerning its clinical benefit and in combination with other therapeutic agents (Hochholzer *et al*., 2011). The majority of clinical data that exists pre-dates the statin era (Hochholzer *et al*., 2011). This is equally so, with regards to its association with PCSK9, with Khera *et al.,* 2015 being one of the limited studies that reported a slight decrease in LDL-C levels due to this combination (Khera *et al.*, 2015). Therefore, this study aimed to further the knowledge generation on the effect of niacin in reducing LDL levels through PCSK9 interaction. Additionally, the study will further strengthen the case for the use of the captive-bred vervet monkey as a suitable model for CVDs. For the regulation of lipid homeostasis, PCSK9 interacts with several genes. Therefore, the mechanisms by which each of these genes is associated with niacin interaction was assessed.

1.4.3 Interaction between LDLR and PCSK9 leading to atherosclerosis development

The main function of LDLR is to facilitate the movement of LDL molecules by internalizing them within the cells, and by so doing act as pivotal features in the removal of lipoproteins from circulation (Brown and Goldstein, 2006; Goldstein and Brown, 2009). Located at position 19p13.1-p13.3, the *LDLR* gene has a molecular weight of 45 kb with 18 exons (Milasan *et al*., 2016) (Figure 1.7). This protein is generally found in several tissues and it inhabits a peptide

molecule that is made up of 839 amino acids, whose role is to maintain cholesterol equilibrium (Milasan *et al*., 2016). The existing association between 6 well-designed domains of the peptide molecule and the coding regions of the gene has been fully articulated. The first exon of the LDLR is responsible for translating a 21 amino acid signal peptide, which is required for transportation to the cell surface and it is degraded in the course of exiting the ER (Gent and Braakman, 2004). Between exon 2-6, rests a binding conducive domain, the facilitator of lipoproteins interaction. The binding domain encompasses LDL receptor type A repeat (LR) of seven modules and has a similar structure to the nine carbon protein of the complement cascade (Gu and Zhang, 2015). One LR particle contains 40 residues, of these are six preserved cysteine molecules and also comprises of a well-maintained acidic area adjacent to the C-terminal that functions the binding site for calcium (Gu and Zhang, 2015). Experimental investigations have revealed that modules 3-7 are pivotal characters involved in the binding LDL particles (Gu and Zhang, 2015). The other two units, LR5 and LR6 are fundamentally autonomous from each other based on their structure (North and Blacklow, 1999).

Exons 7-14 code for a molecule known as the EGF precursor domain, which is required for LDLR detachment and lipoprotein from the ER (Gu and Zhang, 2015). The two initial particles are attached and detached from the third as the results of a 280 amino acid peptide which encloses five replicates of a preserved motif (YWTD) repeated once for each of 40-60 amino acids (Jeon *et al.*, 2001). The first epidermal growth factor-like repeat (EGFA) in the EGF homology terminal cooperates in a well-coordinated method with PCSK9 (Zhang *et al*., 2007; Kwon *et al.*, 2008). Additionally, PCSK9 can regulate LDLR particles after translation by attaching to them on the surfaces of cells thereby inducing their breaking down (Figure 1.8). Studies have discovered that GOF sequence variants lead to PCSK9 attaching itself to LDLR, leading to increased LDL-C levels, these actions have linked *PCSK9* gene with the development of autosomal dominant hypercholesterolemia (Abifadel *et al*., 2003; Abifadel *et al.*, 2009). When LOF mutations occur in PCSK9, the attraction to attach itself to the LDLR is lost, meaning there are low levels LDL found in circulation (Cohen *et al*., 2005). The structure of exon 15 in the *LDLR* gene contains serines and threonines, which enables the binding of the O-linked sugar. However, this exon was found to not harbour important functions in cultured hamster fibroblasts (Davis *et al*., 1986). Exon 16 comprises of module made up of 22 amino acids, which is responsible for the binding of the

receptor to the cell. The last two exons which have a combined number of 50 amino acids cytoplasmic tails are needed for the process of endocytosis (Jamaldini *et al.*, 2014).

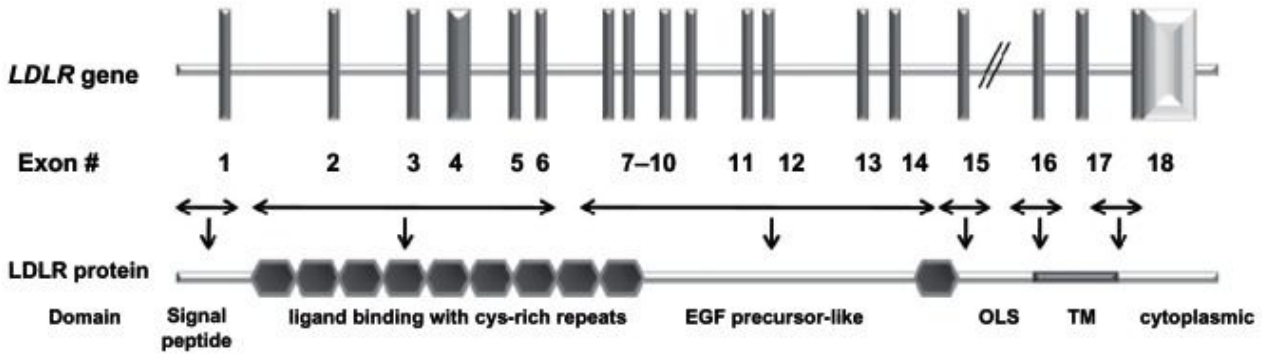


Figure 1.7: Schematic representation of the *LDLR* gene and LDLR protein structures. OLS, O-linked sugar domain; TM, transmembrane (Chmara *et al*., 2010)

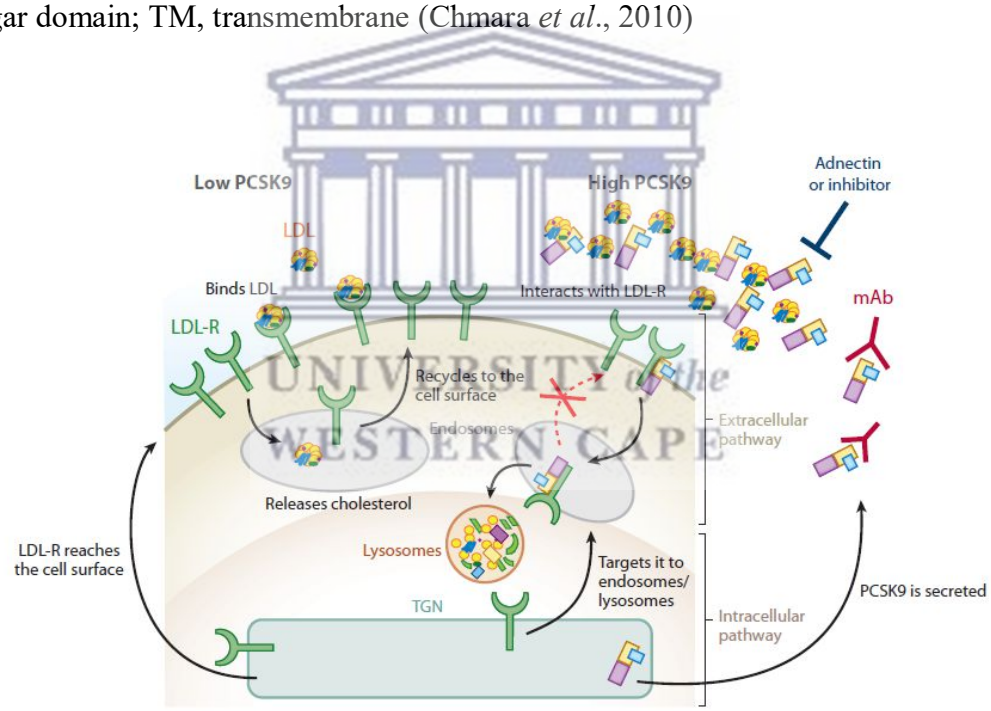


Figure 1.8: The role of PCSK9 in LDLR metabolism (Hess *et al* 2018).

1.4.4 Involvement of SREBP-2 in the lipid metabolism leading to atherosclerosis formation

Sterol regulatory element-binding proteins (SREBPs) represent a class of proteins whose main function is to produce and control the cellular movement of cholesterol and fatty acids (Roy *et al*., 2009). The SREBPs consists of three components such as SREBP1a, SREBP1c, and SREBP-2 (Madison, 2016). The synthesis of cholesterol requires SREBPs to stimulate expression of genes such as HMG-CoA reductase (HMGCR), HMG-CoA synthase (HMGCS) and mevalonate kinase (MVK) (Trapani and Pallottini, 2010; Trapani *et al.*, 2011). The expression of LDLR is then necessary for the uptake of cholesterol (Trapani *et al.*, 2013). For fatty acid biosynthesis to be permitted, the presence of fatty acid synthase (*FASN*) and acetyl-CoA carboxylase (*ACC*) genes must be prompted by SREBPs (Madison, 2016). There is a school of thought that has for a long time perceived SREBP-2 as the major factor in the activation of cholesterol synthesizing genes, as opposed to fatty acid synthesis (Shimano *et al*., 1996; Brown and Goldstein, 1997; Horton *et al*., 1998; Madison, 2016). It has also been revealed that SREBP1c is responsible for the synthesis of fatty acid in the liver, whereas SREBP1a can initiate both pathways in all tissues (Shimano et al., 1996; Brown and Goldstein, 1997; Horton *et al*., 1998; Madison, 2016). Furthermore, the function of SREBP-2 in managing genes that produce cholesterol has been demonstrated (Vergnes *et al.*, 2016). To be able to study the impact of insufficient SREBP-2, an *SREBP-2* knock out murine model was employed in embryos and adult animals at various dosages through a creative conditional allele (Vergnes *et al*., 2016). This conditional allele is a form of SREBP-2 that is genetically engineered by inserting a sequence variant at the intron region, which is made up of a robust connection acceptor and polyadenylation signal that interrupts the connection, leading to a protein molecule that is considerably shortened. The uniqueness of this trap within the first intron of SREBP-2 lies in the fact that it allows the null allele to be transfigured into an allele with a minimal role through Cre-Lox recombination (Madison, 2016). This allele alteration is permitted due to the genetic makeup of this specific gene trap, in which the splice acceptor is sandwiched in between loxP sites, shadowed by the GEO fusion protein/reporter. The splicing of SREBP-2 with this gene trap allele leads to the production of GEO as an alternative to SREBP-2 protein. When there are low levels of intracellular cholesterol, SREBP-2 gets stimulated resulting in greater *LDLR* gene expression, this improves LDL-C removal from circulation (Maxwell *et al*., 2003; Horton *et al*., 2003). SREBP-2 is required for the activation of PCSK9, which prompts LDLR reduction, therefore restricting hepatic cholesterol uptake and increasing circulating LDL-C (Figure 1.9)

(Maxwell *et al*., 2003; Horton *et al.*, 2003). This extremely organized expression arrangement contributes to the internally controlled system avoiding unnecessary cholesterol uptake to maintain cholesterol homeostasis (Urban *et al*., 2013).

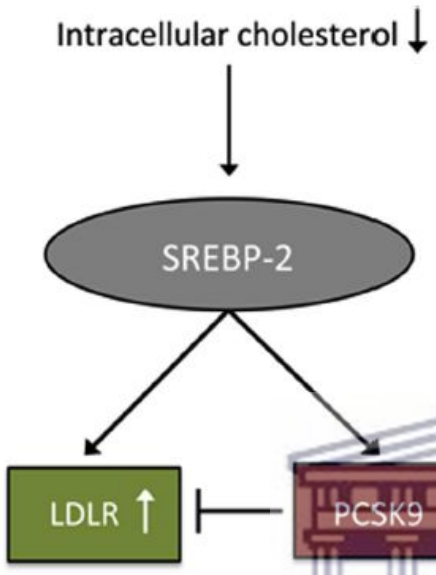


Figure 1.9: Low intracellular cholesterol levels activate SREBP-2 mediated *LDLR* expression, thereby increasing hepatic LDL-C uptake. Concurrently, SREBP-2 induces expression of *PCSK9*, which enhances hepatic LDLR degradation, thus preventing excessive cholesterol uptake to preserve cholesterol homeostasis (Urban *et al* 2013).

1.4.5 The regulation of lipoproteins by CETP

In vitro investigations that were conducted by Nichols and Smith revealed that CETP has plasma activity (Nichols and Smith, 1965). The discovery of this activity led to the subsequent characterization of its biochemistry (Yamashita *et al*., 2000; Inazu *et al*., 2000; Ordovas, 2000; Barter and Rye, 2001). The potential that CETP poses includes modulating both HDL and LDL levels and this has made it one of the key research areas with regards to atherosclerosis (Oliveira and de Faria, 2011). The CETP protein is found in several animals such as birds, mammals, fish and reptiles, however, rats and mice do not harbour this protein (Moghadasian *et al*., 2001). To further characterize CETP, chemical analysis reactions were run, which revealed that it is a plasma glycoprotein made up of 476 amino acids that account for a molar mass of approximately 74 KDa

(Teh *et al*., 1998). This chemical purification paved a way for studies on tissue expression and cDNA cloning (Drayna *et al.*, 1987). The *CETP* gene is expressed in many tissues in primates and humans; however, it mainly resides in the liver (Oliveira and de Faria, 2011). The earliest reported cases of CETP deficiency were found in the Japanese people (Teh *et al*., 1998). These Japanese cases demonstrated an inverse relationship between CETP and HDL-C, whose noticeable increase was revealed as the result of the lack of CETP (Brown *et al*., 1989; Inazu *et al*., 1990). After the discovery of CETP deficiency, genetic engineering techniques were used to study the structure and also map the epitope region (Swenson *et al*., 1989; Wang *et al*., 1993; Roy *et al*., 1996). Thereafter, the human gene was cloned and characterized (Agellon *et al*., 1990), which laid the groundwork for ensuing studies on *CETP* gene regulation (Jiang *et al*., 1992; Oliveira *et al*., 1996; Luo and Tall, 2000). Years later, genetically modified mice strains capable of expressing CETP were created and were found to be predisposed to atherosclerosis. However, the results were somewhat controversial due to the identification of other possible influences that are of a digestive nature. As soon as it was discovered that CETP has a negative effect on HDL-C, investigations have been geared at finding its inhibitors and annotate the molecule's chemical structure. The results of *in vivo* studies which used CETP inhibitors revealed encouraging data in the fight against high cholesterol (Oliveira and de Faria, 2011). The outcomes of the pilot human clinical trials were also promising in producing reduced disease markers (Brousseau *et al*., 2004). However, the transition from pilot to full-scale studies did not produce the desired results due to a high fatality rate associated with torcetrapib, the CETP inhibitor (Barter *et al*., 2007). The molecular structure of CETP was eventually annotated approximately four decades after its discovery (Qiu *et al*., 2007). The annotation was viewed as a breakthrough as it paved a path to uncovering CETP's underlying mechanisms, leading to advanced techniques of drug creation (Oliveira and de Faria, 2011).

1.4.5.1 CETP and atherogenesis

HDL particles are understood to play a starring role in combatting the development of atherosclerosis through RCT, which was conceptualized by Glomset (Glomset, 1968). In essence, the RCT is an HDL reliant pathway whose main function is to remove cholesterol from tissues via the liver to be excreted from the body (Oliveira and de Faria, 2011). Reverse cholesterol transport is a multifaceted system with a carefully coordinated set of reactions which include precise

receptors, cellular transporters, extracellular HDL acceptors and enzymes (Rader *et al.*, 2009). At the beginning of the pathway, HDL molecules interface with ABCA1/G1 membrane transporters leading to the elimination of cholesterol from cell membranes (Oliveira and de Faria, 2011) (Figure 1. 10). The removed cholesterol is then esterified by LCAT on the exterior of the HDL particles, after esterification the cholesterol enters into the water-repelling centre of the HDL particle, this results in the ejection of unesterified cholesterol. Two major molecules control the fate of HDL-cholesteryl ester upon exiting the HDL core: firstly, scavenger receptor class B type I (SRBI) mediates the transportation to tissues such as liver, adrenal, and gonadal, or CETP facilitates the transfer to VLDL and LDL (Oliveira and de Faria, 2011). The CETP enabled pathway leads to the movement of these lipoproteins to the liver with the help of LDLRs. This pathway is referred to as indirect RCT. Upon entering the liver, the cholesterol is stored in the bile and is eliminated from the body through faeces. The CETP protein is suggested to have a two prone effect on cholesterol as it acts as a further path for the supply of cholesterol to the liver, additionally, CETP encourages the buildup of LDL-cholesterol in the plasma, consequently leading to atheroma formation (Oliveira and de Faria, 2011).

As mentioned before, genetically engineered mice strains were created to study the role played by CETP in the development of atherosclerosis, the results of these investigations revealed that *CETP* expression can induce or prevent atherosclerosis predisposition (Oliveira and de Faria, 2011; Tsai *et al*., 2009). Additionally, it has been reported that *CETP* expression prevents the development of many disorders such as hypertriglyceridemia (Hayek *et al.*, 1995), overexpression of LCAT (Föger *et al*., 1999; Berti *et al*., 2005), the combination of surplus APOB with lipoprotein lipase deficiency and diabetes (Kako *et al*., 2002), diabetes and obesity (MacLean *et al*., 2003), castration (Cazita *et al*., 2003; Casquero *et al.*, 2006) and SRBI deficiency (Harder *et al*., 2007).

On the other hand, studies have reported that an increase in *CETP* expression leads to the development of many disorders such as atherosclerosis (Marotti *et al*., 1993), high cholesterol due to defects in *LDLR* or *APOE* genes (Plump *et al*., 1999) and hypertension in rodents (Herrera *et al*., 1999). Furthermore, rabbits fed a high-fat diet produced a substantial decrease in atherosclerotic lesions after a CETP vaccine (Rittershaus *et al*., 2000), an antisense oligonucleotide (Sugano *et al*., 1998), or drug inhibition of CETP was administered (Okamoto *et al*., 2000).

Recently, *in vivo* experimental results agree with the notion that RCT, which is specifically macrophage-linked is systematically associated with atherogenesis (Rader *et al.*, 2009). Studies have also discovered data which suggests that CETP may enhance the reduction of lipid buildup in arterial foam cells (Glass and Witztum, 2001). This was further verified by the addition of CETP to the media of foam cells or smooth muscle that led to the excretion of cholesterol from the cells (Glass and Witztum, 2001). There was a previous report that made observations about CETP expressing mouse peritoneal macrophages accumulating a lower amount of cholesterol from acetylated LDL and this action was seen as a major contributor to atherogenesis (Van Eck *et al*., 2007). Therefore, this further outlined the importance of understanding events that occur at the macrophage level and their role in characterizing the severity of different diseases. However, there are contrasting reports about the exact role played by CETP on the development of atherogenesis; therefore more research is still required (Oliveira and de Faria, 2011).

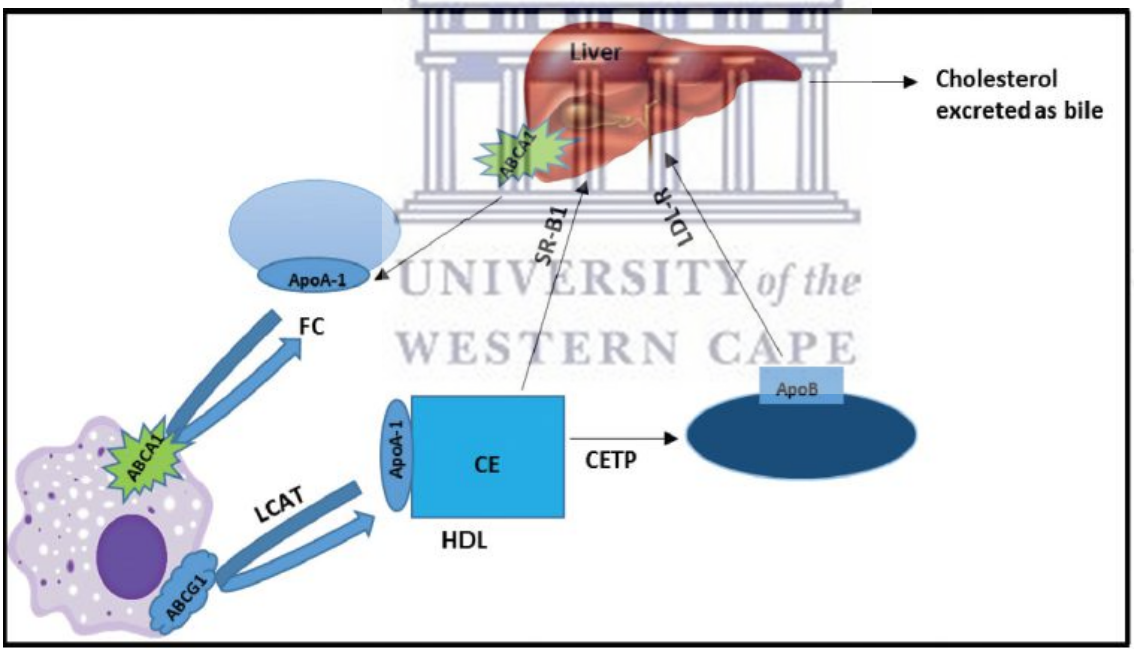


Figure 1.10: Depicts the reverse cholesterol transport (RCT) pathway. This figure details all the key mechanisms of RCT including apolipoprotein A-I (APOA-I), high-density lipoprotein (HDL), lecithin: cholesterol acyltransferase (LCAT), ATP-binding cassette transporter (ABCA1/ABCG1) and cholesterol ester transfer protein (CETP) adopted by (Chauke *et al.*, 2014) from (Rader *et al* 2009).

1.4.6 Involvement of APOB-100 in the lipid metabolism leading to atherosclerosis formation

The processes that modulate the biosynthesis and the storage of APOB remain partially understood, however, its role in the development of CHD, due to APOB-100 being a major component of LDL is well documented (Schaefer *et al*., 1994; Tabas *et al*., 2007; Shapiro and Fazio, 2017)**.** The cleavage of APOB molecule results in the production of well-developed protein particles (550 kDa) APOB-100 or APOB-48 (265 kDa) depending on the size of the signalling amino acids (Whitfield *et al*., 2004). The liver organ contains APOB-100 that exists as a peptide molecule that consists of 4536 amino acids, while APOB-48 is made up of 2,152 amino acids found in the intestines (Hussain *et al*., 2003). The human APOB-100 molecule is predicted to exist as a pentyl configuration that is made up of interchanging alpha helices and beta strands that possesses polar and non-polar ends (Segrest *et al*., 2001). The smaller molecule of APOB encompasses a region that is approximately 1000 amino acids, whose importance is the binding with the microsomal triglyceride transfer protein (MTP) binds during APOB assembly (Figure 1.11)(Segrest *et al*., 1999; Hussain *et al*., 2003). Furthermore, the main purpose of the relationship between MTP and the APOB domain is reported to be the formation of the lipid pocket, which enables lipid collection during lipoprotein assembly (Segrest *et al*., 1999).

Additionally, APOB-100 is the main element of LDL particles, which regulates LDL-C in plasma, therefore this protein is also key in the development of hyperlipidemia and atherosclerosis (Vrablik *et al*., 2001). Furthermore, APOB contains a carboxyl-terminal (site B) responsible for housing the LDLR binding domain (Boren *et al*., 1998; Hussain *et al*., 2003). This APOB carboxyl-terminal occurs as a strictly guarded entity in several species and harbours features that are comparable to the receptor-binding region in APOE (Hussain *et al.*, 2003). The discovery of a direct association that exists between plasma cholesterol-containing APOB attached to lipoproteins and atherosclerosis, led to the development of statins (Shapiro and Fazio, 2017). The incorporation of APOB into the lipoproteins takes place in two phases, firstly nascent APOB polypeptide merges with the lipoprotein particle in the rough ER; followed by lipid droplets that also attach themselves, this can happen in the ER or Golgi apparatus (Levy *et al.*, 2002; Gusarova *et al*., 2003; Swift *et al*., 2003). These two processes are facilitated by MTP in corporation with an ER enzyme, protein-disulfide isomerase (Wetterau *et al*., 1997; Berriot-Varoqueaux *et al*., 2000).

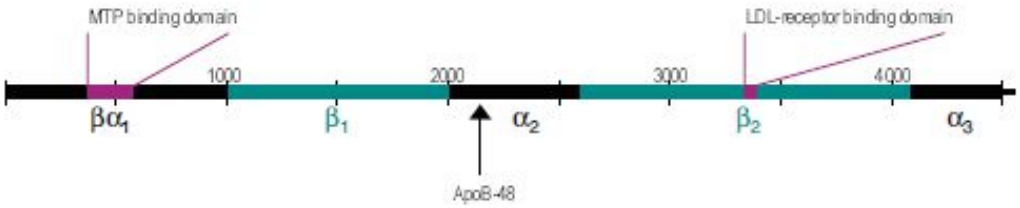


Figure 1.11: Structure of APOB-100, is made up of interchanging α helices and β strands that possesses polar and non-polar ends. Regions of the MTP and LDLR binding domains are also depicted. The length of APOB-48 is indicated by the arrow (Whitfield *et al* 2004).

1.4.6.1 The mechanism of APOB-100 biosynthesis

For VLDL particles to be able to accumulate in the hepatocyte, APOB-100 and lipids are required to be synthesized in a coordinated manner (Figure 1.12) (Burnett *et al.*, 2007). The APOB particle is produced in the rough ER, thereafter it travels to the lumen of the ER, as it is being translated at the same time (Whitfield *et al*., 2004). In the ER, this protein can be found to exist as a structural support that covers the surface area of atherogenic lipoproteins (Shapiro and Fazio, 2017). Disulfide bonds are formed to be able to aid the accurate folding of the amino terminus, this is required to enable lipid transfer to nascent APOB (Huang and Shelness, 1997; Tran *et al*., 1998; Rustaeus *et al*., 1998). It is reported that the first step in the APOB and phospholipids merger may not require MTP facilitation, as this particle is not necessary for the storage of APOB-48 (Liu *et al*., 2010). However, MTP plays an important role in the translocation of mature APOB-100 (Herscovitz *et al*., 1995; Rusiñol *et al*., 1997; Sellers and Shelness, 2001). Furthermore, MTP seems to be necessary for the eventual maturation of VLDL, hence, it can be found in the Golgi apparatus as well in the ER (Wang *et al*., 1997; Pan *et al*., 2002; Swift *et al*., 2003; Larsson *et al.*, 2004).

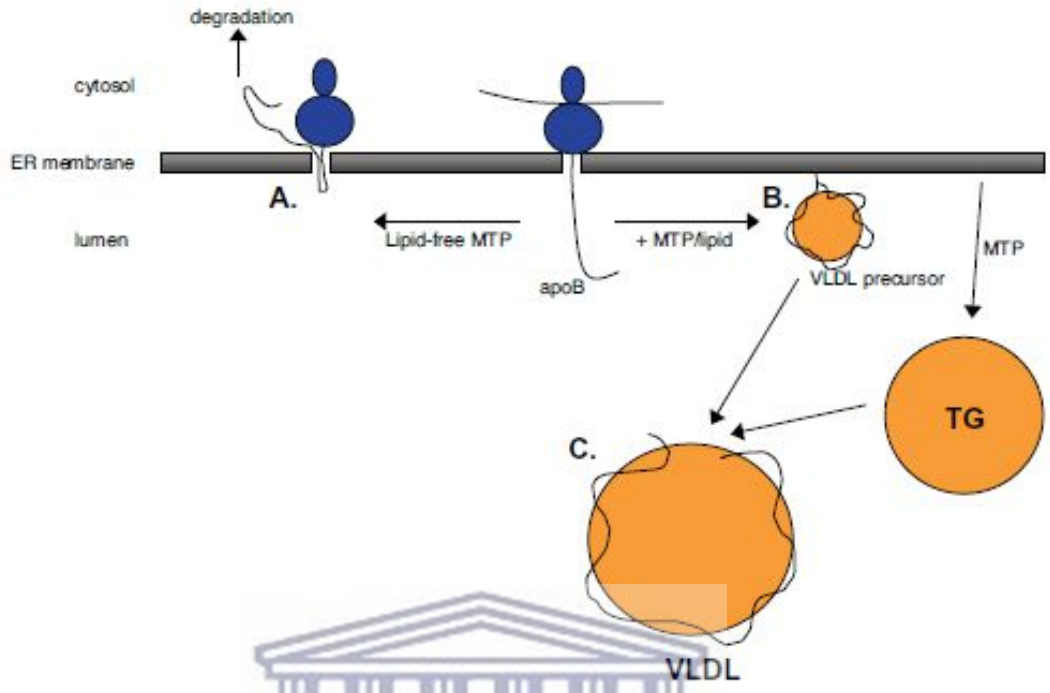


Figure 1.12: The diagram depicts the interaction of APOB and VLDL. Emerging APOB particles associate themselves with lipid-free MTP, leading to a detachment from the lumen of the ER into the cytosol (**A**) and thereafter degraded. On the other hand, the interaction of naïve APOB particles with MTP-lipid complexes results in the formation of VLDL precursor (**B**). A complete VLDL molecule is formed when the VLDL precursor is merged with a triglyceride (*TG*) rich lipid droplet (**C**) (Whitfield *et al* 2004).

1.4.7 The use of non-human primates (NHPs) as research models

A number of laboratory studies directed at understanding these biological and genetic atherosclerotic concepts that are outlined in this manuscript, have been carried out in the past decade (Glass and Witztum, 2001; Libby, 2003; Sanz and Fayad, 2008). Despite these great strides, the challenge has been to translate the experimental animal findings to human atherosclerosis and clinical applications. As much as experimental work has assisted to reveal some of the principles of atherosclerosis pathophysiology, there remain clinical treatment challenges. There is a need to bridge the gap between experimental findings and their translation to meaningful treatment in order achieve the full promise of scientific advances in atherosclerosis prevention and treatment (Libby *et al*., 2011). It is therefore of utmost importance that an appropriate model that is utilized to study atherosclerosis is as genetically close as possible to humans.

The non-human primates (NHPs) have been employed as models to study the pathogenesis and progression of CVDs for decades (Cox *et al*., 2017). Two research focus areas have been carried out in the NHPs. Initial studies were focused on describing the metabolic and physiologic aspects of CVDs, these were followed by investigations into genetic and epigenetic mechanisms influencing CVD initiation and progression. The NHPs that have been extensively used include baboons (*Papio hamadryas*), rhesus macaques *(Macaca mulatta*), cynomolgus macaques (*Macaca fascicularis*), snow monkeys (*Macaca fuscata*), and vervet monkeys (*Chlorocebus aethiops sabaeus*). NHPs present numerous advantages such as genetic, metabolic, and physiologic similarities with humans (Cox *et al*., 2017). They also present an equally important ability to be able to control diet, environment and breeding (Cox *et al*., 2017). Furthermore, these animals are known to be genetically and phenotypically heterogeneous. The Old World NHP species mentioned above, individually bring distinctive strengths as models of human CVDs. Their ability to not require genetic manipulation for the development of CVD provides a chance to use different models that might reveal genetic variants that influence CVD risk. It has been reported that age-related diseases such as dyslipidemia and diabetes progress at a rate that is about 3 to 4 times faster in NHPs than in humans (Cox *et al*., 2013). Another added advantage is due to their larger body size which allows longitudinal studies to be conducted and making the collection of biopsy samples possible in healthy and ill animals at various time points. Although imaging technologies

have emerged, their emergence however has not nullified biopsy sampling in an NHP model (Cox *et al*., 2017).

One of the first investigations on atherosclerotic lesions in NHPs was conducted in baboons by McGill *et al*. (1960). The concentrations of triglycerides and plasma cholesterol have been noted to respond similarly to dietary cholesterol, fat and carbohydrates as observed in humans (Kushwaha *et al*., 1994; Kushwaha and McGill, 1998; Higgins *et al*., 2010). When baboons are fed high fat diet continuously over some time, they do develop fatty streaks and atherosclerotic plaques (McGill *et al*., 1981). Above that, these animals also experience increased body fat, triglyceride concentrations, altered adipokine concentrations, and altered glucose metabolism, when they are on a diet high in simple carbohydrates, observations that are also seen in humans (Higgins *et al*., 2010). The development of insulin resistance, dyslipidemia, atherosclerosis, and CVD in baboons is also observed even when given normal diet (Kamath *et al*., 2011; Higgins *et al*., 2014; Mamtani *et al*., 2014).

Monkeys such as rhesus macaques, cynomolgus macaques, vervets and snow also do progress to atherosclerosis when on a high-carbohydrate diet (Eggen, 1974; Rudel *et al*., 1990). Their relatively smaller size in comparison to baboons makes housing and husbandry more manageable for some research facilities. However, there are some disadvantages to using NHPs models to study CVDs. NHPs are known to live longer than the commonly used rodents such as mice and rats. Additionally, their larger body sizes make them more expensive to maintain in captivity. Their experimental period is usually longer, which can be costly as more reagents will be required. Some NHP species do not have their genomes fully described, posing a challenge in finding and confirming genetic modifications that may influence CVD initiation and progression (Cox *et al*., 2017). However, this can be overcome by making use of comparative genomic analysis and annotation tools, such as those available through the UCSC Genome Browser (Rosenbloom *et al*., 2014). It is envisaged that such limitations will be minimized in future as annotation of NHP genomes improves (Cox *et al*., 2017).

1.4.7.1 Vervet monkey as an animal model of atherosclerosis

The vervet monkey has long been viewed as an invaluable NHP model for biomedical research, with some of the diseases that have been investigated including brain, behaviour, metabolism, and immunity (Jasinska *et al*., 2013). The progression of human atherosclerosis can be modelled morphologically in vervet monkeys by employing light microscopy which uses seven well-defined stages (Stary, 1992; Fincham *et al.*, 1996). Vervets can also portray vital features of human plasma lipid profiles, in addition to important associations between atherosclerosis and lipids in diets and plasma. Furthermore, the vervet monkey model is also capable of detailing the movement of plasma LDL (Fincham *et al.*, 1987a; Fincham *et al.*, 1987b; Benadé *et al*., 1988; Weight *et al.*, 1988; Wynchank *et al*., 1989; Fincham *et al*., 1991; Smuts *et al*., 1992; Kruger *et al*., 1992). Both genders of these monkeys develop atherosclerosis based on the same principles. Susceptible monkeys develop advanced lesions that have aspects of human atherosclerosis types IV-VII after being fed a high-fat diet for approximately four years (Fincham *et al*., 1987b; Fincham *et al*., 1991; Stary, 1992; Fincham *et al*., 1996).

Traditionally, rodents have been extensively used in preclinical research for CVD related studies (Russell and Proctor, 2006), however, they lack cholesteryl ester transport protein (CETP), an important biocatalyst responsible for the transportation of plasma cholesterol that hands over cholesteryl ester (CE) from HDL to APOB-100 containing lipoproteins such as LDL and VLDL (Moghadasian *et al*., 2001). Animals such as rats, dogs, and pigs also have very little or no CETP activity, however, they predominantly have HDL-C and poorly distributed LDL-C that is similar to mice, which is associated with a lowered risk of CVD (Straarup *et al*., 2010). Therefore, studies in a more relevant model, such as NHPs are required. The vervet monkey has been reported as an excellent model to study cholesterol metabolism, as it similarly responds to dietary cholesterol as humans (Weight *et al*., 1988; Suckling and Jackson, 1993). In a previous study conducted at PUDAC using a vervet monkey model, niacin treatment produced a significant increase in HDL (49%), ApoA-1 (34%) and a decrease in LDL (43%) concentrations (Chauke *et al*., 2014).

1.5 Research Objectives

1.5.1 Aims and specific objectives

The primary aim of this study was to determine the expression levels of PCSK9 in captive-bred vervet monkeys and the effect of niacin administration on PCSK9 activity. The study also aims to determine whether *PCSK9* has gain or loss of function in vervet monkeys.

Study objectives

- Conduct biochemical analysis to determine the levels of total cholesterol, triglycerides, LDL-C and HDL-C in both experimental (niacin administered) and control monkeys.
- Screen for *PCSK9, LDLR, APOB-100, CETP* and *SREBP-2* genes in the vervet monkey model using genotyping and gene expression.

1.5.2 Hypothesis

Niacin administration minimizes the ability of PCSK9 to degrade LDLR in the vervet monkey model, resulting in reduced plasma LDL-C levels.

1.5.3 Study overview

This thesis is laid out into different chapters as follows: Chapter 1: Introduction and Literature Review. Chapter 2: The effects of short-term niacin treatment on plasma lipoproteins in the vervet monkey model. Chapter 3: The impact of PCSK9 in lipid metabolism and its relationship to new therapies for lowering cholesterol in a non-human primate model of atherosclerosis. Chapter 4: Discussion /conclusion: Chapter 5: References: Chapter 6: Appendices.

CHAPTER TWO:

The effects of short-term niacin treatment on plasma lipoproteins in the vervet monkey model (*Chlorocebus aethiops*).

2.1 INTRODUCTION

As much as niacin possesses a long history of being a treatment for dyslipidemia, only a handful of clinical trials have investigated its efficiency in the prevention of impairment and loss of life which are associated with CVDs (Hochholzer *et al*., 2011). Despite being underused due to its unbearable side effects (Hochholzer *et al*., 2011), niacin remains high on the list of lipid-modulating agents, second to statins (Hochholzer *et al.*, 2011). Moreover, the development of new formulations has reduced the severity of flushing symptoms of niacin by 20% (Gibbons *et al*., 1995; Maccubbin *et al.*, 2009). Currently, three niacin formulations are available: Immediate-release (IR), sustained-release (SR), and a relatively newer formulation, niacin extended-release (ER) (McKenney, 2004). These formulations are different based on their efficacy, pharmacokinetic and safety profiles (Pieper, 2002). However, the side effects of niacin therapy are known to be associated with the IR formulation, whereas the SR formulation is linked with hepatotoxicity (Knopp, 2000; Dalton and Berry, 1992). These side effects are said to occur as a result of the absorption rate and subsequent metabolism of niacin as delivered from the different formulations (Pieper, 2002). Furthermore, the delivery system of ER has been described to have improved absorption rates compared to IR and SR (McKenney, 2004).

Niacin dosages (between 1-3 g/day) have been reported to lower the amounts of total plasma cholesterol, APOB, triglycerides, VLDL, LDL and Lp (a) while increasing HDL levels (Ganji *et al*., 2003). The impact of niacin on lipid metabolism was previously investigated by our research group using the vervet monkey as the model of choice (Chauke *et al*., 2014). The findings of this previous study were used as the anchor and reason to further study the genetic basis of niacin therapy in the same colony. The same SR granules formulation was considered over the new and improved ER formulation for this study since it can be administered during the day when clinical observations are more conducive verse the ER which is administered at night.

This study had two research focus areas, with the animal intervention being the first, followed by genomic analysis (genotyping and gene expression). This chapter reports on the effects of the administration SR niacin on plasma lipids (triglycerides, LDL-C, total cholesterol and HDL-C) in healthy vervet monkeys. Niacin was chosen as the therapeutic intervention based on its ability to decrease triglycerides, LDL-C, cholesterol and increase HDL-C (Kamanna and Kashyap, 2000).

2.2 MATERIALS AND METHODS

2.2.1 Animal ethics

This study was approved by the South African Medical Research Council (SAMRC) Ethics Committee for Research on Animals (ECRA) (**Ref: 11/18**) (Appendix A). The selected vervet monkeys were maintained at the Primate Unit and Delft Animal Centre (PUDAC)/SAMRC according to the South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008).

2.2.2 Animal housing, diet and group selection

Sixteen healthy vervet monkeys were selected and housed in single cages throughout the study with regular access to exercise cages and environmental enrichment. The closed indoor environment was maintained at a temperature of 24-26°C, 45% humidity, 15-20 air changes/hour and a photoperiod of 12h. Animals were allowed a period of two weeks to acclimatize to the single cages and baseline samples were taken after the acclimatization period. At the end of the treatment period, all the animals were returned to the colony.

The monkeys were divided into two groups of eight animals consisting of an experimental and a control group (Table 2.1). Each group consisted of four females and four males matched according to lipogram levels (Appendix B, Table B1). The control group received a maintenance diet of pre-cooked maize meal mixed with vitamin and mineral concentrate, egg powder, bean flour and sunflower oil in the morning (PUDAC standard operating procedures # 2019-VO1:# 2). The maintenance diet for the experimental group was mixed with 100 mg/kg of niacin (Chauke *et al*., 2014). The diet for both groups was further supplemented with vitamin C and vitamin D3 and each monkey received 70g (dry weight) of the diet per day. At lunch, the animals received fruit (apples, oranges, mandarins) and were given maize meal, containing milled seeds in the evening. The diet

supplied the monkeys with 2412 kJ/day of energy (range of recommendations for primates is 1380 - 2510): 12% from protein, 20% fat and 75% carbohydrates (Seier, 1986). The animals had access to water *ad lib* via an automatic watering device.

Table 2.1: Experimental design

Groups	Sample size	Treatment	Duration	Washout	Feeding time
Group 1: Control	8 (4 M and 4 F)	30g of maintenance diet	12 weeks (3 months)	1 month	07:00 am
Group 2: Experimental	8 (4 M and 4 F)	Niacin (100 mg/kg/day BW) mixed into 30 g of Maintenance diet	12 weeks (3 months)	1 month	07:00 am

Blood (2-4 ml) was collected at baseline and every four weeks for three months. This treatment period was followed by a four-week washout period.

2.2.3 Compound administration and food consumption

Niacin SR granules formulation was supplied (Sigma, Missouri, USA) and the exact amount of the compound was calculated based on each animal's body weight and mixed to a 30 g portion of the maintenance diet. The treated food was voluntarily consumed by animals in the morning and the rest of the food (70 g) was offered once the treated food was consumed. Food intake and wastage for both experimental and controls were monitored and recorded daily. This was done throughout the treatment period of three months which was followed by four weeks of the washout period. At the end of the washout period, all the animals were returned to the colony.

2.2.4 Clinical observations and body weights

Due to the known severe side effects of niacin, special caution was given to the experimental group although the chosen dose (100 mg/kg) had been reported to produce no adverse effects (Gille *et al.*, 2008, Chauke *et al*., 2014). Each animal was observed daily for general well-being and any signs of illness and recorded weekly (Appendix F). The body weight of each animal was recorded at each blood sampling interval as mentioned above.

2.2.5 Blood collection

Animals were sedated with Ketamine hydrochloride (10 mg/kg) (Kyron laboratories, South Africa) anaesthesia via intramuscular injection. Blood samples (2-4 ml) were obtained via femoral venipuncture into EDTA, SST and PAXgene tubes. During the niacin intervention, blood was collected at baseline, every four weeks during the treatment period and the end of the washout period (Table 2.1 and Appendix C, Table C1). Serum samples were isolated from the EDTA tubes at room temperature (1000 x g, 10 minutes) and stored at -80°C for further genetic analysis. For gene expression, blood (2.5 ml) was collected into PAXgene Blood RNA Tubes (BRT) (Qiagen, Venlo, Netherlands) and maintained at room temperature for two hours before being stored at -80°C.

2.2.6 Biochemistry analysis

Blood collected in SST tubes was analyzed for the following lipogram parameters: Total cholesterol, LDL-C, HDL-C and triglycerides. The analysis of the aforementioned parameters was carried out by PathCare® laboratories (Cape Town, South Africa). This was done for each blood sampling time point (T_0-T_4) and at the end of the study (T_5).

2.3 Statistical analysis

The biochemistry analyses data generated in this study were presented as means ± SD. Multiple comparison analysis was used to determine statistical significance at each time point. Two-way ANOVA in the GraphPad Prism program (California, USA) was used to compare changes from baseline (T_0) to washout period (T_5) between the experimental and control groups. Statistical significance was calculated by using the Student *t*-test, and a value of $P < 0.05$ was considered significant.

2.4 RESULTS

Animals with higher lipogram levels at baseline were selected into the treatment group and received the 100 mg/kg dosage of niacin. Food consumption in both groups was similar, with both groups consuming an average of 100% (Appendix C, Tables C2 and C3). This was correlated with animals body weight (niacin group: 4.69±1.11 kg and controls: 4.76±0.98 kg on average), which remained constant throughout the study (Figure 2.1). None of the animals in the treatment group

showed any signs of niacin side effects during the treatment duration of the three months, and all the animals were returned to the colony at the end of the study. The biochemical analysis showed no significant reduction in cholesterol levels (Figure 2.2), LDL-C (Figure 2.3) and triglycerides (Figure 2.5), while statistically significant increases were observed in the HDL-C of the treated group from time point T_1-T_4 (Figure 2.4).

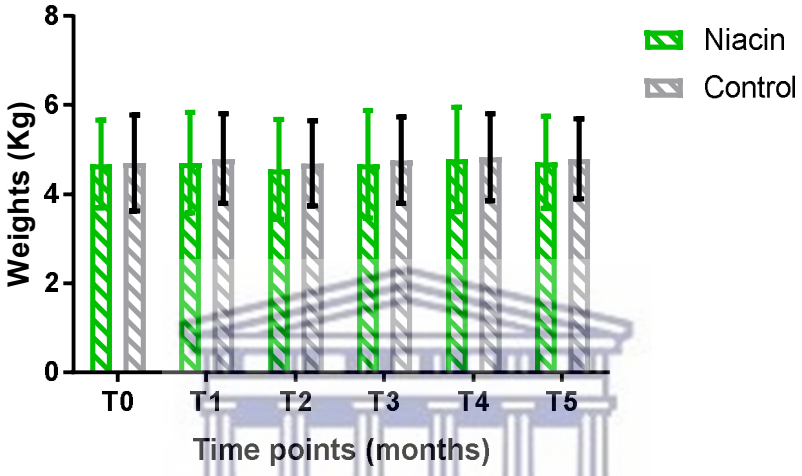


Figure 2.1: Animal body weight (Kg). Mean weight of the animals in the niacin and control group over the study period.

2.4.1 Total cholesterol

Niacin was able to affect the total cholesterol levels of the treated group, while the controls remained relatively unchanged until T_{2-3}, where they were slight fluctuations. The total cholesterol in the treated group was slightly decreased at T_3-T_5, however, this was only statistically significant at T_{4-5} p-values ($T_4 = 0.03$, $T_5 = 0.05$)(Figure 2.2; Appendix E). Additionally, there was a 17.28% reduction from T_0 to T_1 that was observed in the niacin group. There were minimal changes in the levels of cholesterol in the control group throughout the treatment period (T_0-T_4).

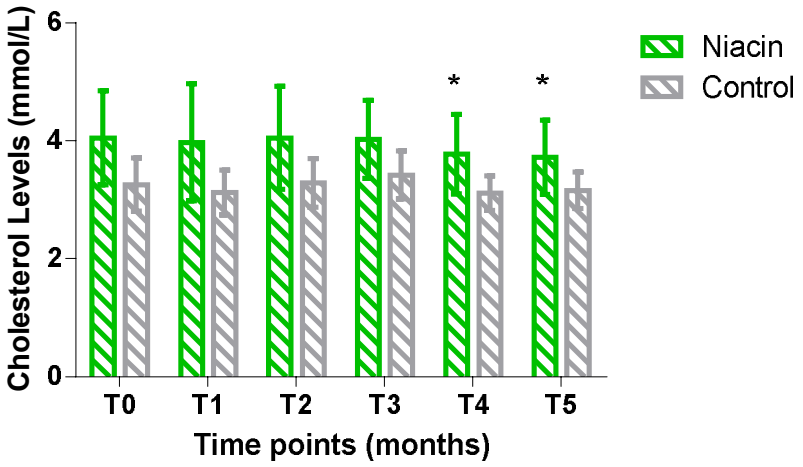


Figure 2.2: Total cholesterol (mmol/L). The total cholesterol expressed as mean ± SD of the niacin and control groups. * indicates statistical significance ($p < 0.05$).

2.4.2 LDL-C levels

The administration of niacin led to an initial 8.62% decrease in LDL-C levels, followed by a slight 9.43% increase at the second month and then decreases at all other time points. All changes to LDL-C levels were however non-significant when compared to initial plasma levels. LDL-C levels in the control group were relatively constant for the duration of the study (Figure 2.3).

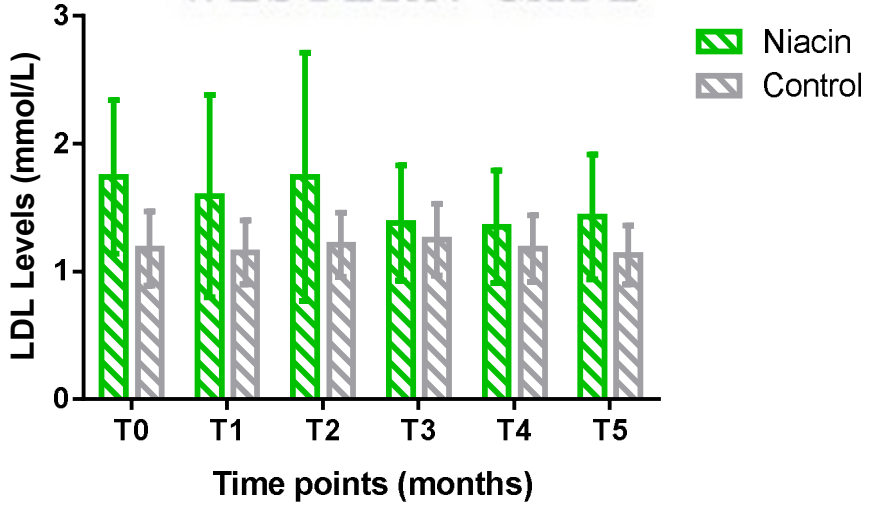


Figure 2.3: LDL-cholesterol (mmol/L). Mean plasma LDL concentration of the animals in the niacin and control groups over the study period.

2.4.3 HDL-C levels

Statistically significant increases in HDL-C levels were observed over the four months of the study in the niacin group, with HDL-C levels dropping back to levels close to those of the baseline after the washout period (Figure 2.4). These statistically significant increases resulted in the following p-values ($T_1 = 0.02$, $T_2 = 0.0018$, $T_3 = 0.0021$ and $T_4 = 0.0004$) (Appendix E). These statistically significant rises resulted in 17.37% and 14.29% increases at T_1 and T_3. In the control animals, no significant changes to HDL-C levels were observed either during the study period or following the washout.

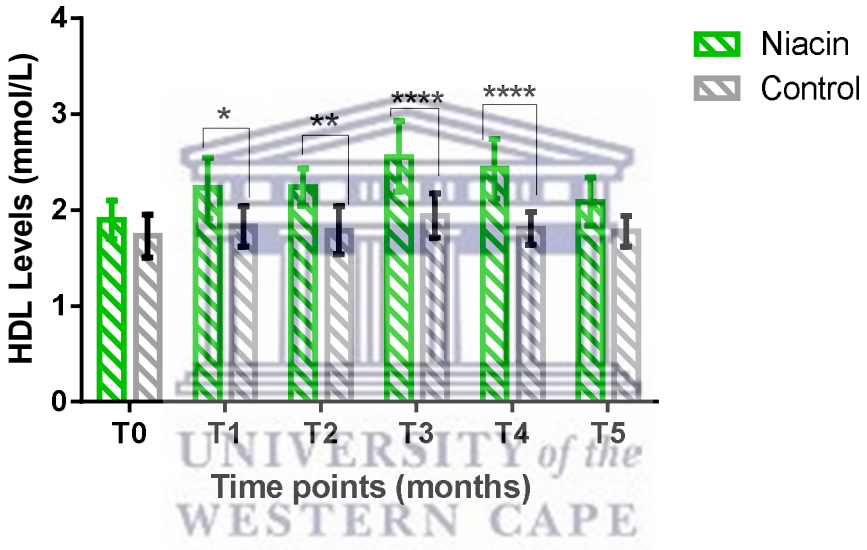


Figure 2.4: HDL-cholesterol (mmol/L). Mean plasma HDL concentration of the animals in the niacin and control groups over the study period. * indicates statistical significance ($p < 0, 05$). ** indicates statistical significance ($p < 0, 005$). **** indicates statistical significance ($p < 0, 0001$).

2.4.4 Triglycerides

There were fluctuations in the levels of both groups which were not statistically significant throughout the study for triglycerides (Figure 2.5).

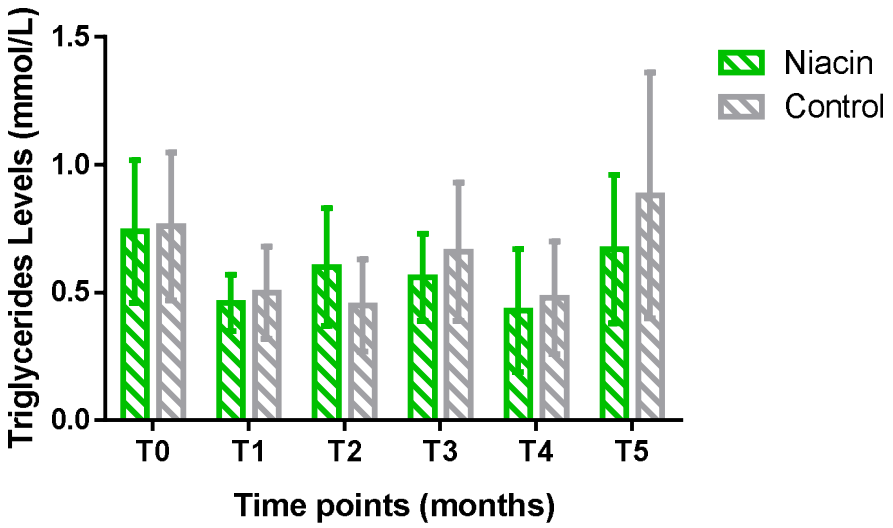


Figure 2.5: Triglycerides (mmol/L). Mean plasma triglycerides concentration expressed as mean ± SD of the animals in the niacin and control groups over the study period.

2.5 DISCUSSION

In this study, the effect of 100 mg/kg dose of niacin on plasma lipids was assessed in the vervet monkey over a treatment period of three months. There were no signs of ill-health or discomfort observed in all the monkeys throughout the study (Appendix F). Furthermore, the results indicated that niacin treatment-induced biochemical changes in the animals were similar to those reported in humans and other primate species (Carlson, 2005; Chauke *et al*., 2014). The dosage of 100 mg/kg of the niacin SR formulation is a relatively low dose used to mitigate the reported side effects (Rader, 2003; Chauke *et al*., 2014). The same SR formulation was utilized in this study as opposed to the ER formulation which is administered at night and not conducive for the clinical observations that have to be conducted during the day (Tavintharan and Kashyap, 2001; Chauke *et al*., 2014).

This study reports that niacin treatment was able to account for slight decreases in cholesterol, triglycerides and LDL-C. Additionally, HDL-C levels increased significantly from T_1 to the end of the treatment (T_4), with a 32,11% upturn after the first month of the administration, even though this percentage increase was not maintained for the duration of the study (Figure 2.4). The statistical significant escalation in the HDL-C levels was anticipated based on the knowledge that

niacin increases HDL-C levels (Hochholzer *et al*., 2011). A significant increase in HDL-C levels is normally attributed primarily to niacin suppressing the hepatic removal of APOA-I, leading to better-quality levels of APOA-I as well as higher APOA-I containing HDL particles (Jin *et al*., 1997; Kamanna and Kashyap, 2000). Although this study did not measure APOA-I levels, the previous study reported a correlation between APOA-I levels and HDL levels (Chauke *et al.*, 2014). Niacin is also reported to enable the hepatic elimination of cholesterol esters, and by so doing maintaining the ability of retained APOA-I to enhance RCT (Morgan *et al*., 2004).

Additionally, clinical studies have reported that niacin treatment elucidates an increase in HDL-C levels of 15-35% and lowers triglycerides levels 20-50%, lipoprotein 24-38%, and LDL-C 5-25% (Carlson *et al.*, 1989; Morgan *et al*., 1996; Detection and Adults, 2002). The results obtained in this study seem to correlate with those of the clinical trials. The slight decrease in LDL-C levels reported in this study can be attributed to the fact that niacin appears to have a modest LDL-C lowering efficiency (Morgan *et al.*, 2004). Under niacin treatment, LDLs are known to increase in particle size, from small LDL to the less atherogenic, large LDL subclasses, (Morgan *et al.*, 2004). One of the niacin mechanisms is to inhibit the biosynthesis of triglycerides by blocking fatty acid build-up and fatty acid esterification, causing a decrease in APOB production, thus decreasing the secretion of APOB containing VLDL particles (Jin *et al*., 1999; Morgan *et al*., 2004). The VLDL particle transforms to IDL and eventually to LDL, therefore a decline in VLDL levels might also result in decreased LDL-C levels (Tavintharan and Kashyap, 2001). In this study, a 22.90% decrease in LDL-C levels was observed, which falls within the range reported in the literature (Carlson *et al*., 1989; Morgan *et al*., 1996; Detection and Adults, 2002). However, this decline was not statistically significant in contrast to the results reported by Chauke and coworkers (2014). This might have occurred due to niacin increasing the LDL particle size more than clearing it, which also leads to a reduced chance of developing CVDs (Lamarche *et al*., 2001).

The other two lipogram parameters measured in this study were total cholesterol and triglycerides, of which only the former had a statistically significant decrease at T_4 and a statistically significant increase at T_5 in plasma concentrations. At T_4 (0.03) decline in total cholesterol was reported, this was followed by a significant increase at T_5 (0.05) (Figure 2.2). There was a 17.28% reduction in

the levels of total cholesterol at T_1, however, a 27.86% increase was observed at the end of T2. The triglycerides were also minimally reduced by 37.84% at T_1. Then there was a 6.44% increase, that was followed by a trend of decreasing values (Figure 2.5).

Hypercholesterolaemia has been recognized for a long time as one of the risks of developing CVDs, especially CHD (Carlson, 2005). At the time when this discovery was made, the only available effective drug treatment to lower cholesterol was niacin (Carlson, 2005). Subsequent studies revealed that cholesterol-lowering was somehow linked to the lowering of LDL, while the reduction of triglycerides was virtually exclusively due to the decline in VLDL (Carlson, 2005). Vervet monkeys are known to respond to dietary cholesterol in a manner that is comparable to humans, which would also explain the trend of a slight decrease in both cholesterol and LDL-C levels reported in this study (Moghadasian *et al.*, 2001). Furthermore, the similarities that appear to exist between humans and vervet monkeys, in terms of the intravascular metabolism of cholesterol are further enhanced by having an active cholesteryl ester transfer system (Fusegawa *et al*., 2001). Niacin administration is reported to result in a significant decrease in triglycerides levels by hindering the movement of fatty acids from adipose tissue as well as hepatic synthesis of fatty acids and triglycerides (Kamanna and Kashyap, 2000). This might not be the case in the vervet monkeys, as they are known to be at the lower end of the triglycerides spectrum (Rudel *et al*., 1995). Additionally, in this study, the triglycerides levels fluctuated throughout the treatment period, this could have also contributed to the final results. In monkeys, the triglycerides for cholesteryl ester exchange system occurs at a lesser rate as in humans, leading to subsequent triglycerides removal via lipoprotein lipase being reduced (Wallace *et al*., 2005). Though LDL-C results were not statistically significant, it was interesting to observe a correlation between the results obtained from this study and those previously reported by Chauke *et al*., 2014, which further validates the vervet monkey as a suitable model for niacin mechanisms.

2.6 CONCLUSION:

In this study, niacin treatment resulted in biochemical changes in the selected vervet monkeys. The results revealed that niacin treatment was responsible for statistically significant reduction in total cholesterol and significant increase in HDL-C levels, while there were minal decreases in LDL-C, and triglycerides. These results further confirm that niacin is the most potent available drug to increase HDLs. Although there have been reported side effects associated with niacin administration, the treatment in this study did not present any observable side effects and there were no signs of ill-health or discomfort. This response validated the efficacy of the 100 mg/kg niacin dose and the suitability of the vervet monkey model to study atherosclerosis. However, these results will be correlated with genomic analysis, which is discussed in subsequent chapters. To be able to understand the shift in LDL particle size when niacin is administered, studies that examine the particle size such as gel electrophoresis quantification are needed. The effect of niacin administration on the expression of genes such as *APOB-100* would be worth studying, as this is a big component of the LDL particle.

CHAPTER THREE:

The effect of PCSK9 on lipid metabolism and the role of its genetic mechanisms on the control of cholesterol in a non-human primate model of atherosclerosis.

3.1 INTRODUCTION

Atherosclerosis involves several genetic factors, as well as established abnormalities of lipid metabolism (Indolfi, 2002). The contribution of environmental factors (diet or smoking) to the development of atherosclerosis cannot be underplayed, however, genetic factors are more significant determinants of atherosclerotic CVD risk (Kovacic and Bakran, 2012). Additionally, the emergence of genome-wide association studies (GWAS) has revealed that genes have a substantial role in the development of CVDs (Lusis, 2012). Various reports have also associated sequence variants identified in candidate genes with LDL and HDL cholesterol levels and being predisposed to CHD (Breslow, 2000). These are mainly genes that have a profound impact on the lipid profile, such as *LDLR*, *CETP*, apolipoproteins (*A-5* or *B* or *C-III* or *E*) and *ABCA1* (Kolovou *et al.,* 2005, Kolovou and Anagnostopoulou, 2007, Kolovou *et al.,* 2007, Wojtczak and Skretkowicz, 2008, Kolovou *et al.,* 2009, Kolovou *et al.,* 2010, Junyent *et al.,* 2010, Kolovou *et al.,* 2012). The number of these candidate genes is continuously growing, leading to the identification of more sequence variants and susceptibility loci aligned with atherosclerotic diseases (Kovacic and Bakran, 2012). Scientists continue to have a substantial interest in uncovering more genetic risk factors and this trend seems to be on the upward trajectory (Kovacic and Bakran, 2012).

Single gene disorders are reported to be some of the most noteworthy instances of the genetic involvement in atherosclerosis (Milewicz and Seidman, 2000). As stated in chapter 1, several monogenic disorders have been implicated in the elevation of plasma levels of LDL by attenuating the activity of hepatic LDL receptor, resulting in a reduction of the clearance of LDLs from plasma (Roy *et al.,* 2009). The *PCSK9* gene has come to prominence in recent times due to its reported function in the clearance of LDLs (Miyake *et al.,* 2008), and it is based on this that this gene is the primary focus for the current study. *PCSK9* has been associated with the formation of hypercholesterolemia resulting from GOF single nucleotide mutations (Miyake *et al.,* 2008). On

the other hand, nonsense mutations account for approximately a 40% reduction in LDL-C (Cohen *et al.,* 2005). Subsequent studies where HepG2 cells were infected with mutant *PCSK9* constructs, revealed that LOF sequence variants identified in *PCSK9* can elevate the levels of LDLR particles in circulation, while the GOF variants are responsible for the drop in LDLR levels (Cameron *et al.,* 2006). A sequence variant (E670G) of *PCSK9* was reported to be a significant determining factor of plasma LDL-C levels and was related to the development of severe coronary atherosclerosis (Chen *et al.,* 2005) and large-vessel atherosclerosis stroke (Abboud *et al.,* 2007). Although there have been a larger number of studies investigating the genetics of PCSK9, not many have studied the effects of its mutations in response to drug treatment.

Several single nucleotide polymorphisms (SNPs) have also been identified in other genes that are involved in lipid metabolism. Sequence variants reported in *APOB-100* gene, play a role in reducing the binding of APOB-100 to LDL receptors, leading to the formation of APOB-100 familial ligand defect disorder (Salek and Marian, 2001). SREBPs are controlled by SREBP cleavage activating protein (SCAP) which mediates cholesterol and fatty acids metabolism (Roy *et al.,* 2009). A mutation identified in *SREBP-2* (G1784C) was reported to be aligned to increased intima-media thickness (IMT) in carotid arteries (Robinet *et al.,* 2003). This mutation predisposes patients to early-onset myocardial infarction (MI), while the *SCAP* (A2386G) polymorphism appears to change the linkages of SREBP-2 genotype with MI (Friedlander *et al.,* 2008). The *CETP* gene is known for facilitating the conversion of cholesteryl esters from triglycerides between HDL and triglycerides-rich lipoproteins (Roy *et al.,* 2009). This gene has a well-known sequence variant, affecting the 277th nucleotide in intron 1, which has been associated with lower levels of plasma CETP, resulting in increased HDL-C levels and lower CVD risk in people with this variant (Ordovas, 2000, Brousseau *et al.,* 2002, McCaskie *et al.,* 2007). However, other studies have revealed that *CETP* SNPs are associated with increased carotid atherosclerosis (Kakko *et al.,* 2000), CVD (Boekholdt *et al.,* 2005, Borggreve *et al.,* 2006) and MI (Meiner *et al.,* 2008).

Non-human primate models that harbour sequence variants, which affect various aspects of the lipid pathway cycle, have become invaluable models to study the genetic and biochemical techniques that are similar to disorders of mankind (Vilahur *et al.,* 2011). Thus far, NHPs have been used in several CVD related studies. However, the aim of most of these investigations is to

acquire a better understanding of biochemical mechanisms and the types of mutations that underscore heritable variations of lipoproteins, as opposed to replicating the exact genetic variants that are found in humans (Chauke, 2012). Therefore, the vervet monkey was further confirmed as a suitable model to study the genetic mechanisms in association with niacin drug intervention (Chauke, 2012). In this study, five genes that are implicated in the development of CVDs were prioritized for downstream analysis. The possible involvement of these prioritized candidate genes and their polymorphisms in CVD was assessed by relating them to biochemical factors such as HDL-C and LDL-C. Drug response (niacin) was also correlated to gene expression or SNPs, which is discussed in detail in this chapter.

3.2 MATERIALS AND METHODS

3.2.1 DNA extraction and quantification

DNA samples were extracted from blood collected from 16 individuals (see chapter 2, section 2.2.5) using NucleoSpin® Blood kit (Macherey-Nagel, Düren, Germany) as per the manufacturer's instructions. The extraction procedure started by adding 200 µl of blood, 25 µl of Proteinase K and 200 µl of lysis buffer into 1.5 ml Eppendorf tubes, then vortexing for 15 seconds, and incubating at 70°C for 15 minutes. Preheated elution buffer (70°C) was added and resulted in 100 µl DNA being eluted. The DNA concentration and purity of the extracted samples were determined by measuring the absorbance at 260/280 nm and 260/230 nm in a Nanodrop 2000 spectrophotometer (Thermo Fisher, Massachusetts, USA). The spectrophotometer was initialized by blanking with 2 µl of nuclease-free water onto the pedestal of the spectrophotometer. Thereafter, 2 µl of the DNA sample was measured and the absorbance determined. Each sample was measured three times then the readings were averaged.

3.2.2 Bioinformatics and candidate gene selection

The prioritized gene sequences were retrieved from National Center for Biotechnology Information (NCBI), University of California Santa Cruz (UCSC) genome browser and Ensembl databases (Table 3.1), these genes were prioritized based on their reported functions in lipid metabolism. Primers for each gene were designed using NCBI primer design tool (Appendix H, Table H1). Since the vervet (*Chlorocebus aethiops*) reference genome has not yet been annotated,

the green monkey (*Chlorocebus sabaeus)* genome sequence from NCBI (https://www.ncbi.nlm.nih.gov/gene) was used as a reference for sequencing alignment. Sequencing results were analyzed using the following bioinformatics tools: Chromas lite software (https://technelysium.com.au/wp/chromas/), CLC DNA Workbench (CLC Bio, Aarhus, Denmark), ClustaW2 (https://www.genome.jp/tools-bin/clustalw) and ExPASy translate tool (https://web.expasy.org/translate/).

Table 3.1: The selected candidate genes

Gene	Species	NCBI accession	Chromosome location	Number of exons
PCSK9	Green monkey	XM_007978607.1	20	12
LDLR	Green monkey	XM_007996082.1	6	13
SREBP-2	Green monkey	XM_007975929.1	19	19
CETP	Green monkey	XM_007993413.1	5	17
APOB-100	Green monkey	XM_007971352.1	14	30

3.2.3 PCR amplification, purification and sequencing analysis

The *PCSK9, CETP, SREBP-2, APOB-100 and LDLR* genes were amplified by polymerase chain reaction (PCR) using GoTaq® green master mix (Promega, Wisconsin, USA) (Table 3.2). The PCR conditions adopted from (Khoza *et al.,* 2019) consisted of the following steps; 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 40-70°C for 30 seconds and 72°C for 1 minute. An extension period of 5 minutes at 72°C completing the procedure (Table 3.3). The PCR products were subjected to gel electrophoresis (2%) to confirm the band size for each exon. The amplicons were further purified using Wizard® SV Gel and PCR clean-up system (Promega, Wisconsin, USA). The purified PCR product was eluted in water and sent for bidirectional Sanger sequencing. The impact of the sequence variants identified was determined by using the following mutation prediction tools: Mutation taster (http://www.mutationtaster.org/), SIFT (http://provean.jcvi.org/index.php) and PolyPhen 2 (http://genetics.bwh.harvard.edu/pph2/).

Table 3.2: PCR reaction used for genotyping

Reagent (Promega)	Volume (µl)	Final Concentration
PCR Master Mix, 2X	12.5	1X
Forward primer, 10µM	1	0.4 µM
Reverse primer, 10µM	1	0.4 µM
DNA template	1	50 ng
Nuclease-Free Water	9.5	N.A
Total	**25**	**N.A**

Table 3.3: PCR program used for genotyping

Step	Temp (° C)	Time	Cycles
Denaturation	95	5 min	1
Denaturation	95	30 sec	30
Annealing	40-70	30 sec	
Extension	72	1 min	
Extension	72	5 min	1

3.2.4 Gene expression

3.2.4.1 RNA extraction, purification and integrity

The PAXgene Blood RNA Kit (Qiagen, Venlo, Netherlands) was used to isolate and purify intracellular RNA from whole blood collected in the PAXgene Blood RNA Tubes (BRT) as per manual instructions. The purification procedure began with a centrifugation step to pellet nucleic acids in the BRT. The pellet was washed and resuspended, followed by RNA purification. The resuspended pellet was incubated in an optimized buffer together with proteinase K to bring about protein digestion. Additional centrifugation through the PAXgene Shreder spin column was carried out to homogenize the cell lysate and remove residual cell debris, and the supernatant of the flow-through fraction was transferred to a fresh micro-centrifuge tube. Ethanol was added to adjust binding conditions, and the lysate was applied to a PAXgene RNA spin column. During brief centrifugation, RNA was selectively bonded to the PAXgene silica membrane as contaminants pass through. Remaining contaminants were removed in several efficient 'wash' steps up until RNA was eluted in elution buffer and heat-denatured at 65°C for 5 minutes.

The TURBO DNase kit (Invitrogen, Massachusetts, USA) was used according to the manufacturer's instructions to remove trace amounts of bound DNA. The concentrations of the purified RNA samples were determined using a Nanodrop 2000 spectrophotometer. The integrity of the RNA samples was further confirmed using Agilent 2100 bioanalyzer (Agilent Technologies, California, USA) in conjunction with the RNA 6000 Nano-kit as recommended by the manufacturer (Appendix I, Table I1). The Agilent 2100 bioanalyzer is an improved analytical tool for total RNA analysis and offers several advantages over agarose gel electrophoresis, the traditional method of assessing RNA quality. The bioanalyzer uses a combination of microfluidics, capillary electrophoresis, and fluorescence to evaluate both RNA concentration and integrity. The software assigns an RNA integrity number (RIN) to the RNA sample, which is a numerical assessment of the integrity of the RNA sample. These RIN values are taking into account the entire electrophoretic pattern of the RNA sample (28S:18S ratio) and the presence or absence of degradation products. The RIN ranges from 1 to 10, with 10 representing high-quality RNA**.**

3.2.4.2 cDNA generation

The purified total RNA samples were reverse transcribed into cDNA using the High-Capacity cDNA kit (Thermo Fisher, Massachusetts, USA). One microgram DNase treated RNA sample was added to 10 µl RNase-free water and placed on ice. A reaction mix consisting of reaction buffer, dNTPs, random primers, RNase-inhibitor (5000 units/ml), reverse transcriptase and nuclease-free water was prepared into two separate tubes labelled RT plus and RT minus (Table 3.4). The RT minus reaction mix tube (negative control) contained the same reaction mix as the RT plus tube, but the reverse transcriptase enzyme was replaced by water. After adding the RT plus and RT minus mix components, the prepared reaction was mixed by pipetting and the tubes centrifuged briefly**.** The conversion of RNA to cDNA was achieved by using a thermocycler, which applied the following conditions: 25°C for 10 minutes followed by 37°C for 120 minutes and 85°C for 5 minutes to complete the cDNA reaction. The converted cDNA was confirmed by PCR as described above using Actin beta (*ACTB*) as a housekeeping gene.

Table 3.4: cDNA reaction mixture

Master mix components	RT plus reaction (µl)	RT minus reaction (µl)
1 µg DNase-treated RNA	10 X rxn	10 X rxn
10 x RT buffer	2	2
25 x dNTP mix	0.8	0.8
10 X random primers	2	2
RNAse inhibitor	1	1
Nuclease Free water	3.2	4.2
Reverse transcriptase	1	0
Total	**10**	**10**

3.2.4.3 Quantitative real-time PCR (qRT-PCR)

The RT^2 qPCR primers assays (Qiagen, Venlo, Netherlands), designed for SYBR® Green-based RT-PCR detection (Applied Biosystems, California, USA), were used for *PCSK9* (PPQ16344A), *CETP* (PPH01386F), *SREBP-2* (PPH00240F), *APOB-100* (PPH02626E) and *LDLR* (PPQ00180A) (Table 3.5). The qRT-PCR standards were prepared using human liver total RNA (Takara, California, USA) and samples were run in duplicates using Applied Biosystems universal cycling conditions; 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Since SYBR green master mix was used, a melting curve was included in the run. Data were normalized using two housekeeping genes; *ACTB* (PPQ00182A) and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*: PPQ08645A). The $2^{-\Delta\Delta Ct}$ method was used to calculate relative mRNA expression levels.

Table 3.5: Selected candidate genes for mRNA expression assays

Gene	Gene Symbol	Species	Map position	Assay ID
Proprotein convertase subtilisin/kexin type 9	*PCSK9*	Rhesus macaque	1p32.3	PPQ16344A
Low-density lipoprotein receptor	*LDLR*	Rhesus macaque	19p13.2	PPQ00180A
Sterol regulatory element-binding protein-2	*SREBP-2*	Human	22q13.2	PPH00240F
Cholesteryl ester transport protein	*CETP*	Human	16q13	PPH01386F
Apolipoprotein-100	*APOB-100*	Human	2p24.1	PPH02626E
Actin beta	*ACTB*	Rhesus macaque	7p22.1	PPQ00182A
Glyceraldehyde 3-phosphate dehydrogenase	*GAPDH*	Rhesus macaque	12p13.31	PPQ08645A

3.2.4.4 Statistical analysis

The gene expression analyses data of *PCSK9, LDLR, SREBP-2, CETP, APOB-100* generated in this study were presented as means ± SD. Multiple comparison analysis was used to determine statistical significance at each time point. Two-way ANOVA in the GraphPad Prism program (California, USA) was used to compare changes from baseline (T_0) to washout period (T_5) between the experimental and control groups. Statistical significance was calculated by using the Student *t*-test, and a value of $P < 0.05$ was considered significant.

3.3 RESULTS

3.3.1 Genotyping analysis

Sanger sequencing results revealed eight missense mutations in *PCSK9* (R148S, H177N), *LDLR* (V19A, D274N), *CETP* (M92L, T413) and *APOB-100* (A647T, F635L); and four silent/synonymous mutations in *PCSK9* (T112T), *CETP* (L63L, T441T) and *SREBP-2* (P565P). Single nucleotide polymorphisms (SNP) were also identified in *PCSK9* (G635G) (Table 3.6).

Table 3.6: Identified sequence variants in the colony of captive-bred vervet monkeys

Genes	Exons	Nucleotide change	Amino acid change	Type of mutation	SIFT	PolyPhen 2	Mutation Taster	Affected animals
PCSK9	1	G337A	T112T	Silent	N/A	N/A	N/A	1
	1	C529A	H177N	Missense	Neutral	Benign	Polymorphism	8
	1	C442A	R148S	Missense	Neutral	Benign	Polymorphism	4
	10	T1905G	G635G	SNP	Neutral	Benign	DC	13
LDLR	1	T56C	V19A	Missense	Neutral	Benign	Polymorphism	16
	6	G820A	D274N	Missense	Deleterious	Benign	DC	13
SREBP-2	9	C1695A	P565P	Silent	Neutral	ND	DC	5
CETP	1	C189T	L63L	Silent	N/A	N/A	N/A	2
	2	A274T	M92L	Missense	Neutral	Benign	Polymorphism	4
	12	A1237G	T413A	Missense	Neutral	PD	Polymorphism	3
	13	C1323T	T441T	Silent	Neutral	ND	Polymorphism	13
APOB-100	14	T1903C	F635L	Missense	Neutral	Benign	DC	5
	14	G1939A	A647T	Missense	Neutral	PD	DC	5

Disease-causing (DC), Possibly Damaging (PD), not determine (ND), not applicable (N/A).

Screening of the *PCSK9* gene indicated three sequence variants in exon 1, the first being a homozygous missense variant (R148S) (Figure 3.1) that resulted from C442>A transversion in four animals (Table 3.6). Three of the four affected animals belong to the control group (Appendix B, Table B1) and this mutation was predicted to be a polymorphism by Mutation taster®. The other mutations in the same exon included H177N which affected eight individuals and a silent (T112T) that occurred as the result of G337>A in one animal (Table 3.6). In exon 10, a homozygous SNP/silent sequence variant (G635G) was identified in 13 individuals.

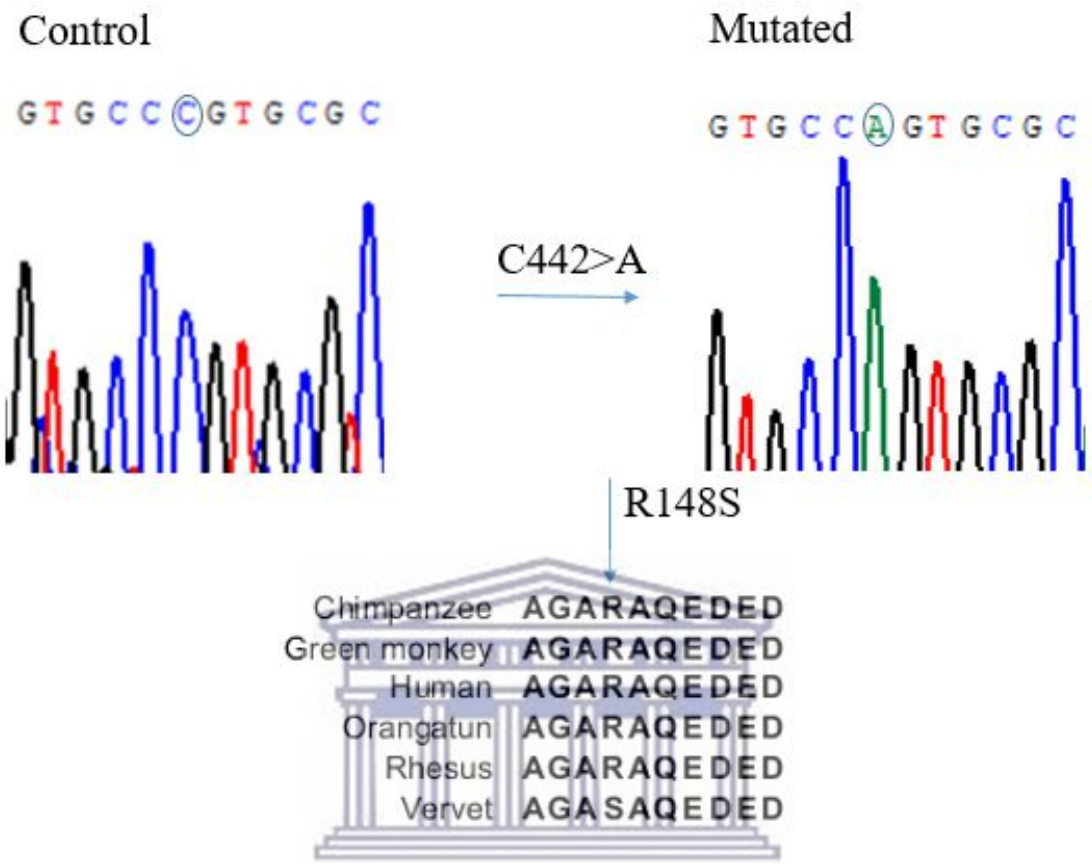


Figure 3.1: Genetic analysis for *PCSK9* in captive-bred vervet monkeys. Sequence chromatogram is showing a nucleotide change at codon 422 (C442>A) located in exon 1. The circles on the chromatographs and the arrow on the protein alignment (R148S) indicate the position of the missense mutation.

Analysis of *LDLR* revealed a homozygous missense mutation/SNP (V19A) which resulted from T56C transition in all the selected animals. This variant was predicted to be neutral by SIFT mutation prediction tool. Another *LDLR* missense sequence variant was identified in exon 6 (G820>A) (Figure 3.2) leading to negatively charged aspartic acid being replaced by polar asparagine at position 274 (D274N). The mutation affected 13 individuals and was predicted to deleterious or diseases causing.

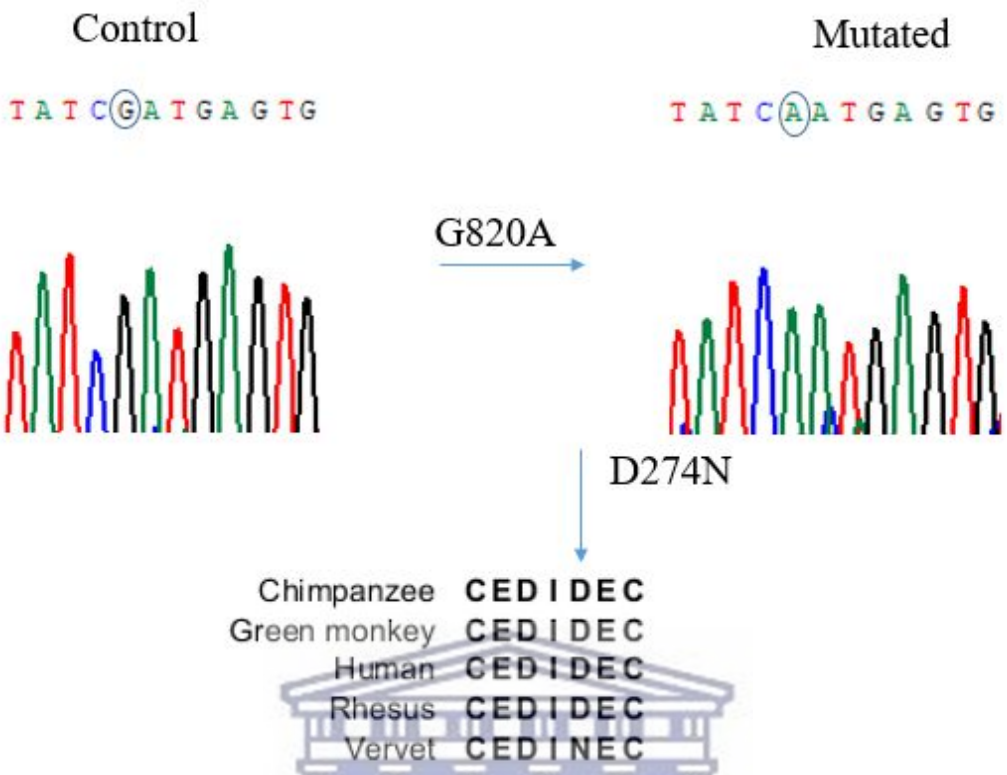


Figure 3.2: Genetic analysis for *LDLR* in captive-bred vervet monkeys. The chromatographs depict a nucleotide change (G820>A) identified in exon 6. The circles on the chromatographs and the arrow on the protein alignment (D274N) indicate the position of the missense mutation.

In *SREBP-2*, one homozygous silent sequence variant in exon 9 (P565P) was identified in five individuals (Table3.6). Mutation taster® predicted that this mutation is disease-causing. The analysis of *CETP* gene revealed two missense mutations in exon 2 (M92L) and exon 12 (T413A) (Figure 3.3), which resulted from A274>T and A1237>G nucleotide changes in four and three individuals, respectively (Table 3.6). Two other sequence variants were also identified in the same gene, these are two silent variants in exon 1 (L63L) and 13 (T441T) (Table3.6) that accounted for two and 13 animals, respectively. Additionally, APOB-100 sequencing analysis indicated two homozygous missense (F635L and A647T) (Figures 3.4 a and b) in exon 14. Both these variants affected the same five animals. They were both found to be polymorphism by the Mutation Taster prediction tool.

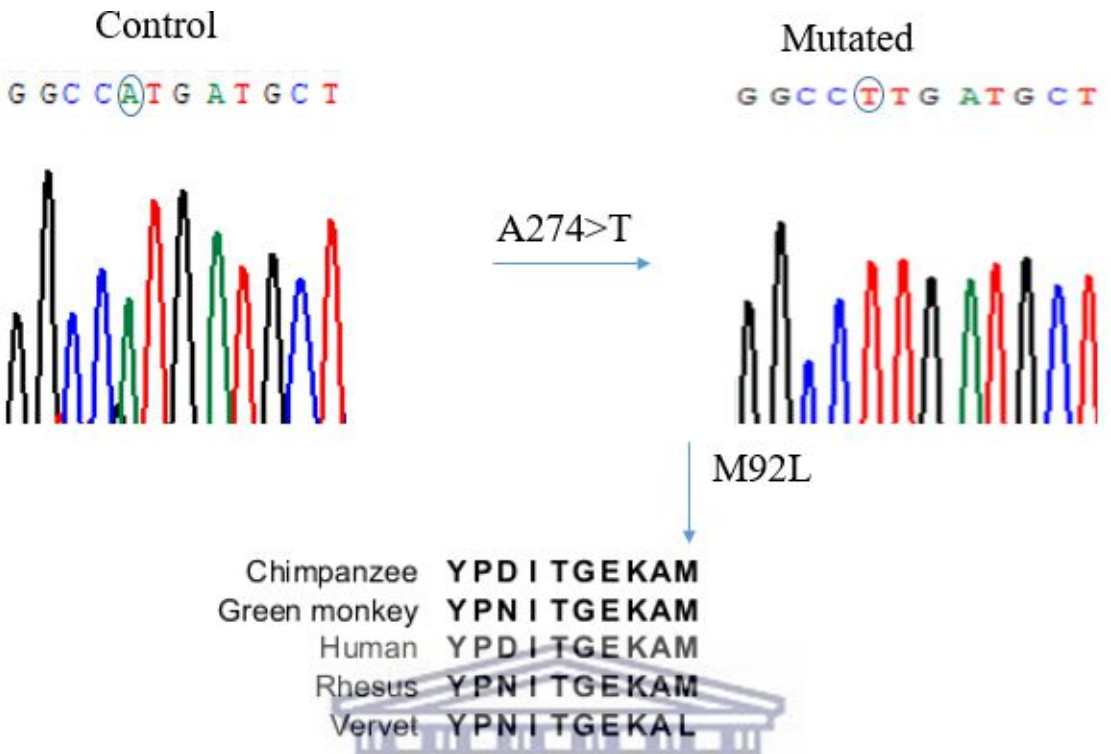


Figure 3.3: Genetic analysis for *CETP* gene in captive-bred vervet monkeys. Sequence chromatogram and protein alignment showing a missense mutation (A274>T) located in exon 2. The circles on the chromatographs and the arrow on the protein alignment (M92L) indicate the position of the mutation.

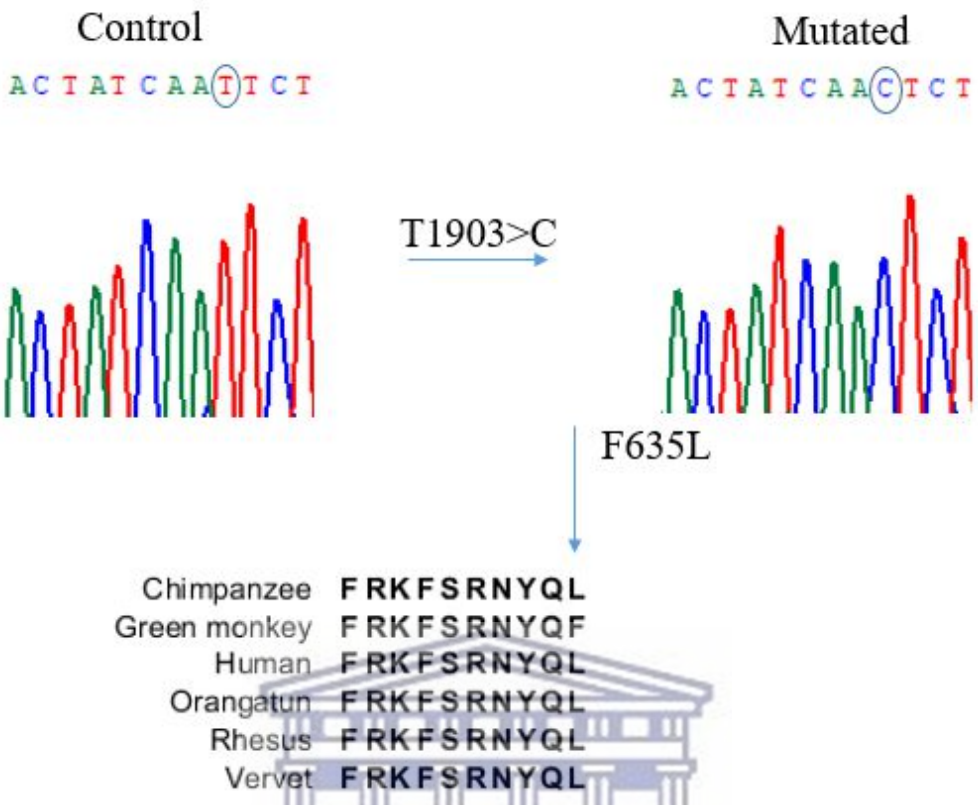


Figure 3.4a: Genetic analysis for *APOB-100* gene in captive-bred vervet monkeys. The chromatograms are showing a nucleotide change in codon 1903 (T1903>C) identified in exon 14. The circles on the chromatographs and the arrow on the protein alignment (F635L) indicate the position of the missense mutation.

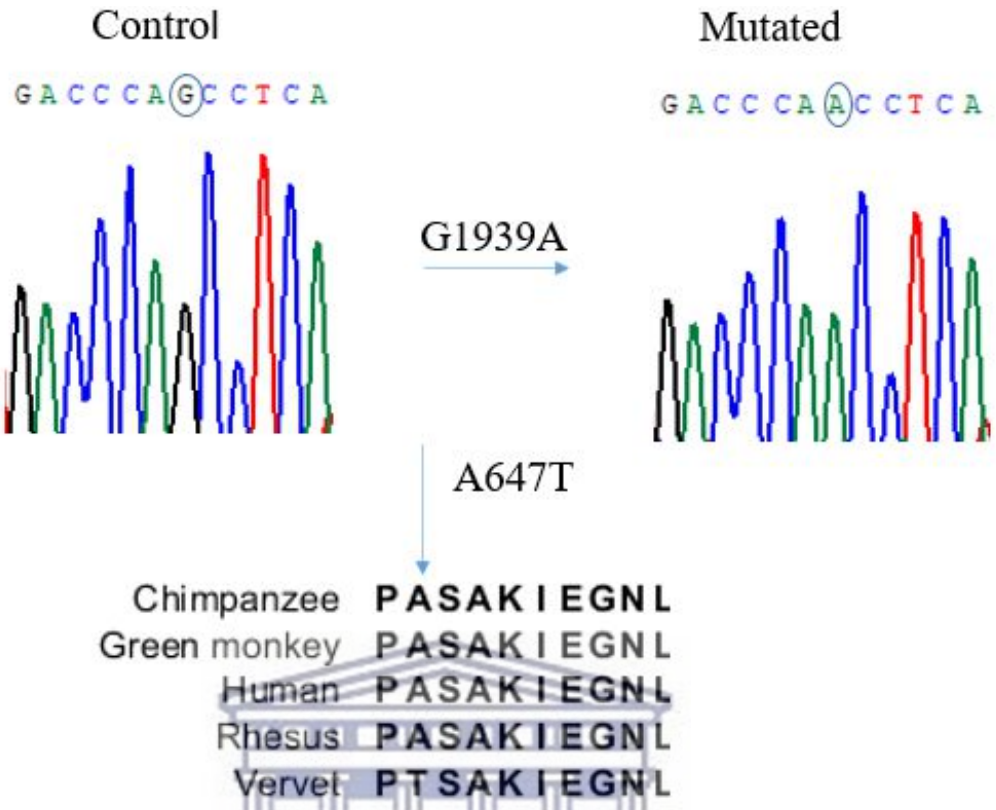


Figure 3.4b: The chromatographs are illustrating a nucleotide change (G1939>A) identified in exon 14 of *APOB-100.* The circles on the chromatographs and the arrow on the protein alignment (A647T) indicate the position of the missense mutation.

3.4 Gene expression analysis

Gene expression findings are discussed in a twofold manner; firstly the niacin treated animals were compared with the controls, then the expression was analyzed based on variant-carrying subjects and wild-type individuals. The niacin SR administration did not produce any statistically significant changes on the expression of *PCSK9* gene in the treated group, with *p*-values of 0.96 and 0.25 at T_1 and T_4 (Appendix J, Table J1a). However, it was able to account for a slight decrease from baseline T_0 to T_3 (14.56%, 7.22% and 5.23%), respectively (Figure 3.5). These respective declines were followed by minimal increases of 3.08 % and 9.96 % from T_4–T_5. The mRNA expression of the untreated group fluctuated throughout the intervention period. The sequence variants identified in this gene further revealed that all affected animals had a decreased *PCSK9* expression when compared with the wild-types. The gene expression of these LOF variants (T112T, H177N and R148S) (Table 3.6), which are depicted in appendix K (Figures K1a-c), were computed for only T_0.

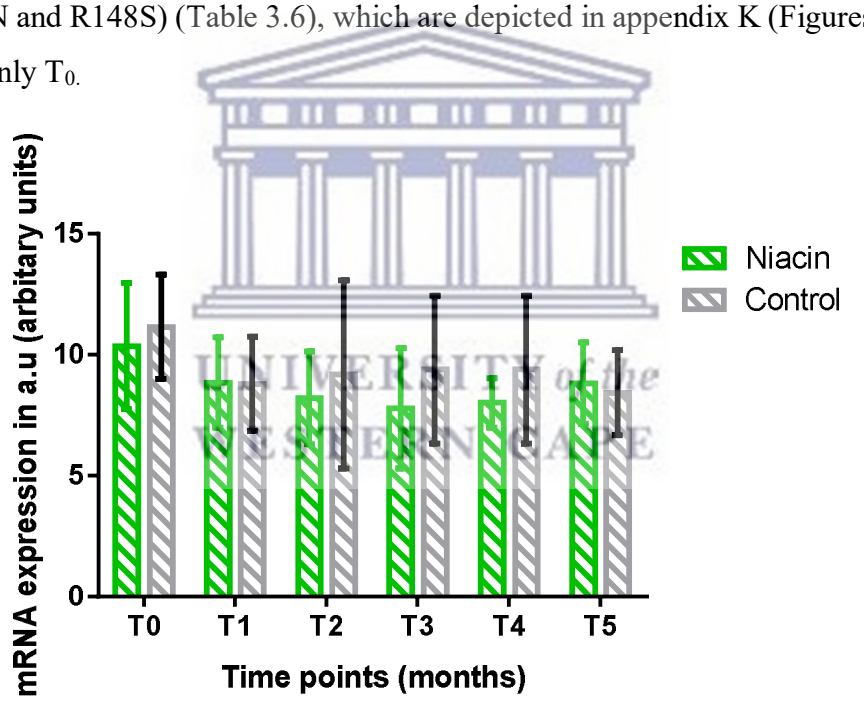


Figure 3.5: *PCSK9* mRNA gene expression in captive-bred vervet monkeys. Gene expression of the control group was compared with the experimental group. The controls were given a maintenance diet while the experimental group received 100 mg/kg of niacin in their food bolus for a period of 12 weeks. The data was expressed as mean ± SD and mRNA expression in a.u. (arbitrary units).

For *LDLR* gene expression, niacin treatment led to a continuous decrease in mRNA expression from T_0-T_4 (Figure 3.6). However, this constant decrease was not statistically significant *p*-values (0.22, 0.50, 0.89, 0.39 and 0.38). The removal of the treatment was responsible for a 35.57% increase in the expression at the end of T_5. The control group had an increase from T_0 to T_1, this was followed by constant decreases until T_4, after which the washout period brought about a slight increase in the expression. Even though there were variations in both groups, this was not statistically significant. Additionally, the deleterious predicted D274N mutation identified in *LDLR* gene resulted in decreased mRNA expression in mutated subjects as compared to the wild-types (Appendix L, Figure L1).

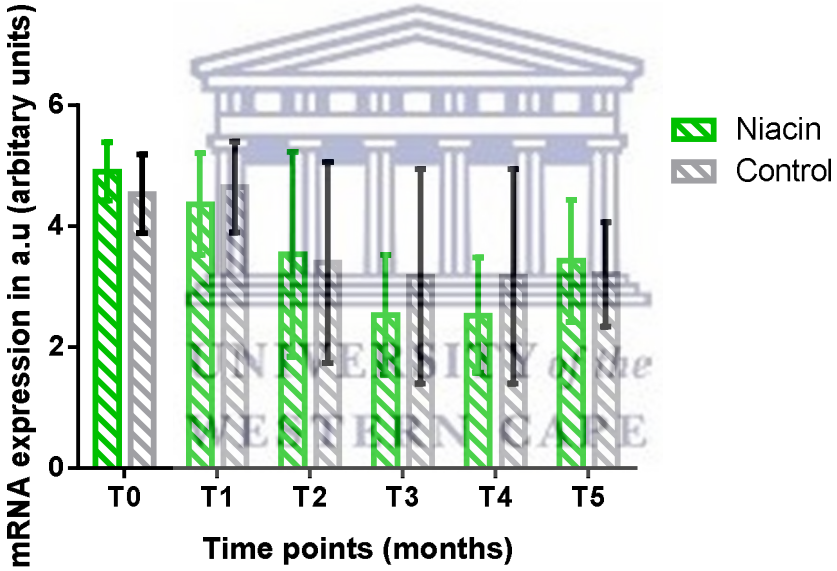


Figure 3.6: *LDLR* mRNA gene expression in vervet monkeys. The control group (maintenance diet) was compared with the experimental group (100 mg/kg of niacin) at T_0-T_5. The data was expressed as mean ± SD and mRNA expression in a.u. (arbitrary units).

Expression analysis of *SREBP-2*, a gene involved in the synthesis and regulation of cholesterol revealed two expression patterns in the treated and control groups (Figure 3.7). In the treated group, a gradual decline occurred between T_0 and T_4. Thereafter, there was a statistically significant (p= 0.05) increase in gene expression after the washout period. In the control group, a gradual decline occurred from T_0 until T_3, while an increase occurred at T_4 and T_5. Furthermore, P565P silent

mutation found in *SREBP-2* resulted in monkeys with this variant having a higher gene expression to those without it (Appendix M, Figure M1).

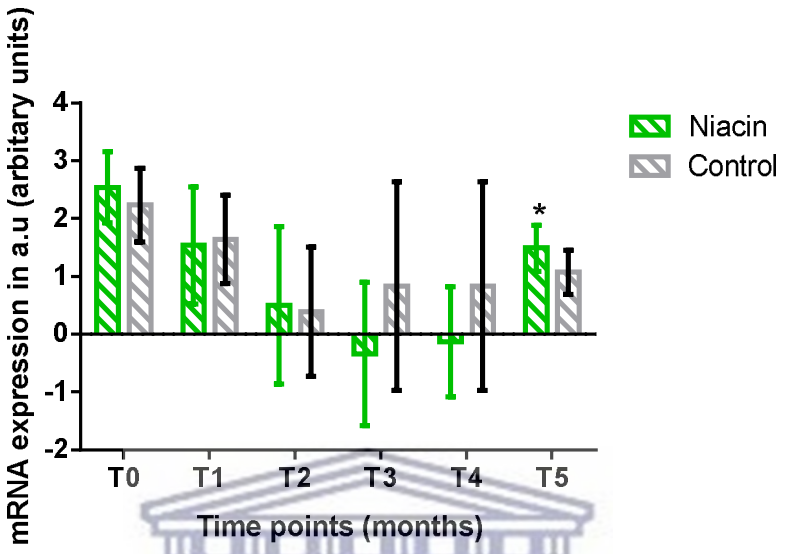


Figure 3.7: ***SREBP-2*** **mRNA gene expression in captive-bred vervet monkeys.** Gene expression of the control group was compared with the experimental group at T_0-T_5. The data was expressed as mean ± SD and mRNA expression in a.u. (arbitrary units).* indicates a statistical significance difference (p < 0, 05).

Expression of the *CETP* gene followed similar patterns for both groups from T_0-T_3, with the experimental animals experiencing declines of 29.75% and 16.94% at T_1 and T_2, respectively, which was statistically significant at T_1 (p=0.01) (Figure 3.8). This was followed by a statistically non-significant (p=0.71) increase at T_3. Thereafter, a statistical significant decrease in expression was observed at T_4 (p=0.01). At the end of T_5, the *CETP* gene expression had increased above the T_0 value. *CETP* gene expression remained constant between T_3 and T_4 and slightly declined at T_5 in the control group. In addition to this, the impact of M92L variant in affected subjects revealed an increased mRNA expression in comparison to the wild-types (Appendix N, Figure N1).

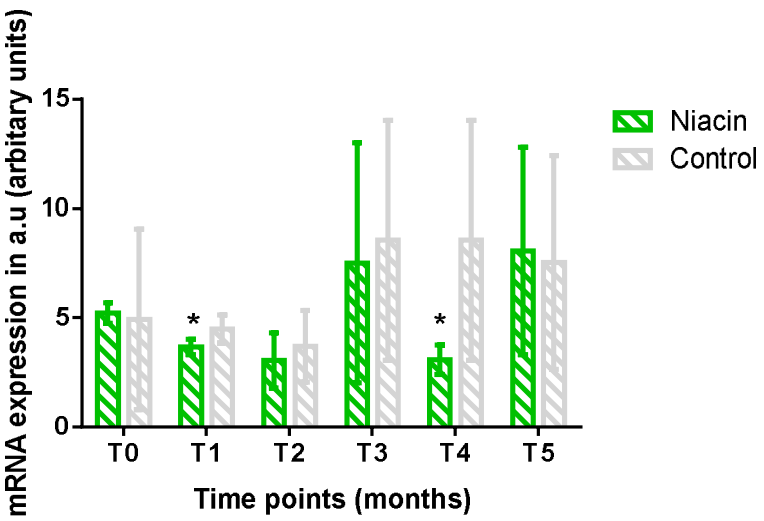


Figure 3.8: ***CETP*** **mRNA gene expression in captive-bred vervet monkeys**. Gene expression of the control group was compared with the experimental group at T_0-T_5. The data was expressed as mean ± SD and mRNA expression in a.u. (arbitrary units). * indicates statistical significance ($p < 0, 05$).

With respect to *APOB-100* gene expression, the niacin treated group had gradual decreases of 12.80% and 20.73% from T_1 until T_2. After that, there was a 24.16% (T_3) increase during the treatment period. Then again an increase of 26.71% at T_4 was observed. During the washout period, the expression levels of *APOB-100* increased by 15.27%. A similar gene expression pattern between the control and the treated group was observed at T_0-T_3 while there were variations in the subsequent time points (Figure 3.9). Two *APOB-100* mutations (F635L and A647T) were further analyzed with regards to gene expression, where animals with the mutations were compared to the wild-types. This analysis revealed that the monkeys affected with these variants had a slightly increased mRNA expression as compared to monkeys without the variants (Appendix O, Figure O1).

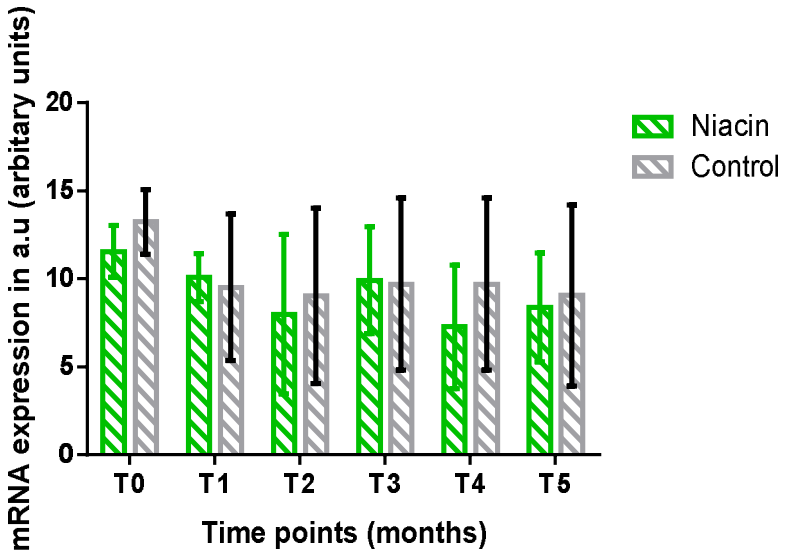


Figure 3.9: *APOB-100* mRNA gene expression in captive-bred vervet monkeys. Gene expression of the control group was compared with the experimental group. The data was expressed as mean ± SD and mRNA expression in a.u. (arbitrary units).

3.5 DISCUSSION

In utilizing a combination of molecular strategies, this study aimed at obtaining findings that would have a synergic understanding. Sixteen vervet monkeys were screened to discover sequence variants that might predispose or shield them from developing CVDs. The screening for mutations in five prioritized genes known to have critical influences in lipid metabolism was coupled with their gene expression analysis, which was correlated with the animal intervention discussed in chapter 2. This is because genes such as *PCSK9, LDLR*, and APOB-*100* are heavily involved in the control of LDL-C, *CETP* is mainly for HDL-C, while *SREBP-2* is involved in the synthesis of both types of cholesterol (HDL and LDL). This study reports 13 sequence variants identified in *PCSK9* (T112T, R148S, H177N, G635G), *LDLR* (V19A, D274N), *SREBP-2* (P565P), *CETP* (L63L, M92L, T413, T441T) and *APOB-100* (A647T, F635L) genes (see Table 3.6).

Sanger sequencing of *PCSK9* gene revealed four mutations, with three of these being found in the first exon. One of these mutations is a missense variant (R148S) that resulted in a positively charged arginine being replaced by a polar serine (Figure 3.1). Mutation Taster® predicted that this variant is a polymorphism, while other tools indicated a neutral or benign impact. The

alignment of human *PCSK9* and the vervet monkey gene revealed that this mutation is found at codon 29 (R29S) in *Homo sapiens*. The first part of exon 1 of *PCSK9* gene is also referred to as the signal peptide (SP), which covers the first 30 amino acids of the gene. The importance of this region lies in the fact that it allows the formation of three subsequent heterodimer proteins with 3 domains (see Chapter 1, Figure 1.5) (Shapiro *et al.,* 2018). A sequence variant identified in the same region was classified as being a minor or relatively common occurrence. This mutation resulted in non-polar leucine being inserted at positions 21 and 22 (L21-22ins), this affected 15 out of 78 individuals that had low LDL-C levels (Miyake *et al.,* 2008). In this study, the R148S variant was also present in three of the control individuals with relatively low LDL-C levels; this suggested that this variant may fall in the category of LOF mutations. According to the SAMRC/PUDAC reference values for our captive-bred vervet monkeys, LDL-C levels above 2.75 mmol/L for both genders is considered high. At the end of T_4, the control and treated groups had LDL-C levels of 1.18 and 1.35 mmol/L, respectively, which further suggests that PCSK9 variants in these monkeys do not increase the chances of being predisposal to CVD related diseases. To further support this notion, the prediction tools could not predict its severity. Another variant found in exon 1 was H177N in the vervet model while it was located in the prodomain region (31-152 amino acids) (H58N) in humans. This region is known to undergo self-induced cleavage while it remains bound to the rest of the molecule (Shapiro *et al.,* 2018). This is the missense that affected eight individuals and was predicted to be a polymorphism by Mutation Taster®. There are two reported variants in the prodomain region which belongs to the LOF mutations (R46L and E57K) (Kotowski *et al.,* 2006). The former is said to be significantly associated with the reduction of LDL-C, this was reported in a study where 301 people carrying R46L variant had a 15% decline in LDL-C levels and had a 44% chance of not developing CVDs in comparison to non-carriers (Cohen *et al.,* 2006). The exchanging of negatively charged glutamic acid with positively charged lysine at position 57 (E57K) was identified only in subjects with low LDL-C (Kotowski *et al.,* 2006). In the current investigation, the H177N (human: H58N) affected five animals which were in the control group. Due to the proximity of these two variants and the fact that both are pronounced in subjects with relatively low LDL-C, we postulate that the H177N variant plays a role in preventing the development of CVDs. The last mutation to be identified in *PCSK9* is a silent/SNP at position 635 (human: 516 (G516G) of exon 10. This homozygous variant is found in the region where the interaction with other cell surface proteins occurs (Farnier, 2011). Two

mutations have been reported in the C-terminal area that is a couple of amino acids away from the SNP reported here. The first variant (A514T) was identified in one person out 96 people who were on anti-hypercholesterolemia medication. To assess the impact of this variant, the *LDLR* gene was investigated and no mutations found, meaning that this variant didn't hinder the function of LDLR (Miyake *et al.,* 2008). At position 522, alanine was replaced by threonine (A522T), and this was found only in individuals who were hypocholesterolemic, while absent in the general public with no underlying conditions, who were also screened for this variant (Fasano *et al.,* 2007). It is therefore postulated that the sequence variants reported in our study in *PSCK9* gene do fall into the category of LOF mutations, which result in the gene losing its function to degrade LDLR. This postulation is further supported by the number of affected individuals, with the control group of animals being the majority. The genotyping outcomes in this study were further correlated with gene expression findings.

One of the reported reasons behind the inability of statins to effectively clear LDL-C in certain individuals is the ability to induce PCSK9 expression (Dong *et al.,* 2010). Niacin has been considered as a mainstream treatment for elevated serum cholesterol levels due to its suppression of the expression of this gene. In this study, niacin administration led to an initial non-significant reduction in gene expression with increases following the washout period (Figure 3.5; Appendix J, Table J1a). The slight increase at the end of the washout period does point to the fact that CVD treatment is continuous, and needs to be taken regularly for quality of life to be improved. Moreover, there was a minor decrease from T_0 to T_1 in the control group, with steady increases observed subsequently until the end of the treatment. Studies have also reported the importance of hepatocyte nuclear factor 1 α (HNF1 α), which is a key inducer of *PCSK9* gene expression (Li *et al.,* 2009). This protein molecule is found 28bp upstream from sterol regulator element (SRE-1), critical sequence motif for PCSK9 transcription (Li *et al.,* 2009). Therefore, it is hypothesized that the SRE-1/HNF1 α complex can be inactivated by sequence variants or by encouraging the depletion of HNF1 α leading to reduced expression (Dong *et al.,* 2010). Furthermore, the R29S mutation (vervets: R148S, see Table 3.6) is found just one amino acid away from the HNF1 α region, this suggests that this mutation might be responsible for attenuating gene expression. Additionally, the gene expression of *PCSK9* in all the mutated animals was lowered as compared to the wild-type individuals for the three variants (R148S, H177N and G635G) (Appendix K,

Figure K1a-c). A decline in the *PCSK9* expression was observed in the monkeys carrying R148S and H177N variants. An investigation revealed that curcumin was able to reduce the expression of HNF1 α that resulted in the downregulation of *PCSK9* gene expression (Tai *et al.,* 2014). Interestingly, both niacin and curcumin are found in the same food items, such as dairy products, green vegetables, and cereal grains, therefore niacin induction may have also contributed to the suppression of HNF1 α. As expected, the removal of niacin was followed by an increase in *PCSK9* mRNA expression of about 0.75 fold-change.

The role played by the *LDLR* gene in the clearing of LDL-C is well documented; it is for this reason that the impact of sequence variants identified in this protein should be assessed. This study reports a homozygous missense mutation (V19A) which resulted from T56C transition in all the selected animals (Table 3.6). The V19A missense variant occurred as the result of two non-polar proteins (alanine replacing valine) at position 19 of exon 1 of *LDLR*, which is also named as the signal peptide just like exon 1 of *PCSK9* gene. This exon is made up of 21 amino acids, which shuttle the protein to the cell surface and is thereafter degraded upon release from the ER (Gent and Braakman, 2004). Additionally, the V19A variant identified in this study (T56C) and the published G20R (G58A) (Komarova *et al.,* 2013) are close to each other even though the latter was associated with FH. However, there is a possibility that the vervet variant (V19A) is not disease-causing especially since it was predicted to be neutral or benign by the prediction tools and the affected individuals showed a decline in LDL-C levels after being treated with niacin. This was the first published data which did not find any association between the G20R mutation and FH development (Komarova *et al.,* 2013). Furthermore, a missense sequence variant (G820A) which was predicted to be diseases causing or deleterious was identified in 13 individuals in exon 6. The identification of mutations in this region should be alarming because it contains the ligand-binding domain that facilitates lipoproteins interaction (Gu and Zhang, 2015). Similarly, C276F which is situated close to the vervet D274N was reported in five-year-old Slovenian children and was also found to be deleterious by SIFT (Klančar *et al.,* 2015). Within the binding domain of LDLR, there is a class A7 molecule, which is where both of these variants are located. The class A7 molecule is critical for the proper functioning of LDLR as it is involved in the binding of LDL particles (Gu and Zhang, 2015). It is, therefore, reasonable to hypothesize that LOF mutations identified in this region may lead to increased levels of LDL-C as the functioning of LDLR will

be distorted. It is worthy to note that LOF mutations are not desirable in the *LDLR* gene as opposed to *PCSK9*. Therefore, it is not surprising that the identified variants were predicted to be deleterious. Furthermore, the identification of deleterious D274N in *LDLR* gene could also account for the decline in gene expression. This variant may have slowed the function of degrading LDL-C and the transcription of the *LDLR*. Gene expression findings showed that the subjects harbouring the D274N variant had a 1.36-fold decline in their expression as compared to the wild-type individuals. This further suggested that the sequence variants identified in *LDLR* may have also led to a decrease in the mRNA expression.

A disease-causing homozygous silent sequence variant in exon 9 (P565P) that affected five individuals was the only mutation reported in *SREBP-2* gene (Table 3.6). This transversion occurred as the result of cytosine being replaced by adenine at position 1695 (C1695>A). Three out of the five affected animals were in the control group. The dual role played by SREBP-2 in controlling the production of cholesterol while also upregulating PCSK9 needs to be taken into account in its analysis. The sequence variant identified in *SREBP-2* was expected to have an impact on cholesterol concentrations. Consequently, the disease-causing assessment of P565P variant confirmed the expectations. This variant was found to be located in the second transmembrane domain, a region reported to be within the essential functional domains covering exons 5- 10 of *SREBP-2* (Muller and Miserez, 2002). Gene expression assessment revealed that the mutated animals were slightly higher (0.67 fold-change) compared to the wild-type group. This elevated expression was associated with the response observed in Chapter 2, where a decline in cholesterol levels was noticed. The predicted impact of P565P variant supported the notion that silent mutations can also induce incorrect mRNA splicing leading to altered protein expression and enzymatic activity by affecting mRNA stability (Cartegni *et al.,* 2002, Nackley *et al.,* 2006).

Furthermore, *LDLR* gene expression is specifically regulated by SREBP-2, as opposed to PCSK9, which is controlled by HNF1α and SREBP-2 (Dong *et al.,* 2010). Therefore, variations in SREBP-2 expression are similar to that of LDLR. In the current study, both *LDLR* and *SREBP-2* mRNA expressions had comparable trends from T_0 to T_3 (Figure 3.6 and 3.7). The decline in LDL-C and *PCSK9* expression may be due to the action of niacin as reported by Khera and colleagues (Khera *et al*., 2015b). Additionally, the intrinsic and intertwined transcriptional relationship that exists

between PCSK9, LDLR and SREBP-2 may account for the decline in LDLR. Both PCSK9 and LDLR are known to possess operational SREs in their promoter regions that get activated by changes in intracellular cholesterol levels via the SREBP mediated pathway (Tai *et al.,* 2014). The variants identified in PCSK9 may have weakened the HNF1α protein, leaving SREBP-2 to be the only transcriptional factor for both PCSK9 and LDLR. Furthermore, the reported declines in total cholesterol and LDL-C would have triggered SREBP-2 to stimulate more cholesterol synthesis. This study, therefore hypothesizes that this might have put too much strain on SREBP-2, leading to its decreased expression which culminated in downregulation at T_3-T_4. Additionally, niacin treatment could also be the reason as the gene reported a statistically significant increase at the end of the washout period. This entangled affiliation between these three genes is one of the drawbacks of statins, as they can shift the position of SREBP-2 in a way that allows upregulation of both PCSK9 and LDLR. Consequently, this leads to increased levels of circulating LDL-C (Tai *et al.,* 2014). Niacin administration is suggested in this current study to reduce both LDL-C and PCSK9 expression, this should encourage researchers to further investigate and possibly find ways to counter the decrease of LDLR.

With the ongoing conflicting findings of the role played by the *CETP* gene in the increase/decrease of HDL-C and eventually the development or lack thereof of CVDs, a lot of investigations are still required. In this study, two missense variants in exons 2 (M92L) and 12 (T413A) were identified in the *CETP* gene. The M92L variant resulted from non-polar methionine replacing a non-polar leucine at position 92 (M92L) and this affected both groups (Figure 3.3). Exon 1-2 of *CETP* fall within the promoter region of the gene which is responsible for the initiation of CETP translation (Agellon *et al.,* 1990). These two variants were predicted to be polymorphisms by Mutation Taster, while T413A was probably damaging according to Polyphen2. This observation was further supported by the gene expression analysis that revealed that the mutated animals had a slight increase compared to the wild-type subjects (Appendix N, Figure N1). The statistically significant increase in HDL-C levels mentioned in the previous chapter was associated with the attributes of niacin, it can also be attributed to the predicted impact of T413A variant. It is also understood that CETP can either cause or prevent the development of atherosclerosis (Shah, 2007), and it is believed that in this study it might have a protective effect since two of the affected experimental animals also showed a significant increase in HDL-C.

This study suggests that niacin treatment had a role in the decrease of *CETP* mRNA expression (T_0-T_2 and T_4) in the treated animals compared to the control. Nonetheless, there were two-time points (T_1 and T_4) with statistically significant differences between the niacin treated and control group (Figure 3.8). The other time points were not able to produce statistically significant differences, even though there was a decline in the expression except for T_3, where an increase, possibly occurred due to skewed transfer of CE from HDL to APOB as opposed to the normal equal transfer that involves the movement of triglycerides from VLDL and LDL(Dong *et al.,* 2014). This is further supported by the observed increase in *APOB-100* expression at the same time point (Figure 3.9). Niacin treatment was able to account for statistically significant changes in the up-regulation of HDL-C at T_1 (see Figure 2.4, Chapter 2) and down-regulation of *CETP* gene expression at T_1 and T_4. Therefore, the results of *CETP* expression do support the HDL-C hypothesis.

Furthermore, screening of the *APOB-100* gene indicated two disease-causing homozygous missense variants (F635L and A647T) identified in five animals. The F635L variant occurred due to the T1903>C nucleotide change, which resulted in phenylalanine changing to leucine at position 635. It is known that APOB-100 is the main element of LDL particles, which regulates LDL-C in the plasma and this protein is critical for the development of hyperlipidemia and atherosclerosis (Vrablik *et al.,* 2001). Therefore, it can be hypothesized that a decrease in LDL-C will also cause a decrease in *APOB-100* expression. The majority of mutations reported to cause hyperlipidemia in this gene are found in exon 26, which is the biggest in this gene (Henderson *et al.,* 2016). This exon contains a carboxyl-terminal (site B) responsible for housing the LDLR binding domain (Boren *et al.,* 1998, Hussain *et al.,* 2003). Since both variants are found further upstream and all the affected animals have LDL-C values below 2.75 mmol/L, they may not be predisposed to CVD development. Additionally, *APOB-100* gene expression showed a gradual decrease from T_0-T_2 in the experimental group (Figure 3.9). The control animals indicated a slight decline from T_0 to T_1 and remained relatively unchanged thereafter. The decrease in *APOB-100* mRNA expression may be predominantly due to the actions of niacin since it is specifically reported to induce APOB-100 breakdown by hindering the synthesis of triglycerides , while not interfering with MTP-mediated intracellular APOB-100 processing (Jin *et al.,* 1999). The presence of sequence variants may have interfered with the proper binding of LDLR and APOB-100, even though this did not change the

association completely. This is suggested since the mutated animals had an increase in the expression of *APOB-100* compared to the wild-type. This could account for the lack of statistically significant in the observed gradual decline.

3.6 CONCLUSION

The current chapter aimed at determining the impact of niacin and sequence variants in correlation with their gene expression activity. Sequencing analysis of the selected genes revealed eight missense and five silent variants as illustrated in Table 3.6. The findings suggest that that niacin induction slightly decreased the expression of *PCSK9,* even though it was not significant. This observed decline was also correlated with the identification of LOF mutations in the gene. These sequence variants were shown to repress *PCSK9* expression in affected animals. Therefore, it would be of interest to study the effects of niacin on HNF1 α molecule of PCSK9 in future studies. Furthermore, a deleterious mutation (D274N) identified in the *LDLR* gene was responsible for the loss of function, which culminated in its repression. However, it is possible that other underlying mechanisms led to the observed decrease in the mRNA expression, and thus require further investigations. The current study was also able to highlight the importance of the *SREBP-2* gene in the cholesterol pathway, as evidenced in *SREBP-2* down-regulation in an attempt to balance its multifaceted roles. The silent sequence variant (P565P) identified in this gene was predicted to be disease-causing and animals with this variant had a higher gene expression as compared with the wild-type individuals. It was encouraging to note that *APOB-100* expression was suppressed for almost all the treatment time points except for T_3. This suppression correlated with that of LDL-C, which is reported in the previous chapter, as anticipated due to their connected existence. Furthermore, the statistically significant downregulation of *CETP* ($T_1$and T_4) suggested that our vervet monkey model can support the HDL-C hypothesis. The results of the current study appear to correlate with those previously reported in this vervet colony. Future studies that are aimed at supporting these findings may use other molecular techniques such as Western blots and ELISA.

CHAPTER FOUR:
OVERALL DISCUSSION/CONCLUSION

The prevailing knowledge about the mechanisms of atherosclerosis development and the role played by LDL-C has been accumulated mostly through studies conducted in humans and NHPs. This is primarily due to the well-documented similarities between these two organisms. These similarities include plasma lipoprotein characteristics and genetic susceptibility (Lane, 2000), which served as two critical parameters in this investigation. There are some disadvantages to utilizing NHP in lipid metabolism and atherosclerosis studies; these include high costs and difficulties in handling them (Moghadasian *et al*., 2001b). However, the SAMRC/PUDAC facility is fortunate as it has been breeding NHPs for more than 30 years for biomedical research purposes and has qualified staff members who have experience in all aspects of NHP maintenance. Studies which modelled diet-induced atherosclerosis have reported the importance of the African green monkey within the NHP research community (Moghadasian *et al*., 2001a). The significance of this species was further enhanced by some investigations that were carried out at SAMRC/PUDAC, particularly, the niacin study conducted by Chauke and colleagues. This study was therefore formulated to further verify this model and potentially discover new aspects of LDL-C modification by niacin. The major difference between those two studies is the determination of *PCSK9* expression levels and the subsequent interactions with niacin. The presence of the *CETP* gene in NHPs and humans places them ahead of some laboratory research models such as rodents in atherosclerosis research although in the past rodents have been utilized using gene manipulation techniques. The benchmark of any successful CVD related research is therefore the ability to elucidate LDL-C underlying methods.

Although there are several different theories about the exact mechanisms that precipitate the development of CVDs, but the starring role played by lipids in these mechanisms cannot be disputed. Lipid metabolism pathways are very complex, requiring well-coordinated maintenance and regulation processes. The maintenance of lipid equilibrium in these pathways is critical in preventing the development of CVDs such as dyslipidemia and atherosclerosis. It is for these

reasons that this investigation conducted a biochemical analysis, determining the levels of total cholesterol, triglycerides, LDL-C, and HDL-C in plasma.

The administration of niacin resulted in a statistically significant increase in HDL-C throughout the treatment intervention period. The statistically significant increase at T_1-T_4 was anticipated based on the knowledge that niacin has previously been reported to increase the levels of HDL-C (Hochholzer *et al*., 2011). The observed findings further demonstrate the importance of niacin in combating CVDs. The ability of niacin to achieve this role is highly important due to the reported intricate nature of HDL-C metabolism. The complexity occurs as a result of the vast differences in the particles in terms of their magnitude and configuration (Zhou *et al*., 2015). Studies on the complexity of HDL particles have revealed that in addition to increasing the levels of HDL-C, the functionality of HDL particles is equally important (Kardassis *et al*., 2014). The importance of HDL particle functionality is attributed to their ability to promote RCT and due to their pleiotropic nature, they can affect inflammation, homeostasis and apoptosis (Ali *et al*., 2012). In addition to increasing HDL-C levels, niacin has been reported to contribute to the shifting of HDL subclass distribution towards larger HDL particles (Investigators, 2011, Bays *et al*., 2012, Toth *et al*., 2012, Franceschini *et al*., 2013). The increase of HDL-C and mechanisms underpinning the increase are also referred to as the HDL-C hypothesis. Another component of increasing HDL-C is the RCT pathway, which is mediated by *CETP*, amongst other genes. The expression analysis of this gene is discussed further down this section. The statistically significant increase in HDL-C was followed by a slight decrease in the levels of LDL-C. This may not have been the expected outcome in terms of the magnitude, however, even the minimal decrease in LDL-C is of importance. This is because LDL particles are generally termed as 'bad' cholesterol, as opposed to the 'good' HDL molecules. In this study, niacin administration resulted in a 31.47% reduction of LDL-C levels. Furthermore, there was statistically significant decrease in total cholesterol levels (T_4), while triglycerides levels fluctuated throughout the period of niacin administration. These biochemical results proved that the vervet monkey model is a suitable model for investigations of this nature. Additionally, the 100 mg/kg niacin SR dose was able to elicit changes in the plasma levels of major molecules that are involved in the cholesterol pathway. Therefore, moving forward, niacin compounds such as the ER formulation require further investigation. Should niacin shortfalls be addressed, this drug

could be among those that are used to effectively treat hypercholesterolemia for patients who are unresponsive to currently available treatments (Zeman *et al*., 2015).

The biochemical parameters are not the only important determinants that predispose individuals to the development of atherosclerosis. Other critical determinants are those of a genetic nature. Therefore, the second focus area was to use genotyping and gene expression to screen the vervet monkey model for genes involved in cholesterol synthesis and regulation. These genes included *PCSK9, LDLR, SREBP-2, CETP* and *APOB-100.* The importance of *PCSK9* gene has been stated in previous chapters of this study, and for the investigation to be successful, the sequence variants identified in this gene needed to fall in the category of LOF with downregulation of the mRNA expression. The screening of this gene revealed four mutations with two of them being missense. After the analysis, two missense variants were reported to be LOF mutations, and this was supported by the majority of affected animals belonging to the control group. Individuals harbouring these two variants had a suppressed mRNA expression as compared to wild-type. Moreover, the SNP/silent mutation (G635G) was predicted to be disease-causing by Mutation Taster and was found in the C-terminal region of the gene. It is also postulated that G635G may have played a part in *PCSK9* losing its function to degrade LDLR. This hypothesis is supported by the disease-causing prediction of this variant, and the mutated animals had a decreased mRNA expression. Furthermore, nine of the 13 subjects with this mutation belong to the control group. It is, therefore, reasonable to believe that the *PCSK9* variants were LOF and led to the suppressed mRNA expression in addition to the decreasing action of niacin.

Another gene that revealed sequence variants that may fall in the category of LOF is *LDLR,* although these mutations are not desired in this gene as they are for *PCSK9*. The loss of function in *LDLR* gene may hinder its core function of breaking down LDLs. The D274N mutation was predicted to be deleterious and disease-causing by SIFT and Mutation Taster, respectively. These findings were postulated to be the major cause of the repression in *LDLR* gene expression. Ordinarily, the decline in gene expression of *PCSK9* leads to increased *LDLR* mRNA expression. However, in this current study, this was not the case and this might be due to the variants observed and niacin administration. This was evidenced by the increased mRNA expression at the end of the washout period. The decrease in *LDLR* gene expression in this investigation may not have

predisposed the animals to the development of CVDs because of the decrease in LDL-C levels reported in chapter 2. Additionally, the decrease in *APOB-100* gene expression highlighted in the previous chapter can also support this notion as it is a major component of LDL. This study was further able to confirm the critical multifaceted role of *SREBP-2* gene in the cholesterol pathways (see Figure 9, chapter 1). The administration of niacin resulted in down-regulation of mRNA expression at T_3 and T_4 and a statistically significant increase after its removal at T_5. The statistically significant decreases at T_1 and T_4 in the gene expression of *CETP* correlated with the statistically significant increase in HDL-C levels at the same time points. These results confirmed the importance of CETP inhibition in the upregulation of HDL-C. This also highlighted that niacin administration supports the HDL hypothesis.

Therefore, the two main objectives of this study were achieved, based on the results discussed above. Although, the findings were not statistically significant, the hypothesis that niacin administration minimizes the ability of PCSK9 to degrade LDLR in the vervet monkey model, resulting in reduced plasma LDL-C levels cannot be disregarded. This is informed by the decreased mRNA expression of *PCSK9* coupled with LOF mutations identified in this gene. If this postulation were to be rejected, it would have to be expected that *PCSK9* would have shown elevated gene expression and increased levels of LDL-C. The reported decrease in *LDLR* mRNA expression was as a result of its sequence variants as opposed to gain of function of PCSK9, therefore the hypothesis still stands. The only thing that might have been affected by the lowered *LDLR* mRNA expression is the margins by which LDL-C levels would have been lowered.

Certain limitations were encountered during this study. The current investigation was non-terminal; therefore the mRNA expression levels of the genes at the tissue level (liver) could not be measured. The study was also non-invasive, which meant techniques such as liver biopsies were not performed. One of the drawbacks of this is that PCSK9 expression is the highest in the liver as opposed to other tissues that express the gene (Frank-Kamenetsky *et al*., 2008). Therefore, it is speculated that the reported expression results could be an underestimation of expressions at the liver. Nevertheless, measurement of circulating levels of biochemical parameters and gene expression is still considered to be useful.

Healthy vervet monkeys were employed to evaluate the niacin induced response due to the ability of dietary mediations to only modify LDL(Grundy and Denke, 1990). To the best knowledge of this current investigation, there is no existing NHP model of naturally low HDL. Additionally, to induce atherosclerotic lesions similar to humans, these monkeys would require an average of three years on the high-fat diet. The 100 mg/kg dose of niacin was chosen based on the reports that higher doses caused side effects. Therefore, considering the welfare of the animals the safest dose which showed significant results in a previous study was used. The omission of molecular techniques such as Western blots and ELISA may be another limitation of this study. It is reported that PCSK9 has a truncated form which is about 52-55 kDa as opposed to the 74 kDa normal form (Han *et al.,* 2014). Using specific antibodies that bind to each form and quantification of the size by Western blots, could have further complemented the findings of this study. However, the results of qRT-PCR were able to provide important insights in terms of the gene expression.

To be able to overcome some of the above-mentioned limitations, this study recommends that future investigations consider obtaining liver biopsy samples to evaluate tissue gene expression. Future studies should also include an extended intervention period of at least four months provided the health and welfare of the animals are not compromised. It is also postulated that had the intervention period been longer by at least a month the results may have been far more significant. This is informed by looking at graphs of LDL-C and HDL-C where at T_4 there seems to be a continuing decrease and increase, respectively. Therefore, it would have been interesting to find out if this was the case and what changes it would have made to the final results. To able to ascertain the exact form of vervet PCSK9, ELISA and Western blots should be included.

In conclusion, the purpose of the current investigation was to determine the expression levels of PCSK9 in captive-bred vervet monkeys and its interaction with niacin administration. The genetic screening of *PCSK9* and its associated genes *LDLR, SREBP-2, CETP* and *APOB-100* assisted in determining whether *PCSK9* gained or lost function in captive-bred vervet monkeys. The aims of this study were accomplished by firstly conducting a three-month-long niacin mediated animal intervention. Niacin successfully produced statistically significant increases in four-time points ($T_{1, 2, 3}$ and $_4$) which led to an overall increase in plasma HDL-C, thereby further proving that it is a leading HDL-C up-regulator. This animal intervention also resulted in another statistically

significant decline in total cholesterol levels and minimal decreases in LDL-C. Furthermore, the combination of genotyping and gene expression was able to identify sequence variants that impacted the gene expression of these selected genes. Three identified *PCSK9* variants were categorized as LOF mutations, this was supported by a decreased expression in animals harbouring the variants. The *LDLR* also had LOF variants that were the reason for its decreased mRNA expression. Additionally, *SREBP-2* proved to be a key mediator of cholesterol pathways. The importance of the *CETP* gene in HDL-C metabolism was also highlighted. The statistically significant declines in *CETP* mRNA expression in two-time points (T_1 and $_4$) correlated with other statistically significant increases at the same time points in HDL-C. In addition to lowering LDL-C, niacin also decreased LDL's main component, the APOB-*100* mRNA expression. Although a limited number of studies had investigated the impact of niacin on PCSK9 activity, this is the first time that niacin treatment is reported to suppress the gene expression of *PCSK9* coupled with LOF variants. The only other similar report came from a study in which niacin was administered in combination with other drugs such as fibrates. It is therefore anticipated that the results of this study have reinforced some of the existing knowledge while also discovering new insights into niacin mechanisms in combating CVD development.

REFERENCES

ABBOUD, S., KARHUNEN, P. J., LÜTJOHANN, D., GOEBELER, S., LUOTO, T., FRIEDRICHS, S., LEHTIMAKI, T., PANDOLFO, M. & LAAKSONEN, R. 2007. Proprotein convertase subtilisin/kexin type 9 (PCSK9) gene is a risk factor of large-vessel atherosclerosis stroke. *PloS one,* 2**,** e1043.

ABIFADEL, M., RABÈS, J. P., DEVILLERS, M., MUNNICH, A., ERLICH, D., JUNIEN, C., VARRET, M. & BOILEAU, C. 2009. Mutations and polymorphisms in the proprotein convertase subtilisin kexin 9 (PCSK9) gene in cholesterol metabolism and disease. *Human mutation,* 30**,** 520-529.

ABIFADEL, M., VARRET, M., RABÈS, J.-P., ALLARD, D., OUGUERRAM, K., DEVILLERS, M., CRUAUD, C., BENJANNET, S., WICKHAM, L. & ERLICH, D. 2003. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nature genetics,* 34**,** 154.

AGELLON, L. B., QUINET, E. M., GILLETTE, T. G., DRAYNA, D. T., BROWN, M. L. & TALL, A. R. 1990. Organization of the human cholesteryl ester transfer protein gene. *Biochemistry,* 29**,** 1372-1376.

AKRAM, O. N., BERNIER, A., PETRIDES, F., WONG, G. & LAMBERT, G. 2010. Beyond LDL cholesterol, a new role for PCSK9. Am Heart Assoc.

ALI, K. M., WONNERTH, A., HUBER, K. & WOJTA, J. 2012. Cardiovascular disease risk reduction by raising HDL cholesterol–current therapies and future opportunities. *British journal of pharmacology,* 167**,** 1177-1194.

ALWAN, A. 2011. *Global status report on noncommunicable diseases 2010*, World Health Organization.

ALWAN, A., MACLEAN, D. R., RILEY, L. M., D'ESPAIGNET, E. T., MATHERS, C. D., STEVENS, G. A. & BETTCHER, D. 2010. Monitoring and surveillance of chronic non-communicable diseases: progress and capacity in high-burden countries. *The Lancet,* 376**,** 1861-1868.

ARMITAGE, J., BOWMAN, L., WALLENDSZUS, K., BULBULIA, R., RAHIMI, K., HAYNES, R., PARISH, S., PETO, R. & COLLINS, R. 2010. Intensive lowering of LDL cholesterol with 80 mg versus 20 mg simvastatin daily in 12,064 survivors of myocardial infarction: a double-blind randomised trial. *Lancet (London, England),* 376**,** 1658-1669.

AUSTIN, M. A. & KRAUSS, R. M. 1995. LDL density and atherosclerosis. *Jama,* 273**,** 115-115.

BADIMON, J. J., BADIMON, L. & FUSTER, V. 1990. Regression of atherosclerotic lesions by high density lipoprotein plasma fraction in the cholesterol-fed rabbit. *The Journal of clinical investigation,* 85**,** 1234-1241.

BADIMON, L. & VILAHUR, G. 2012. LDL-cholesterol versus HDL-cholesterol in the atherosclerotic plaque: inflammatory resolution versus thrombotic chaos. *Annals of the New York Academy of Sciences,* 1254**,** 18-32.

BAIGENT, C., BLACKWELL, L., EMBERSON, J., HOLLAND, L., REITH, C., BHALA, N., PETO, R., BARNES, E., KEECH, A. & SIMES, J. 2010. Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170,000 participants in 26 randomised trials. Elsevier.

BARTER, P. J., CAULFIELD, M., ERIKSSON, M., GRUNDY, S. M., KASTELEIN, J. J., KOMAJDA, M., LOPEZ-SENDON, J., MOSCA, L., TARDIF, J.-C. & WATERS, D. D. 2007. Effects of torcetrapib in patients at high risk for coronary events. *New England journal of medicine,* 357**,** 2109-2122.

BARTER, P. J. & RYE, K.-A. 2001. Cholesteryl ester transfer protein, high density lipoprotein and arterial disease. *Current opinion in lipidology,* 12**,** 377-382.

BARTH, J. L. & ARGRAVES, W. S. 2001. Cubilin and megalin: partners in lipoprotein and vitamin metabolism. *Trends in cardiovascular medicine,* 11**,** 26-31.

BAYS, H., GIEZEK, H., MCKENNEY, J. M., O'NEILL, E. A. & TERSHAKOVEC, A. M. 2012. Extended-release niacin/laropiprant effects on lipoprotein subfractions in patients with type 2 diabetes mellitus. *Metabolic syndrome and related disorders,* 10**,** 260-266.

BENADÉ, A., FINCHAM, J., SMUTS, C., TUNG, M. T. L., CHALTON, D., KRUGER, M., WEIGHT, M., DAUBITZER, A. K. & TICHELAAR, H. 1988. Plasma low density lipoprotein composition in relation to atherosclerosis in nutritionally defined Vervet monkeys. *Atherosclerosis,* 74**,** 157-168.

BENJANNET, S., RHAINDS, D., ESSALMANI, R., MAYNE, J., WICKHAM, L., JIN, W., ASSELIN, M.-C., HAMELIN, J., VARRET, M. & ALLARD, D. 2004. NARC-1/PCSK9 and its natural mutants zymogen cleavage and effects on the low density lipoprotein (LDL) receptor and LDL cholesterol. *Journal of Biological Chemistry,* 279**,** 48865-48875.

BERRIOT-VAROQUEAUX, N., AGGERBECK, L., SAMSON-BOUMA, M.-E. & WETTERAU, J. 2000. The role of the microsomal triglygeride transfer protein in abetalipoproteinemia. *Annual review of nutrition,* 20**,** 663-697.

BERTI, J., DE FARIA, E. & OLIVEIRA, H. 2005. Atherosclerosis in aged mice over-expressing the reverse cholesterol transport genes. *Brazilian journal of medical and biological research,* 38**,** 391-398.

BIRJMOHUN, R. S., HUTTEN, B. A., KASTELEIN, J. J. & STROES, E. S. 2005. Efficacy and safety of high-density lipoprotein cholesterol-increasing compounds: a meta-analysis of randomized controlled trials. *Journal of the American College of Cardiology,* 45**,** 185-197.

BLACHER, J., LEVY, B. I., MOURAD, J.-J., SAFAR, M. E. & BAKRIS, G. 2016. From epidemiological transition to modern cardiovascular epidemiology: hypertension in the 21st century. *The Lancet,* 388**,** 530-532.

BODOR, E. & OFFERMANNS, S. 2008. Nicotinic acid: an old drug with a promising future. *British journal of pharmacology,* 153**,** S68-S75.

BOEKHOLDT, S., SACKS, F., JUKEMA, J., SHEPHERD, J., FREEMAN, D., MCMAHON, A., CAMBIEN, F., NICAUD, V., DE GROOTH, G. & TALMUD, P. 2005. Cholesteryl ester transfer protein TaqIB variant, high-density lipoprotein cholesterol levels, cardiovascular risk, and efficacy of pravastatin treatment: individual patient meta-analysis of 13 677 subjects. *Circulation,* 111**,** 278-287.

BOREN, J., LEE, I., ZHU, W., ARNOLD, K., TAYLOR, S. & INNERARITY, T. L. 1998. Identification of the low density lipoprotein receptor-binding site in apolipoprotein B100 and the modulation of its binding activity by the carboxyl terminus in familial defective apo-B100. *The Journal of clinical investigation,* 101**,** 1084-1093.

BORGGREVE, S. E., HILLEGE, H. L., WOLFFENBUTTEL, B. H., DE JONG, P. E., ZUURMAN, M. W., VAN DER STEEGE, G., VAN TOL, A., DULLAART, R. P. & GROUP, P. S. 2006. An increased coronary risk is paradoxically associated with common cholesteryl ester transfer protein gene variations that relate to higher high-density lipoprotein cholesterol: a population-based study. *The Journal of Clinical Endocrinology & Metabolism,* 91**,** 3382-3388.

BRESLOW, J. L. 2000. Genetics of lipoprotein abnormalities associated with coronary heart disease susceptibility. *Annual review of genetics,* 34**,** 233-254.

BROUSSEAU, M. E., O'CONNOR JR, J. J., ORDOVAS, J. M., COLLINS, D., OTVOS, J. D., MASSOV, T., MCNAMARA, J. R., RUBINS, H. B., ROBINS, S. J. & SCHAEFER, E. J. 2002. Cholesteryl Ester Transfer Protein Taq I B2B2 Genotype Is Associated With Higher HDL Cholesterol Levels and Lower Risk of Coronary Heart Disease End Points in Men With HDL Deficiency: Veterans Affairs HDL Cholesterol Intervention Trial. *Arteriosclerosis, thrombosis, and vascular biology,* 22**,** 1148-1154.

BROUSSEAU, M. E., SCHAEFER, E. J., WOLFE, M. L., BLOEDON, L. T., DIGENIO, A. G., CLARK, R. W., MANCUSO, J. P. & RADER, D. J. 2004. Effects of an inhibitor of cholesteryl ester transfer protein on HDL cholesterol. *New England Journal of Medicine,* 350**,** 1505-1515.

BROWN, M. L., INAZU, A., HESLER, C. B., AGELLON, L. B., MANN, C., WHITLOCK, M. E., MARCEL, Y. L., MILNE, R. W., KOIZUMI, J. & MABUCHI, H. 1989. Molecular basis of lipid transfer protein deficiency in a family with increased high-density lipoproteins. *Nature,* 342**,** 448.

BROWN, M. S. & GOLDSTEIN, J. L. 1997. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell,* 89**,** 331-340.

BROWN, M. S. & GOLDSTEIN, J. L. 2006. Lowering LDL--not only how low, but how long? *Science,* 311**,** 1721-1723.

BURKE, A. C., DRON, J. S., HEGELE, R. A. & HUFF, M. W. 2017. PCSK9: regulation and target for drug development for dyslipidemia. *Annual review of pharmacology and toxicology,* 57**,** 223-244.

BURNETT, J. R., ZHONG, S., JIANG, Z. G., HOOPER, A. J., FISHER, E. A., MCLEOD, R. S., ZHAO, Y., BARRETT, P. H. R., HEGELE, R. A. & VAN BOCKXMEER, F. M. 2007. Missense mutations in APOB within the βα1 domain of human APOB-100 result in impaired secretion of ApoB and ApoB-containing lipoproteins in familial hypobetalipoproteinemia. *Journal of Biological Chemistry,* 282**,** 24270-24283.

CAMERON, J., HOLLA, Ø. L., RANHEIM, T., KULSETH, M. A., BERGE, K. E. & LEREN, T. P. 2006. Effect of mutations in the PCSK9 gene on the cell surface LDL receptors. *Human molecular genetics,* 15**,** 1551-1558.

CARIOU, B., LE MAY, C. & COSTET, P. 2011a. Clinical aspects of PCSK9. *Atherosclerosis,* 216**,** 258-265.

CARIOU, B., LE MAY, C. & COSTET, P. 2011b. Clinical aspects of PCSK9. *Atherosclerosis,* 216**,** 258-265.

CARLSON, L., HAMSTEN, A. & ASPLUND, A. 1989. Pronounced lowering of serum levels of lipoprotein Lp (a) in hyperlipidaemic subjects treated with nicotinic acid. *Journal of internal medicine,* 226**,** 271-276.

CARLSON, L. A. 2005. Nicotinic acid: the broad-spectrum lipid drug. A 50th anniversary review. *Journal of internal medicine,* 258**,** 94-114.

CARLSON, L. A. 2006. Nicotinic acid and other therapies for raising high-density lipoprotein. *Current opinion in cardiology,* 21**,** 336-344.

CARTEGNI, L., CHEW, S. L. & KRAINER, A. R. 2002. Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nature reviews genetics,* 3**,** 285-298.

CASQUERO, A., BERTI, J., SALERNO, A., BIGHETTI, E., CAZITA, P., KETELHUTH, D., GIDLUND, M. & OLIVEIRA, H. 2006. Atherosclerosis is enhanced by testosterone deficiency and attenuated by CETP expression in transgenic mice. *Journal of lipid research,* 47**,** 1526-1534.

CAZITA, P. M., BERTI, J. A., AOKI, C., GIDLUND, M., HARADA, L. M., NUNES, V. S., QUINTÃO, E. C. & OLIVEIRA, H. C. 2003. Cholesteryl ester transfer protein expression attenuates atherosclerosis in ovariectomized mice. *Journal of lipid research,* 44**,** 33-40.

CHAN, J. C., PIPER, D. E., CAO, Q., LIU, D., KING, C., WANG, W., TANG, J., LIU, Q., HIGBEE, J. & XIA, Z. 2009. A proprotein convertase subtilisin/kexin type 9 neutralizing antibody reduces serum cholesterol in mice and nonhuman primates. *Proceedings of the National Academy of Sciences,* 106**,** 9820-9825.

CHAPMAN, M. J., GINSBERG, H. N., AMARENCO, P., ANDREOTTI, F., BORÉN, J., CATAPANO, A. L., DESCAMPS, O. S., FISHER, E., KOVANEN, P. T. & KUIVENHOVEN, J. A. 2011. Triglyceride-rich lipoproteins and high-density lipoprotein cholesterol in patients at high risk of cardiovascular disease: evidence and guidance for management. *European heart journal,* 32**,** 1345-1361.

CHAUDHARY, R., GARG, J., SHAH, N. & SUMNER, A. 2017. PCSK9 inhibitors: A new era of lipid lowering therapy. *World journal of cardiology,* 9**,** 76.

CHAUHAN, S. & AERI, B. T. 2013. Prevalence of cardiovascular disease in India and it is economic impact-A review. *International Journal of Scientific and Research Publications,* 3**,** 1-5.

CHAUKE, C. G. 2012. *Defining the African green monkey (Chlorocebus Aethiops): expression behaviour of selected lipid metabolism genes in response to niacin.* University of Western Cape.

CHAUKE, C. G., ARIEFF, Z., KAUR, M. & SEIER, J. V. 2014. Effects of short-term niacin treatment on plasma lipoprotein concentrations in African green monkeys (Chlorocebus aethiops). *Lab animal,* 43**,** 58.

CHEN, S. N., BALLANTYNE, C. M., GOTTO, A. M., TAN, Y., WILLERSON, J. T. & MARIAN, A. J. 2005. A common PCSK9haplotype, encompassing the E670G coding single nucleotide polymorphism, is a novel genetic marker for plasma low-density lipoprotein cholesterol levels and severity of coronary atherosclerosis. *Journal of the American College of Cardiology,* 45**,** 1611-1619.

CHEN, Y., COPELAND, W. K., VEDANTHAN, R., GRANT, E., LEE, J. E., GU, D., GUPTA, P. C., RAMADAS, K., INOUE, M. & TSUGANE, S. 2013. Association between body mass index and cardiovascular disease mortality in east Asians and south Asians: pooled analysis of prospective data from the Asia Cohort Consortium. *Bmj,* 347**,** f5446.

CHMARA, M., WASĄG, B., ŻUK, M., KUBALSKA, J., WĘGRZYN, A., BEDNARSKA-MAKARUK, M., PRONICKA, E., WEHR, H., DEFESCHE, J. & RYNKIEWICZ, A. 2010. Molecular characterization of Polish patients with familial hypercholesterolemia: novel and recurrentLDLR mutations. *Journal of applied genetics,* 51**,** 95-106.

CHUNG, M., LICHTENSTEIN, A. H., IP, S., LAU, J. & BALK, E. M. 2009. Comparability of methods for LDL subfraction determination: a systematic review. *Atherosclerosis,* 205**,** 342-348.

COHEN, J., PERTSEMLIDIS, A., KOTOWSKI, I. K., GRAHAM, R., GARCIA, C. K. & HOBBS, H. H. 2005. Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9. *Nature genetics,* 37**,** 161.

COHEN, J. C., BOERWINKLE, E., MOSLEY JR, T. H. & HOBBS, H. H. 2006. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. *New England Journal of Medicine,* 354**,** 1264-1272.

COHEN, J. D., BRINTON, E. A., ITO, M. K. & JACOBSON, T. A. 2012. Understanding Statin Use in America and Gaps in Patient Education (USAGE): an internet-based survey of 10,138 current and former statin users. *Journal of clinical lipidology,* 6**,** 208-215.

COX, L. A., COMUZZIE, A. G., HAVILL, L. M., KARERE, G. M., SPRADLING, K. D., MAHANEY, M. C., NATHANIELSZ, P. W., NICOLELLA, D. P., SHADE, R. E. & VORUGANTI, S. 2013. Baboons as a model to study genetics and epigenetics of human disease. *ILAR journal,* 54**,** 106-121.

COX, L. A., OLIVIER, M., SPRADLING-REEVES, K., KARERE, G. M., COMUZZIE, A. G. & VANDEBERG, J. L. 2017. Nonhuman primates and translational research—cardiovascular disease. *ILAR journal,* 58**,** 235-250.

CUNNINGHAM, D., DANLEY, D. E., GEOGHEGAN, K. F., GRIFFOR, M. C., HAWKINS, J. L., SUBASHI, T. A., VARGHESE, A. H., AMMIRATI, M. J., CULP, J. S. & HOTH, L. R. 2007a. Structural and biophysical studies of PCSK9 and its mutants linked to familial hypercholesterolemia. *Nature Structural and Molecular Biology,* 14**,** 413.

CUNNINGHAM, D., DANLEY, D. E., GEOGHEGAN, K. F., GRIFFOR, M. C., HAWKINS, J. L., SUBASHI, T. A., VARGHESE, A. H., AMMIRATI, M. J., CULP, J. S. & HOTH, L. R. 2007b. Structural and biophysical studies of PCSK9 and its mutants linked to familial hypercholesterolemia. *Nature structural & molecular biology,* 14**,** 413.

DALTON, T. A. & BERRY, R. S. 1992. Hepatotoxicity associated with sustained-release niacin. *The American journal of medicine,* 93**,** 102-104.

DAVIES, J. T., DELFINO, S. F., FEINBERG, C. E., JOHNSON, M. F., NAPPI, V. L., OLINGER, J. T., SCHWAB, A. P. & SWANSON, H. I. 2016. Current and emerging uses of statins in clinical therapeutics: a review. *Lipid insights,* 9**,** LPI. S37450.

DAVIGNON, J., DUBUC, G. & SEIDAH, N. G. 2010. The influence of PCSK9 polymorphisms on serum low-density lipoprotein cholesterol and risk of atherosclerosis. *Current atherosclerosis reports,* 12**,** 308-315.

DAVIS, C. G., ELHAMMER, A., RUSSELL, D., SCHNEIDER, W., KORNFELD, S., BROWN, M. & GOLDSTEIN, J. 1986. Deletion of clustered O-linked carbohydrates does not impair function of low density lipoprotein receptor in transfected fibroblasts. *Journal of Biological Chemistry,* 261**,** 2828-2838.

DENIS, M., MARCINKIEWICZ, J., ZAID, A., GAUTHIER, D., POIRIER, S., LAZURE, C., SEIDAH, N. G. & PRAT, A. 2012. Gene inactivation of proprotein convertase subtilisin/kexin type 9 reduces atherosclerosis in mice. *Circulation,* 125**,** 894-901.

DETECTION, N. C. E. P. E. P. O. & ADULTS, T. O. H. B. C. I. 2002. *Third report of the National Cholesterol Education Program (NCEP) Expert Panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III)*, International Medical Pub.

DI STASI, S. L., MACLEOD, T. D., WINTERS, J. D. & BINDER-MACLEOD, S. A. 2010. Effects of statins on skeletal muscle: a perspective for physical therapists. *Physical therapy,* 90**,** 1530-1542.

DONG, B., SINGH, A. B., FUNG, C., KAN, K. & LIU, J. 2014. CETP inhibitors downregulate hepatic LDL receptor and PCSK9 expression in vitro and in vivo through a SREBP2 dependent mechanism. *Atherosclerosis,* 235**,** 449-462.

DONG, B., WU, M., LI, H., KRAEMER, F. B., ADELI, K., SEIDAH, N. G., PARK, S. W. & LIU, J. 2010. Strong induction of PCSK9 gene expression through HNF1α and SREBP2: mechanism for the resistance to LDL-cholesterol lowering effect of statins in dyslipidemic hamsters. *Journal of lipid research,* 51**,** 1486-1495.

DRAYNA, D., JARNAGIN, A. S., MCLEAN, J., HENZEL, W., KOHR, W., FIELDING, C. & LAWN, R. 1987. Cloning and sequencing of human cholesteryl ester transfer protein cDNA. *Nature,* 327**,** 632.

DUBUC, G., CHAMBERLAND, A., WASSEF, H., DAVIGNON, J., SEIDAH, N. G., BERNIER, L. & PRAT, A. 2004. Statins upregulate PCSK9, the gene encoding the proprotein convertase neural apoptosis-regulated convertase-1 implicated in familial hypercholesterolemia. *Arteriosclerosis, thrombosis, and vascular biology,* 24**,** 1454-1459.

EGGEN, D. A. 1974. Cholesterol metabolism in rhesus monkey, squirrel monkey, and baboon. *Journal of lipid research,* 15**,** 139-145.

ENDO, A. 2010. A historical perspective on the discovery of statins. *Proceedings of the Japan Academy, Series B,* 86**,** 484-493.

EXPERT PANEL ON DETECTION, E. 2001. Executive summary of the third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III). *Jama,* 285**,** 2486.

FARNIER, M. 2011. The role of proprotein convertase subtilisin/kexin type 9 in hyperlipidemia. *American Journal of Cardiovascular Drugs,* 11**,** 145-152.

FASANO, T., CEFALU, A. B., DI LEO, E., NOTO, D., POLLACCIA, D., BOCCHI, L., VALENTI, V., BONARDI, R., GUARDAMAGNA, O. & AVERNA, M. 2007. A novel loss of function mutation of PCSK9 gene in white subjects with low-plasma low-density lipoprotein cholesterol. *Arteriosclerosis, thrombosis, and vascular biology,* 27**,** 677-681.

FEINGOLD, K. R. & GRUNFELD, C. 2018. Introduction to lipids and lipoproteins. *Endotext [Internet].* MDText. com, Inc.

FERENCE, B. A., MAJEED, F., PENUMETCHA, R., FLACK, J. M. & BROOK, R. D. 2015. Effect of naturally random allocation to lower low-density lipoprotein cholesterol on the risk of coronary heart disease mediated by polymorphisms in NPC1L1, HMGCR, or both: a 2× 2 factorial Mendelian randomization study. *Journal of the American College of Cardiology,* 65**,** 1552-1561.

FIELDING, C. J. & FIELDING, P. E. 1995. Molecular physiology of reverse cholesterol transport. *Journal of lipid research,* 36**,** 211-228.

FINCHAM, J., FABER, M., WEIGHT, M., LABADARIOS, D., TALJAARD, J., STEYTLER, J., JACOBS, P. & KRITCHEVSKY, D. 1987a. Diets realistic for westernised people significantly effect lipoproteins,

calcium, zinc, vitamins C, E, B6 and haematology in Vervet monkeys. *Atherosclerosis,* 66**,** 191-203.

FINCHAM, J., GOUWS, E., WOODROOF, C., VAN WYK, M., KRUGER, M., SMUTS, C., VAN JAARSVELD, P., TALJAARD, J., SCHALL, R. & STRAUSS, J. 1991. Atherosclerosis. Chronic effects of fish oil and a therapeutic diet in nonhuman primates. *Arteriosclerosis and thrombosis: a journal of vascular biology,* 11**,** 719-732.

FINCHAM, J., QUACK, G., WÜLFROTH, P. & BENADE, A. 1996. Confirmation of efficacy of etofibrate against peripheral atherosclerosis in non-human primates which model human lesion types I-VII. *Arzneimittel-Forschung,* 46**,** 519-525.

FINCHAM, J. E., WOODROOF, C. W., VAN WYK, M. J., CAPATOS, D., WEIGHT, M. J., KRITCHEVSKY, D. & ROSSOUW, J. E. 1987b. Promotion and regression of atherosclerosis in vervet monkeys by diets realistic for Westernised people. *Atherosclerosis,* 66**,** 205-213.

FIORANELLI, M., BOTTACCIOLI, A. G., BOTTACCIOLI, F., BIANCHI, M., ROVESTI, M. & ROCCIA, M. G. 2018. Stress and inflammation in coronary artery disease: a review psychoneuroendocrineimmunology-based. *Frontiers in immunology,* 9**,** 2031.

FISHER, T. S., SURDO, P. L., PANDIT, S., MATTU, M., SANTORO, J. C., WISNIEWSKI, D., CUMMINGS, R. T., CALZETTA, A., CUBBON, R. M. & FISCHER, P. A. 2007. Effects of pH and low density lipoprotein (LDL) on PCSK9-dependent LDL receptor regulation. *Journal of Biological Chemistry,* 282**,** 20502-20512.

FÖGER, B., CHASE, M., AMAR, M. J., VAISMAN, B. L., SHAMBUREK, R. D., PAIGEN, B., FRUCHART-NAJIB, J., PAIZ, J. A., KOCH, C. A. & HOYT, R. F. 1999. Cholesteryl ester transfer protein corrects dysfunctional high density lipoproteins and reduces aortic atherosclerosis in lecithin cholesterol acyltransferase transgenic mice. *Journal of Biological Chemistry,* 274**,** 36912-36920.

FRANCESCHINI, G., FAVARI, E., CALABRESI, L., SIMONELLI, S., BONDIOLI, A., ADORNI, M. P., ZIMETTI, F., GOMARASCHI, M., COUTANT, K. & ROSSOMANNO, S. 2013. Differential effects of fenofibrate and extended-release niacin on high-density lipoprotein particle size distribution and cholesterol efflux capacity in dyslipidemic patients. *Journal of clinical lipidology,* 7**,** 414-422.

FRANK-KAMENETSKY, M., GREFHORST, A., ANDERSON, N. N., RACIE, T. S., BRAMLAGE, B., AKINC, A., BUTLER, D., CHARISSE, K., DORKIN, R. & FAN, Y. 2008. Therapeutic RNAi targeting PCSK9 acutely lowers plasma cholesterol in rodents and LDL cholesterol in nonhuman primates. *Proceedings of the National Academy of Sciences,* 105**,** 11915-11920.

FRIEDLANDER, Y., SCHWARTZ, S. M., DURST, R., MEINER, V., ROBERTSON, A. S., EREZ, G., LEITERSDORF, E. & SISCOVICK, D. S. 2008. SREBP-2 and SCAP isoforms and risk of early onset myocardial infarction. *Atherosclerosis,* 196**,** 896-904.

FUSEGAWA, Y., KELLEY, K. L., SAWYER, J. K., SHAH, R. N. & RUDEL, L. L. 2001. Influence of dietary fatty acid composition on the relationship between CETP activity and plasma lipoproteins in monkeys. *Journal of lipid research,* 42**,** 1849-1857.

GANJI, S. H., KAMANNA, V. S. & KASHYAP, M. L. 2003. Niacin and cholesterol: role in cardiovascular disease. *The Journal of nutritional biochemistry,* 14**,** 298-305.

GANJI, S. H., TAVINTHARAN, S., ZHU, D., XING, Y., KAMANNA, V. S. & KASHYAP, M. L. 2004. Niacin noncompetitively inhibits DGAT2 but not DGAT1 activity in HepG2 cells. *Journal of lipid research,* 45**,** 1835-1845.

GENT, J. & BRAAKMAN, I. 2004. Low-density lipoprotein receptor structure and folding. *Cellular and Molecular Life Sciences CMLS,* 61**,** 2461-2470.

GIBBONS, L. W., GONZALEZ, V., GORDON, N. & GRUNDY, S. 1995. The prevalence of side effects with regular and sustained-release nicotinic acid. *The American journal of medicine,* 99**,** 378-385.

GILLE, A., BODOR, E. T., AHMED, K. & OFFERMANNS, S. 2008. Nicotinic acid: pharmacological effects and mechanisms of action. *Annu. Rev. Pharmacol. Toxicol.,* 48**,** 79-106.

GLASS, C. K. & WITZTUM, J. L. 2001. Atherosclerosis: the road ahead. *Cell,* 104**,** 503-516.
GLOMSET, J. A. 1968. The plasma lecithin: cholesterol acyltransferase reaction. *Journal of lipid research,* 9**,** 155-167.
GOEL, H. & DUNBAR, R. L. 2016. Niacin alternatives for dyslipidemia: fool's gold or gold mine? Part II: novel niacin mimetics. *Current atherosclerosis reports,* 18**,** 17.
GOLDSTEIN, J. L. & BROWN, M. S. 2009. The LDL receptor. *Arteriosclerosis, thrombosis, and vascular biology,* 29**,** 431-438.
GOUNI-BERTHOLD, I. & K BERTHOLD, H. 2011. Antisense oligonucleotides for the treatment of dyslipidemia. *Current pharmaceutical design,* 17**,** 950-960.
GRAHAM, M. J., LEMONIDIS, K. M., WHIPPLE, C. P., SUBRAMANIAM, A., MONIA, B. P., CROOKE, S. T. & CROOKE, R. M. 2007. Antisense inhibition of proprotein convertase subtilisin/kexin type 9 reduces serum LDL in hyperlipidemic mice. *Journal of lipid research,* 48**,** 763-767.
GRUNDY, S. M. & DENKE, M. A. 1990. Dietary influences on serum lipids and lipoproteins. *Journal of lipid research,* 31**,** 1149-1172.
GU, H.-M. & ZHANG, D.-W. 2015. Hypercholesterolemia, low density lipoprotein receptor and proprotein convertase subtilisin/kexin-type 9. *Journal of biomedical research,* 29**,** 356.
GUPTA, N., FISKER, N., ASSELIN, M.-C., LINDHOLM, M., ROSENBOHM, C., ØRUM, H., ELMÉN, J., SEIDAH, N. G. & STRAARUP, E. M. 2010. A locked nucleic acid antisense oligonucleotide (LNA) silences PCSK9 and enhances LDLR expression in vitro and in vivo. *PloS one,* 5**,** e10682.
GUSAROVA, V., BRODSKY, J. L. & FISHER, E. A. 2003. Apolipoprotein B100 exit from the endoplasmic reticulum (ER) is COPII-dependent, and its lipidation to very low density lipoprotein occurs post-ER. *Journal of Biological Chemistry,* 278**,** 48051-48058.
HAN, B., EACHO, P. I., KNIERMAN, M. D., TROUTT, J. S., KONRAD, R. J., YU, X. & SCHROEDER, K. M. 2014. Isolation and characterization of the circulating truncated form of PCSK9. *Journal of lipid research,* 55**,** 1505-1514.
HANDELSMAN, Y. & SHAPIRO, M. D. 2016. Triglycerides, atherosclerosis, and cardiovascular outcome studies: focus on omega-3 fatty acids. *Endocrine Practice,* 23**,** 100-112.
HARDER, C., LAU, P., MENG, A., WHITMAN, S. C. & MCPHERSON, R. 2007. Cholesteryl ester transfer protein (CETP) expression protects against diet induced atherosclerosis in SR-BI deficient mice. *Arteriosclerosis, thrombosis, and vascular biology,* 27**,** 858-864.
HARPER, C. R. & JACOBSON, T. A. 2007. The broad spectrum of statin myopathy: from myalgia to rhabdomyolysis. *Current opinion in lipidology,* 18**,** 401-408.
HAYEK, T., MASUCCI-MAGOULAS, L., JIANG, X., WALSH, A., RUBIN, E., BRESLOW, J. L. & TALL, A. R. 1995. Decreased early atherosclerotic lesions in hypertriglyceridemic mice expressing cholesteryl ester transfer protein transgene. *The Journal of clinical investigation,* 96**,** 2071-2074.
HENDERSON, R., O'KANE, M., MCGILLIGAN, V. & WATTERSON, S. 2016. The genetics and screening of familial hypercholesterolaemia. *Journal of biomedical science,* 23**,** 39.
HERMAN, R. J. 1999. Drug interactions and the statins. *Cmaj,* 161**,** 1281-1286.
HERRERA, V. L., MAKRIDES, S. C., XIE, H. X., ADARI, H., KRAUSS, R. M., RYAN, U. S. & RUIZ-OPAZO, N. 1999. Spontaneous combined hyperlipidemia, coronary heart disease and decreased survival in Dahl salt-sensitive hypertensive rats transgenic for human cholesteryl ester transfer protein. *Nature medicine,* 5**,** 1383.
HERRINGTON, W., LACEY, B., SHERLIKER, P., ARMITAGE, J. & LEWINGTON, S. 2016. Epidemiology of atherosclerosis and the potential to reduce the global burden of atherothrombotic disease. *Circulation research,* 118**,** 535-546.
HERSCOVITZ, H., KRITIS, A., TALIANIDIS, I., ZANNI, E., ZANNIS, V. & SMALL, D. M. 1995. Murine mammary-derived cells secrete the N-terminal 41% of human apolipoprotein B on high density

lipoprotein-sized lipoproteins containing a triacylglycerol-rich core. *Proceedings of the National Academy of Sciences,* 92**,** 659-663.

HIGGINS, P. B., BASTARRACHEA, R. A., LOPEZ-ALVARENGA, J. C., GARCIA-FOREY, M., PROFFITT, J. M., VORUGANTI, V. S., TEJERO, M. E., MATTERN, V., HAACK, K. & SHADE, R. E. 2010. Eight week exposure to a high sugar high fat diet results in adiposity gain and alterations in metabolic biomarkers in baboons (Papio hamadryas sp.). *Cardiovascular diabetology,* 9**,** 71.

HIGGINS, P. B., RODRIGUEZ, P. J., VORUGANTI, V. S., MATTERN, V., BASTARRACHEA, R. A., RICE, K., RAABE, T. & COMUZZIE, A. G. 2014. Body composition and cardiometabolic disease risk factors in captive baboons (Papio hamadryas sp.): Sexual dimorphism. *American journal of physical anthropology,* 153**,** 9-14.

HOBBS, F. 2004. Cardiovascular disease: different strategies for primary and secondary prevention? *Heart,* 90**,** 1217-1223.

HOCHHOLZER, W., BERG, D. D. & GIUGLIANO, R. P. 2011. The facts behind niacin. *Therapeutic advances in cardiovascular disease,* 5**,** 227-240.

HOEKSTRA, M., VAN BERKEL, T. J. & VAN ECK, M. 2010. Scavenger receptor BI: a multi-purpose player in cholesterol and steroid metabolism. *World journal of gastroenterology: WJG,* 16**,** 5916.

HOLLA, Ø. L., CAMERON, J., BERGE, K. E., RANHEIM, T. & LEREN, T. P. 2007. Degradation of the LDL receptors by PCSK9 is not mediated by a secreted protein acted upon by PCSK9 extracellularly. *BMC cell biology,* 8**,** 9.

HOMEISTER, J. W. & WILLIS, M. S. 2010. Atherosclerosis: Pathogenesis, Genetics and Experimental Models. *eLS*.

HORTON, J. D., SHAH, N. A., WARRINGTON, J. A., ANDERSON, N. N., PARK, S. W., BROWN, M. S. & GOLDSTEIN, J. L. 2003. Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. *Proceedings of the National Academy of Sciences,* 100**,** 12027-12032.

HORTON, J. D., SHIMOMURA, I., BROWN, M. S., HAMMER, R. E., GOLDSTEIN, J. L. & SHIMANO, H. 1998. Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. *The Journal of clinical investigation,* 101**,** 2331-2339.

HUANG, X. F. & SHELNESS, G. S. 1997. Identification of cysteine pairs within the amino-terminal 5% of apolipoprotein B essential for hepatic lipoprotein assembly and secretion. *Journal of Biological Chemistry,* 272**,** 31872-31876.

HUSSAIN, M. M., SHI, J. & DREIZEN, P. 2003. Microsomal triglyceride transfer protein and its role in apoB-lipoprotein assembly. *Journal of lipid research,* 44**,** 22-32.

INAZU, A., BROWN, M. L., HESLER, C. B., AGELLON, L. B., KOIZUMI, J., TAKATA, K., MARUHAMA, Y., MABUCHI, H. & TALL, A. R. 1990. Increased high-density lipoprotein levels caused by a common cholesteryl-ester transfer protein gene mutation. *New England Journal of Medicine,* 323**,** 1234-1238.

INAZU, A., KOIZUMI, J. & MABUCHI, H. 2000. Cholesteryl ester transfer protein and atherosclerosis. *Current opinion in lipidology,* 11**,** 389-396.

INDOLFI, C. 2002. Genetic factors in atherosclerosis: status and perspectives. *European Heart Journal Supplements,* 4**,** B14-B16.

INVESTIGATORS, A.-H. 2011. The role of niacin in raising high-density lipoprotein cholesterol to reduce cardiovascular events in patients with atherosclerotic cardiovascular disease and optimally treated low-density lipoprotein cholesterol: rationale and study design. The Atherothrombosis Intervention in Metabolic syndrome with low HDL/high triglycerides: Impact on Global Health outcomes (AIM-HIGH). *American heart journal,* 161**,** 471-477. e2.

ISTVAN, E. S. & DEISENHOFER, J. 2001. Structural mechanism for statin inhibition of HMG-CoA reductase. *Science,* 292**,** 1160-1164.

JACOBSON, T. A. Lipoprotein (a), cardiovascular disease, and contemporary management. Mayo Clinic Proceedings, 2013. Elsevier, 1294-1311.

JAMALDINI, S. H., BABANEJAD, M., MOZAFFARI, R., NIKZAT, N., JALALVAND, K., BADIEI, A., SANATI, H., SHAKERIAN, F., AFSHARI, M. & KAHRIZI, K. 2014. Association of polymorphisms at LDLR locus with coronary artery disease independently from lipid profile. *Acta Medica Iranica***,** 352-359.

JASINSKA, A. J., SCHMITT, C. A., SERVICE, S. K., CANTOR, R. M., DEWAR, K., JENTSCH, J. D., KAPLAN, J. R., TURNER, T. R., WARREN, W. C. & WEINSTOCK, G. M. 2013. Systems biology of the vervet monkey. *ILAR journal,* 54**,** 122-143.

JEON, H., MENG, W., TAKAGI, J., ECK, M. J., SPRINGER, T. A. & BLACKLOW, S. C. 2001. Implications for familial hypercholesterolemia from the structure of the LDL receptor YWTD-EGF domain pair. *Nature Structural & Molecular Biology,* 8**,** 499.

JIANG, X. C., AGELLON, L. B., WALSH, A., BRESLOW, J. L. & TALL, A. 1992. Dietary cholesterol increases transcription of the human cholesteryl ester transfer protein gene in transgenic mice. Dependence on natural flanking sequences. *The Journal of clinical investigation,* 90**,** 1290-1295.

JIN, F.-Y., KAMANNA, V. S. & KASHYAP, M. L. 1997. Niacin decreases removal of high-density lipoprotein apolipoprotein AI but not cholesterol ester by Hep G2 cells: implication for reverse cholesterol transport. *Arteriosclerosis, thrombosis, and vascular biology,* 17**,** 2020-2028.

JIN, F.-Y., KAMANNA, V. S. & KASHYAP, M. L. 1999. Niacin accelerates intracellular ApoB degradation by inhibiting triacylglycerol synthesis in human hepatoblastoma (HepG2) cells. *Arteriosclerosis, thrombosis, and vascular biology,* 19**,** 1051-1059.

JØRGENSEN, A. B., FRIKKE-SCHMIDT, R., WEST, A. S., GRANDE, P., NORDESTGAARD, B. G. & TYBJÆRG-HANSEN, A. 2012. Genetically elevated non-fasting triglycerides and calculated remnant cholesterol as causal risk factors for myocardial infarction. *European heart journal,* 34**,** 1826-1833.

JUNYENT, M., GILABERT, R., JARAUTA, E., NÚÑEZ, I., COFÁN, M., CIVEIRA, F., POCOVÍ, M., MALLÉN, M., ZAMBÓN, D. & ALMAGRO, F. 2010. Impact of low-density lipoprotein receptor mutational class on carotid atherosclerosis in patients with familial hypercholesterolemia. *Atherosclerosis,* 208**,** 437-441.

KAKKO, S., TAMMINEN, M., PÄIVÄNSALO, M., KAUMA, H., RANTALA, A., LILJA, M., REUNANEN, A., KESÄNIEMI, Y. & SAVOLAINEN, M. 2000. Cholesteryl ester transfer protein gene polymorphisms are associated with carotid atherosclerosis in men. *European journal of clinical investigation,* 30**,** 18-25.

KAKO, Y., MASSÉ, M., HUANG, L.-S., TALL, A. R. & GOLDBERG, I. J. 2002. Lipoprotein lipase deficiency and CETP in streptozotocin-treated apoB-expressing mice. *Journal of lipid research,* 43**,** 872-877.

KAMANNA, V. 2008. Kashyap ML. *Mechanism of action of niacin. Am J Cardiol,* 101**,** 20B-26B.

KAMANNA, V. S. & KASHYAP, M. L. 2000. Mechanism of action of niacin on lipoprotein metabolism. *Current atherosclerosis reports,* 2**,** 36-46.

KAMATH, S., CHAVEZ, A. O., GASTALDELLI, A., CASIRAGHI, F., HALFF, G. A., ABRAHAMIAN, G. A., DAVALLI, A. M., BASTARRACHEA, R. A., COMUZZIE, A. G. & GUARDADO-MENDOZA, R. 2011. Coordinated defects in hepatic long chain fatty acid metabolism and triglyceride accumulation contribute to insulin resistance in non-human primates. *PLoS One,* 6**,** e27617.

KANG, I., KIM, S.-W. & YOUN, J. H. 2011. Effects of nicotinic acid on gene expression: potential mechanisms and implications for wanted and unwanted effects of the lipid-lowering drug. *The Journal of Clinical Endocrinology & Metabolism,* 96**,** 3048-3055.

KARDASSIS, D., MOSIALOU, I., KANAKI, M., TINIAKOU, I. & THYMIAKOU, E. 2014. Metabolism of HDL and its regulation. *Current medicinal chemistry,* 21**,** 2864-2880.

KASSNER, U., SCHLABS, T., ROSADA, A. & STEINHAGEN-THIESSEN, E. 2015. Lipoprotein (a)–An independent causal risk factor for cardiovascular disease and current therapeutic options. *Atherosclerosis Supplements,* 18**,** 263-267.

KHERA, A. V., QAMAR, A., REILLY, M. P., DUNBAR, R. L. & RADER, D. J. 2015a. Effects of niacin, statin, and fenofibrate on circulating proprotein convertase subtilisin/kexin type 9 levels in patients with dyslipidemia. *American Journal of Cardiology,* 115**,** 178-182.

KHERA, A. V., QAMAR, A., REILLY, M. P., DUNBAR, R. L. & RADER, D. J. 2015b. Effects of niacin, statin, and fenofibrate on circulating proprotein convertase subtilisin/kexin type 9 levels in patients with dyslipidemia. *The American journal of cardiology,* 115**,** 178-182.

KHETARPAL, S. A. & RADER, D. J. 2015. Triglyceride-rich lipoproteins and coronary artery disease risk: new insights from human genetics. *Arteriosclerosis, thrombosis, and vascular biology,* 35**,** e3-e9.

KHOZA, S., NGQANEKA, T., MAGWEBU, Z. E. & CHAUKE, C. G. 2019. Nonketotic hyperglycinemia in captive-bred Vervet monkeys (Chlorocebus aethiops) with cataracts. *Journal of medical primatology,* 48**,** 161-165.

KLANČAR, G., GROŠELJ, U., KOVAČ, J., BRATANIČ, N., BRATINA, N., PODKRAJŠEK, K. T. & BATTELINO, T. 2015. Universal screening for familial hypercholesterolemia in children. *Journal of the American College of Cardiology,* 66**,** 1250-1257.

KNOPP, R. H. 2000. Evaluating niacin in its various forms. *The American journal of cardiology,* 86**,** 51-56.

KOLOVOU, G., STAMATELATOU, M., ANAGNOSTOPOULOU, K., KOSTAKOU, P., KOLOVOU, V., MIHAS, C., VASILIADIS, I., DIAKOUMAKOU, O., MIKHAILIDIS, D. P. & COKKINOS, D. V. 2010. Cholesteryl ester transfer protein gene polymorphisms and longevity syndrome. *The open cardiovascular medicine journal,* 4**,** 14.

KOLOVOU, G. D. & ANAGNOSTOPOULOU, K. K. 2007. Apolipoprotein E polymorphism, age and coronary heart disease. *Ageing research reviews,* 6**,** 94-108.

KOLOVOU, G. D., ANAGNOSTOPOULOU, K. K., KOSTAKOU, P., GIANNAKOPOULOU, V., MIHAS, C., HATZIGEORGIOU, G., VASILIADIS, I. K., MIKHAILIDIS, D. P. & COKKINOS, D. V. 2009. Apolipoprotein E gene polymorphism and obesity status in middle-aged men with coronary heart disease. *in vivo,* 23**,** 33-39.

KOLOVOU, G. D., ANAGNOSTOPOULOU, K. K., MIKHAILIDIS, D. P., PANAGIOTAKOS, D. B., PILATIS, N. D., CARIOLOU, M. A., YIANNAKOURIS, N., DEGIANNIS, D., STAVRIDIS, G. & COKKINOS, D. V. 2005. Association of apolipoprotein E genotype with early onset of coronary heart disease in Greek men. *Angiology,* 56**,** 663-670.

KOLOVOU, G. D., DEDOUSSIS, G. V., ANAGNOSTOPOULOU, K. K., HATZIGEORGIOU, G. C., SALPEA, K. D., CHOUMERIANOU, D. M., RAMMOS, S., MIKHAILIDIS, D. P. & COKKINOS, D. V. 2007. Management of a patient with a null low-density lipoprotein receptor mutation: a case report. *Angiology,* 57**,** 729-732.

KOLOVOU, V., MARVAKI, A., KARAKOSTA, A., VASILOPOULOS, G., KALOGIANI, A., MAVROGENI, S., DEGIANNIS, D., MARVAKI, C. & KOLOVOU, G. 2012. Association of gender, ABCA1 gene polymorphisms and lipid profile in Greek young nurses. *Lipids in health and disease,* 11**,** 62.

KOMAROVA, T. Y., KORNEVA, V. A., KUZNETSOVA, T. Y., GOLOVINA, A. S., VASILYEV, V. B. & MANDELSHTAM, M. Y. 2013. Familial hypercholesterolemia mutations in Petrozavodsk: no similarity to St. Petersburg mutation spectrum. *BMC medical genetics,* 14**,** 128.

KOTOWSKI, I. K., PERTSEMLIDIS, A., LUKE, A., COOPER, R. S., VEGA, G. L., COHEN, J. C. & HOBBS, H. H. 2006. A spectrum of PCSK9 alleles contributes to plasma levels of low-density lipoprotein cholesterol. *The American Journal of Human Genetics,* 78**,** 410-422.

KOVACIC, S. & BAKRAN, M. 2012. Genetic susceptibility to atherosclerosis. *Stroke research and treatment,* 2012.

KOZYRAKI, R., FYFE, J., KRISTIANSEN, M., GERDES, C., JACOBSEN, C., CUI, S., CHRISTENSEN, E. I., AMINOFF, M., DE LA CHAPELLE, A. & KRAHE, R. 1999. The intrinsic factor–vitamin B 12 receptor, cubilin, is a high-affinity apolipoprotein AI receptor facilitating endocytosis of high-density lipoprotein. *Nature medicine,* 5**,** 656.

KRAUSS, R. M. 1994. Heterogeneity of plasma low-density lipoproteins and atherosclerosis risk. *Current opinion in lipidology,* 5**,** 339-349.

KRUGER, M., SMUTS, C., BENADÉ, A., FINCHAM, J., LOMBARD, C., ALBERTSE, E. & VAN DER MERWE, K. 1992. Comparison of the effect of the amount and degree of unsaturation of dietary fat on plasma low density lipoproteins in vervet monkeys. *Lipids,* 27**,** 733-739.

KUSHWAHA, R. S. & MCGILL, H. 1998. Diet, plasma lipoproteins and experimental atherosclerosis in baboons (Papio sp.). *Human Reproduction Update,* 4**,** 420-429.

KUSHWAHA, R. S., REARDON, C. A., GETZ, G., LEWIS, D., RICE, K. S., CAREY, K. & MCGILL, H. 1994. Metabolic mechanisms for responses to dietary cholesterol and fat in high and low LDL responding baboons (Papio sp.). *Journal of lipid research,* 35**,** 633-643.

KWON, H. J., LAGACE, T. A., MCNUTT, M. C., HORTON, J. D. & DEISENHOFER, J. 2008. Molecular basis for LDL receptor recognition by PCSK9. *Proceedings of the National Academy of Sciences,* 105**,** 1820-1825.

LABARTHE, D. R. 2010. *Epidemiology and prevention of cardiovascular diseases: a global challenge*, Jones & Bartlett Publishers.

LAGACE, T. A., CURTIS, D. E., GARUTI, R., MCNUTT, M. C., PARK, S. W., PRATHER, H. B., ANDERSON, N. N., HO, Y., HAMMER, R. E. & HORTON, J. D. 2006. Secreted PCSK9 decreases the number of LDL receptors in hepatocytes and inlivers of parabiotic mice. *The Journal of clinical investigation,* 116**,** 2995-3005.

LAMARCHE, B., ST-PIERRE, A. C., RUEL, I. L., CANTIN, B., DAGENAIS, G. R. & DESPRÉS, J.-P. 2001. A prospective, population-based study of low density lipoprotein particle size as a risk factor for ischemic heart disease in men. *The Canadian journal of cardiology,* 17**,** 859-865.

LANE, M. A. 2000. Nonhuman primate models in biogerontology. *Experimental gerontology,* 35**,** 533-541.

LARSSON, S. L., SKOGSBERG, J. & BJÖRKEGREN, J. 2004. The low density lipoprotein receptor prevents secretion of dense apoB100-containing lipoproteins from the liver. *Journal of Biological Chemistry,* 279**,** 831-836.

LEVY, E., STAN, S., DELVIN, E., MÉNARD, D., SHOULDERS, C., GAROFALO, C., SLIGHT, I., SEIDMAN, E., MAYER, G. & BENDAYAN, M. S. 2002. Localization of Microsomal Triglyceride Transfer Protein in the Golgi POSSIBLE ROLE IN THE ASSEMBLY OF CHYLOMICRONS. *Journal of Biological Chemistry,* 277**,** 16470-16477.

LI, H., DONG, B., PARK, S. W., LEE, H.-S., CHEN, W. & LIU, J. 2009. HNF1α plays a critical role in PCSK9 gene transcription and regulation by a natural hypocholesterolemic compound berberine. *Journal of Biological Chemistry***,** jbc. M109. 052407.

LIANG, H., CHAPARRO-RIGGERS, J., STROP, P., GENG, T., SUTTON, J. E., TSAI, D., BAI, L., ABDICHE, Y., DILLEY, J. & YU, J. 2012. Proprotein convertase substilisin/kexin type 9 antagonism reduces low-density lipoprotein cholesterol in statin-treated hypercholesterolemic nonhuman primates. *Journal of Pharmacology and Experimental Therapeutics,* 340**,** 228-236.

LIBBY, P. 2003. Vascular biology of atherosclerosis: overview and state of the art. *The American journal of cardiology,* 91**,** 3-6.

LIBBY, P., RIDKER, P. M. & HANSSON, G. K. 2011. Progress and challenges in translating the biology of atherosclerosis. *Nature,* 473**,** 317.

LIBBY, P., TABAS, I., FREDMAN, G. & FISHER, E. A. 2014. Inflammation and its resolution as determinants of acute coronary syndromes. *Circulation research,* 114**,** 1867-1879.

LINDHOLM, M. W., ELMÉN, J., FISKER, N., HANSEN, H. F., PERSSON, R., MØLLER, M. R., ROSENBOHM, C., ØRUM, H., STRAARUP, E. M. & KOCH, T. 2012. PCSK9 LNA antisense oligonucleotides induce sustained reduction of LDL cholesterol in nonhuman primates. *Molecular Therapy,* 20**,** 376-381.

LIU, Y., MANCHEKAR, M., SUN, Z., RICHARDSON, P. E. & DASHTI, N. 2010. Apolipoprotein B-containing lipoprotein assembly in microsomal triglyceride transfer protein-deficient McA-RH7777 cells. *Journal of lipid research,* 51**,** 2253-2264.

LOPEZ, A. D., MATHERS, C. D., EZZATI, M., JAMISON, D. T. & MURRAY, C. J. 2006. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. *The Lancet,* 367**,** 1747-1757.

LUO, Y. & TALL, A. R. 2000. Sterol upregulation of human CETP expression in vitro and in transgenic mice by an LXR element. *The Journal of clinical investigation,* 105**,** 513-520.

LUSIS, A. J. 2012. Genetics of atherosclerosis. *Trends in Genetics,* 28**,** 267-275.

MACCUBBIN, D., KOREN, M. J., DAVIDSON, M., GAVISH, D., PASTERNAK, R. C., MACDONELL, G., MALLICK, M., SISK, C. M., PAOLINI, J. F. & MITCHEL, Y. 2009. Flushing profile of extended-release niacin/laropiprant versus gradually titrated niacin extended-release in patients with dyslipidemia with and without ischemic cardiovascular disease. *The American journal of cardiology,* 104**,** 74-81.

MACLEAN, P. S., BOWER, J. F., VADLAMUDI, S., OSBORNE, J. N., BRADFIELD, J. F., BURDEN, H. W., BENSCH, W. H., KAUFFMAN, R. F. & BARAKAT, H. A. 2003. Cholesteryl ester transfer protein expression prevents diet-induced atherosclerotic lesions in male db/db mice. *Arteriosclerosis, thrombosis, and vascular biology,* 23**,** 1412-1415.

MADISON, B. B. 2016. Srebp2: a master regulator of sterol and fatty acid synthesis. *Journal of lipid research,* 57**,** 333-335.

MAMTANI, M., MEIKLE, P. J., KULKARNI, H., WEIR, J. M., BARLOW, C. K., JOWETT, J. B., BELLIS, C., DYER, T. D., ALMASY, L. & MAHANEY, M. C. 2014. Plasma dihydroceramide species associate with waist circumference in Mexican American families. *Obesity,* 22**,** 950-956.

MANUEL, D. G., LIM, J., TANUSEPUTRO, P., ANDERSON, G. M., ALTER, D. A., LAUPACIS, A. & MUSTARD, C. A. 2006. Revisiting Rose: strategies for reducing coronary heart disease. *Bmj,* 332**,** 659-662.

MARDUEL, M., CARRIÉ, A., SASSOLAS, A., DEVILLERS, M., CARREAU, V., DI FILIPPO, M., ERLICH, D., ABIFADEL, M., MARQUES-PINHEIRO, A. & MUNNICH, A. 2010. Molecular spectrum of autosomal dominant hypercholesterolemia in France. *Human mutation,* 31**,** E1811-E1824.

MAROTTI, K. R., CASTLE, C. K., BOYLE, T. P., LIN, A. H., MURRAY, R. W. & MELCHIOR, G. W. 1993. Severe atherosclerosis in transgenic mice expressing simian cholesteryl ester transfer protein. *Nature,* 364**,** 73.

MARTINEZ, L. O., JACQUET, S., ESTEVE, J.-P., ROLLAND, C., CABEZÓN, E., CHAMPAGNE, E., PINEAU, T., GEORGEAUD, V., WALKER, J. E. & TERCÉ, F. 2003. Ectopic β-chain of ATP synthase is an apolipoprotein AI receptor in hepatic HDL endocytosis. *Nature,* 421**,** 75.

MAXWELL, K. N. & BRESLOW, J. L. 2004. Adenoviral-mediated expression of Pcsk9 in mice results in a low-density lipoprotein receptor knockout phenotype. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 7100-7105.

MAXWELL, K. N., FISHER, E. A. & BRESLOW, J. L. 2005. Overexpression of PCSK9 accelerates the degradation of the LDLR in a post-endoplasmic reticulum compartment. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 2069-2074.

MAXWELL, K. N., SOCCIO, R. E., DUNCAN, E. M., SEHAYEK, E. & BRESLOW, J. L. 2003. Novel putative SREBP and LXR target genes identified by microarray analysis in liver of cholesterol-fed mice. *Journal of lipid research,* 44**,** 2109-2119.

MAYER, G., POIRIER, S. & SEIDAH, N. G. 2008. Annexin A2 is a C-terminal PCSK9-binding protein that regulates endogenous low density lipoprotein receptor levels. *Journal of Biological Chemistry,* 283**,** 31791-31801.
MCCASKIE, P. A., BEILBY, J. P., CHAPMAN, C. M., HUNG, J., MCQUILLAN, B. M., THOMPSON, P. L. & PALMER, L. J. 2007. Cholesteryl ester transfer protein gene haplotypes, plasma high-density lipoprotein levels and the risk of coronary heart disease. *Human genetics,* 121**,** 401-411.
MCGILL JR, H. C., MCMAHAN, C. A., KRUSKI, A. W. & MOTT, G. E. 1981. Relationship of lipoprotein cholesterol concentrations to experimental atherosclerosis in baboons. *Arteriosclerosis: An Official Journal of the American Heart Association, Inc.,* 1**,** 3-12.
MCKENNEY, J. 2004. New perspectives on the use of niacin in the treatment of lipid disorders. *Archives of internal medicine,* 164**,** 697-705.
MCNUTT, M. C., LAGACE, T. A. & HORTON, J. D. 2007. Catalytic activity is not required for secreted PCSK9 to reduce low density lipoprotein receptors in HepG2 cells. *Journal of Biological Chemistry,* 282**,** 20799-20803.
MEINER, V., FRIEDLANDER, Y., MILO, H., SHARON, N., BEN-AVI, L., SHPITZEN, S., LEITERSDORF, E., SISCOVICK, D. S. & SCHWARTZ, S. M. 2008. Cholesteryl ester transfer protein (CETP) genetic variation and early onset of non-fatal myocardial infarction. *Annals of human genetics,* 72**,** 732-741.
MENDIS, S., PUSKA, P., NORRVING, B. & ORGANIZATION, W. H. 2011. *Global atlas on cardiovascular disease prevention and control*, Geneva: World Health Organization.
MILASAN, A., DALLAIRE, F., MAYER, G. & MARTEL, C. 2016. Effects of LDL receptor modulation on lymphatic function. *Scientific reports,* 6**,** 27862.
MILEWICZ, D. M. & SEIDMAN, C. E. 2000. Genetics of cardiovascular disease. *Circulation,* 102**,** Iv-103-Iv-111.
MILLER, M. 2015. Is tryglyceride therapy worth the effort. *Cleve Clin J Med,* 82**,** 162-166.
MIYAKE, Y., KIMURA, R., KOKUBO, Y., OKAYAMA, A., TOMOIKE, H., YAMAMURA, T. & MIYATA, T. 2008. Genetic variants in PCSK9 in the Japanese population: rare genetic variants in PCSK9 might collectively contribute to plasma LDL cholesterol levels in the general population. *Atherosclerosis,* 196**,** 29-36.
MOGHADASIAN, M. H., FROHLICH, J. J. & MCMANUS, B. M. 2001a. Advances in experimental dyslipidemia and atherosclerosis. *Laboratory Investigation,* 81**,** 1173-1183.
MOGHADASIAN, M. H., FROHLICH, J. J. & MCMANUS, B. M. 2001b. Advances in experimental dyslipidemia and atherosclerosis. *Laboratory Investigation,* 81**,** 1173.
MORGAN, J., CAPUZZI, D., GUYTON, J., CENTOR, R., GOLDBERG, R., ROBBINS, D., DIPETTE, D., JENKINS, S. & MARCOVINA, S. 1996. Treatment effect of Niaspan, a controlled-release niacin, in patients with hypercholesterolemia: a placebo-controlled trial. *Journal of cardiovascular pharmacology and therapeutics,* 1**,** 195-202.
MORGAN, J. M., CAREY, C. M., LINCOFF, A. & CAPUZZI, D. M. 2004. The effects of niacin on lipoprotein subclass distribution. *Preventive cardiology,* 7**,** 182-189.
MORRISH, N., WANG, S.-L., STEVENS, L., FULLER, J., KEEN, H. & GROUP, W. M. S. 2001. Mortality and causes of death in the WHO Multinational Study of Vascular Disease in Diabetes. *Diabetologia,* 44**,** S14.
MOTA, R., HOMEISTER, J. W., WILLIS, M. S. & BAHNSON, E. M. 2001. Atherosclerosis: Pathogenesis, Genetics and Experimental Models. *eLS***,** 1-10.
MOZAFFARIAN, D., BENJAMIN, E. J., GO, A. S., ARNETT, D. K., BLAHA, M. J., CUSHMAN, M., DAS, S. R., DE FERRANTI, S., DESPRÉS, J. P. & FULLERTON, H. J. 2016. Heart disease and stroke statistics-2016 update a report from the American Heart Association. *Circulation,* 133**,** e38-e48.

MULLER, P. & MISEREZ, A. 2002. Identification of mutations in the gene encoding sterol regulatory element binding protein (SREBP)-2 in hypercholesterolaemic subjects. *Journal of medical genetics,* 39**,** 271-275.

NACKLEY, A. G., SHABALINA, S., TCHIVILEVA, I., SATTERFIELD, K., KORCHYNSKYI, O., MAKAROV, S., MAIXNER, W. & DIATCHENKO, L. 2006. Human catechol-O-methyltransferase haplotypes modulate protein expression by altering mRNA secondary structure. *Science,* 314**,** 1930-1933.

NASSOURY, N., BLASIOLE, D. A., TEBON OLER, A., BENJANNET, S., HAMELIN, J., POUPON, V., MCPHERSON, P. S., ATTIE, A. D., PRAT, A. & SEIDAH, N. G. 2007. The cellular trafficking of the secretory proprotein convertase PCSK9 and its dependence on the LDLR. *Traffic,* 8**,** 718-732.

NATARAJAN, P., RAY, K. K. & CANNON, C. P. 2010. High-density lipoprotein and coronary heart disease: current and future therapies. *Journal of the American College of Cardiology,* 55**,** 1283-1299.

NAURECKIENE, S., MA, L., SREEKUMAR, K., PURANDARE, U., LO, C. F., HUANG, Y., CHIANG, L. W., GRENIER, J. M., OZENBERGER, B. A. & JACOBSEN, J. S. 2003. Functional characterization of Narc 1, a novel proteinase related to proteinase K. *Archives of Biochemistry and Biophysics,* 420**,** 55-67.

NI, Y. G., CONDRA, J. H., ORSATTI, L., SHEN, X., DI MARCO, S., PANDIT, S., BOTTOMLEY, M. J., RUGGERI, L., CUMMINGS, R. T. & CUBBON, R. M. 2010. A proprotein convertase subtilisin-like/kexin type 9 (PCSK9) C-terminal domain antibody antigen-binding fragment inhibits PCSK9 internalization and restores low density lipoprotein uptake. *Journal of Biological Chemistry,* 285**,** 12882-12891.

NI, Y. G., DI MARCO, S., CONDRA, J. H., PETERSON, L. B., WANG, W., WANG, F., PANDIT, S., HAMMOND, H. A., ROSA, R. & CUMMINGS, R. T. 2011. A PCSK9-binding antibody that structurally mimics the EGF (A) domain of LDL-receptor reduces LDL cholesterol in vivo. *Journal of lipid research,* 52**,** 78-86.

NICHOLS, A. & SMITH, L. 1965. Effect of very low-density lipoproteins on lipid transfer in incubated serum. *Journal of lipid research,* 6**,** 206-210.

NORDESTGAARD, B. G., BENN, M., SCHNOHR, P. & TYBJÆRG-HANSEN, A. 2007. Nonfasting triglycerides and risk of myocardial infarction, ischemic heart disease, and death in men and women. *Jama,* 298**,** 299-308.

NORDESTGAARD, B. G. & VARBO, A. 2014. Triglycerides and cardiovascular disease. *The Lancet,* 384**,** 626-635.

NORTH, C. L. & BLACKLOW, S. C. 1999. Structural independence of ligand-binding modules five and six of the LDL receptor. *Biochemistry,* 38**,** 3926-3935.

OKAMOTO, H., YONEMORI, F., WAKITANI, K., MINOWA, T., MAEDA, K. & SHINKAI, H. 2000. A cholesteryl ester transfer protein inhibitor attenuates atherosclerosis in rabbits. *Nature,* 406**,** 203.

OLIVEIRA, H. C., CHOUINARD, R. A., AGELLON, L. B., BRUCE, C., MA, L., WALSH, A., BRESLOW, J. L. & TALL, A. R. 1996. Human Cholesteryl Ester Transfer Protein Gene Proximal Promoter Contains Dietary Cholesterol Positive Responsive Elements and Mediates Expression in Small Intestine and Periphery While Predominant Liver and Spleen Expression Is Controlled by 5'-distal Sequences CIS-ACTING SEQUENCES MAPPED IN TRANSGENIC MICE. *Journal of Biological Chemistry,* 271**,** 31831-31838.

OLIVEIRA, H. C. & DE FARIA, E. C. 2011. Cholesteryl ester transfer protein: the controversial relation to atherosclerosis and emerging new biological roles. *IUBMB life,* 63**,** 248-257.

ORDOVAS, J. M. 2000. Genetic polymorphisms and activity of cholesterol ester transfer protein (CETP): should we be measuring them? *Clinical chemistry and laboratory medicine,* 38**,** 945-949.

ORGANIZATION, W. H. 2002. *The world health report 2002: reducing risks, promoting healthy life*, World Health Organization.

ORGANIZATION, W. H. 2003. *Prevention of recurrent heart attacks and strokes in low and middle income populations: evidence-based recommendations for policy-makers and health professionals*, World Health Organization.
ORGANIZATION, W. H. 2007. *Prevention of cardiovascular disease*, World Health Organization.
ORGANIZATION, W. H. 2008. The global burden of disease: 2004 update.
ORGANIZATION, W. H. 2014. Global health estimates: deaths by cause, age, sex and country, 2000-2012. *Geneva, WHO,* 9.
P MIKHAILIDIS, D., ELISAF, M., RIZZO, M., BERNEIS, K., GRIFFIN, B., ZAMBON, A., ATHYROS, V., DE GRAAF, J., MARZ, W. & G PARHOFER, K. 2011. "European panel on low density lipoprotein (LDL) subclasses": a statement on the pathophysiology, atherogenicity and clinical significance of LDL subclasses. *Current vascular pharmacology,* 9**,** 533-571.
PALMER-SMITH, H. & BASAK, A. 2010. Regulatory effects of peptides from the pro and catalytic domains of proprotein convertase subtilisin/kexin 9 (PCSK9) on low-density lipoprotein receptor (LDL-R). *Current medicinal chemistry,* 17**,** 2168-2182.
PAN, M., LIANG, J.-S., FISHER, E. A. & GINSBERG, H. N. 2002. The late addition of core lipids to nascent apolipoprotein B100, resulting in the assembly and secretion of triglyceride-rich lipoproteins, is independent of both microsomal triglyceride transfer protein activity and new triglyceride synthesis. *Journal of Biological Chemistry,* 277**,** 4413-4421.
PARK, S. W., MOON, Y.-A. & HORTON, J. D. 2004. Post-transcriptional regulation of low density lipoprotein receptor protein by proprotein convertase subtilisin/kexin type 9a in mouse liver. *Journal of Biological Chemistry,* 279**,** 50630-50638.
PARKS, J. S., CHUNG, S. & SHELNESS, G. S. 2012. Hepatic ABC transporters and triglyceride metabolism. *Current opinion in lipidology,* 23**,** 196.
PHILLIPS, C., OWENS, D., COLLINS, P. & TOMKIN, G. H. 2005. Low density lipoprotein non-esterified fatty acids and lipoprotein lipase in diabetes. *Atherosclerosis,* 181**,** 109-114.
PIEPER, J. A. 2002. Understanding niacin formulations. *The American journal of managed care,* 8**,** S308-14.
PLUMP, A. S., MASUCCI-MAGOULAS, L., BRUCE, C., BISGAIER, C. L., BRESLOW, J. L. & TALL, A. R. 1999. Increased atherosclerosis in ApoE and LDL receptor gene knock-out mice as a result of human cholesteryl ester transfer protein transgene expression. *Arteriosclerosis, thrombosis, and vascular biology,* 19**,** 1105-1110.
QIAN, Y.-W., SCHMIDT, R. J., ZHANG, Y., CHU, S., LIN, A., WANG, H., WANG, X., BEYER, T. P., BENSCH, W. R. & LI, W. 2007. Secreted PCSK9 downregulates low density lipoprotein receptor through receptor-mediated endocytosis. *Journal of lipid research,* 48**,** 1488-1498.
QIU, X., MISTRY, A., AMMIRATI, M. J., CHRUNYK, B. A., CLARK, R. W., CONG, Y., CULP, J. S., DANLEY, D. E., FREEMAN, T. B. & GEOGHEGAN, K. F. 2007. Crystal structure of cholesteryl ester transfer protein reveals a long tunnel and four bound lipid molecules. *Nature structural & molecular biology,* 14**,** 106.
RADER, D. J. 2003. Regulation of reverse cholesterol transport and clinical implications. *The American journal of cardiology,* 92**,** 42-49.
RADER, D. J., ALEXANDER, E. T., WEIBEL, G. L., BILLHEIMER, J. & ROTHBLAT, G. H. 2009. The role of reverse cholesterol transport in animals and humans and relationship to atherosclerosis. *Journal of lipid research,* 50**,** S189-S194.
RADER, D. J. & HOVINGH, G. K. 2014. HDL and cardiovascular disease. *The Lancet,* 384**,** 618-625.
RADER, D. J. & MAUGEAIS, C. 2000. Genes influencing HDL metabolism: new perspectives and implications for atherosclerosis prevention. *Molecular Medicine Today,* 6**,** 170-175.
RASHID, S., CURTIS, D. E., GARUTI, R., ANDERSON, N. N., BASHMAKOV, Y., HO, Y., HAMMER, R. E., MOON, Y.-A. & HORTON, J. D. 2005a. Decreased plasma cholesterol and hypersensitivity to

statins in mice lacking Pcsk9. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 5374-5379.
RASHID, S., CURTIS, D. E., GARUTI, R., ANDERSON, N. N., BASHMAKOV, Y., HO, Y., HAMMER, R. E., MOON, Y.-A. & HORTON, J. D. 2005b. Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. *Proceedings of the National Academy of Sciences,* 102**,** 5374-5379.
REICHL, D. & MILLER, N. E. 1989. Pathophysiology of reverse cholesterol transport. Insights from inherited disorders of lipoprotein metabolism. *Arteriosclerosis: An Official Journal of the American Heart Association, Inc.,* 9**,** 785-797.
REINER, Ž. 2014. Resistance and intolerance to statins. *Nutrition, Metabolism and Cardiovascular Diseases,* 24**,** 1057-1066.
RHAINDS, D., ARSENAULT, B. J. & TARDIF, J. C. 2012. PCSK9 inhibition and LDL cholesterol lowering: the biology of an attractive therapeutic target and critical review of the latest clinical trials. *Clinical Lipidology,* 7**,** 621-640.
RITTERSHAUS, C. W., MILLER, D. P., THOMAS, L. J., PICARD, M. D., HONAN, C. M., EMMETT, C. D., PETTEY, C. L., ADARI, H., HAMMOND, R. A. & BEATTIE, D. T. 2000. Vaccine-induced antibodies inhibit CETP activity in vivo and reduce aortic lesions in a rabbit model of atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology,* 20**,** 2106-2112.
ROBINET, P., VÉDIE, B., CHIRONI, G., GARIÉPY, J., SIMON, A., MOATTI, N. & PAUL, J.-L. 2003. Characterization of polymorphic structure of SREBP-2 gene: role in atherosclerosis. *Atherosclerosis,* 168**,** 381-387.
ROSENBLOOM, K. R., ARMSTRONG, J., BARBER, G. P., CASPER, J., CLAWSON, H., DIEKHANS, M., DRESZER, T. R., FUJITA, P. A., GURUVADOO, L. & HAEUSSLER, M. 2014. The UCSC genome browser database: 2015 update. *Nucleic acids research,* 43**,** D670-D681.
ROUSSET, X., VAISMAN, B., AMAR, M., SETHI, A. A. & REMALEY, A. T. 2009. Lecithin: cholesterol acyltransferase: from biochemistry to role in cardiovascular disease. *Current opinion in endocrinology, diabetes, and obesity,* 16**,** 163.
ROY, H., BHARDWAJ, S. & YLA-HERTTUALA, S. 2009. Molecular genetics of atherosclerosis. *Human genetics,* 125**,** 467-491.
ROY, P., MACKENZIE, R., HIRAMA, T., JIANG, X., KUSSIE, P., TALL, A., RASSART, E. & MILNE, R. 1996. Structure-function relationships of human cholesteryl ester transfer protein: analysis using monoclonal antibodies. *Journal of lipid research,* 37**,** 22-34.
RUBIN, E. M., KRAUSS, R. M., SPANGLER, E. A., VERSTUYFT, J. G. & CLIFT, S. M. 1991. Inhibition of early atherogenesis in transgenic mice by human apolipoprotein AI. *Nature,* 353**,** 265.
RUDEL, L. L., HAINES, J. L. & SAWYER, J. K. 1990. Effects on plasma lipoproteins of monounsaturated, saturated, and polyunsaturated fatty acids in the diet of African green monkeys. *Journal of lipid research,* 31**,** 1873-1882.
RUDEL, L. L., PARKS, J. S. & SAWYER, J. K. 1995. Compared with dietary monounsaturated and saturated fat, polyunsaturated fat protects African green monkeys from coronary artery atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology,* 15**,** 2101-2110.
RUSIÑOL, A. E., JAMIL, H. & VANCE, J. E. 1997. In vitro reconstitution of assembly of apolipoprotein B48-containing lipoproteins. *Journal of Biological Chemistry,* 272**,** 8019-8025.
RUSSELL, J. C. & PROCTOR, S. D. 2006. Small animal models of cardiovascular disease: tools for the study of the roles of metabolic syndrome, dyslipidemia, and atherosclerosis. *Cardiovascular pathology,* 15**,** 318-330.
RUSTAEUS, S., STILLEMARK, P., LINDBERG, K., GORDON, D. & OLOFSSON, S.-O. 1998. The microsomal triglyceride transfer protein catalyzes the post-translational assembly of apolipoprotein B-100 very low density lipoprotein in McA-RH7777 cells. *Journal of Biological Chemistry,* 273**,** 5196-5203.

SAKAI, T., KAMANNA, V. S. & KASHYAP, M. L. 2001. Niacin, but not gemfibrozil, selectively increases LP-AI, a cardioprotective subfraction of HDL, in patients with low HDL cholesterol. *Arteriosclerosis, thrombosis, and vascular biology,* 21**,** 1783-1789.

SALEK, L. & MARIAN, A. Genetic basis of coronary atherosclerosis. 2nd Virtual Congress of Cardiology. Argent Fed Cardiol, 2001.

SANZ, J. & FAYAD, Z. A. 2008. Imaging of atherosclerotic cardiovascular disease. *Nature,* 451**,** 953.

SARASWATHI, V. & HASTY, A. H. 2006. The role of lipolysis in mediating the proinflammatory effects of very low density lipoproteins in mouse peritoneal macrophages. *Journal of lipid research,* 47**,** 1406-1415.

SCHAEFER, E. J., LAMON-FAVA, S., COHN, S. D., SCHAEFER, M. M., ORDOVAS, J., CASTELLI, W. & WILSON, P. 1994. Effects of age, gender, and menopausal status on plasma low density lipoprotein cholesterol and apolipoprotein B levels in the Framingham Offspring Study. *Journal of lipid research,* 35**,** 779-792.

SCRIVER, C. R. 2001. *The metabolic & molecular bases of inherited disease*, New York; Montreal: McGraw-Hill.

SEGATTO, M., TRAPANI, L., MARINO, M. & PALLOTTINI, V. 2011. Age-and sex-related differences in extra-hepatic low-density lipoprotein receptor. *Journal of cellular physiology,* 226**,** 2610-2616.

SEGREST, J. P., JONES, M. K. & DASHTI, N. 1999. N-terminal domain of apolipoprotein B has structural homology to lipovitellin and microsomal triglyceride transfer protein: a "lipid pocket" model for self-assembly of apoB-containing lipoprotein particles. *Journal of lipid research,* 40**,** 1401-1416.

SEGREST, J. P., JONES, M. K., DE LOOF, H. & DASHTI, N. 2001. Structure of apolipoprotein B-100 in low density lipoproteins. *Journal of lipid research,* 42**,** 1346-1367.

SEIDAH, N. G. 2009. PCSK9 as a therapeutic target of dyslipidemia. *Expert opinion on therapeutic targets,* 13**,** 19-28.

SEIDAH, N. G., BENJANNET, S., WICKHAM, L., MARCINKIEWICZ, J., JASMIN, S. B., STIFANI, S., BASAK, A., PRAT, A. & CHRÉTIEN, M. 2003. The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation. *Proceedings of the National Academy of Sciences,* 100**,** 928-933.

SEIDAH, N. G. & PRAT, A. 2012. The biology and therapeutic targeting of the proprotein convertases. *Nature reviews Drug discovery,* 11**,** 367.

SEIER, J. 1986. Breeding vervet monkeys in a closed environment. *Journal of medical primatology,* 15**,** 339-349.

SELLERS, J. A. & SHELNESS, G. S. 2001. Lipoprotein assembly capacity of the mammary tumor-derived cell line C127 is due to the expression of functional microsomal triglyceride transfer protein. *Journal of lipid research,* 42**,** 1897-1904.

SEWRIGHT, K. A., CLARKSON, P. M. & THOMPSON, P. D. 2007. Statin myopathy: incidence, risk factors, and pathophysiology. *Current atherosclerosis reports,* 9**,** 389-396.

SHAH, P. K. 2007. Inhibition of CETP as a novel therapeutic strategy for reducing the risk of atherosclerotic disease. *European heart journal,* 28**,** 5-12.

SHAN, L., PANG, L., ZHANG, R., MURGOLO, N. J., LAN, H. & HEDRICK, J. A. 2008. PCSK9 binds to multiple receptors and can be functionally inhibited by an EGF-A peptide. *Biochemical and biophysical research communications,* 375**,** 69-73.

SHAPIRO, M. D. & FAZIO, S. 2017. Apolipoprotein B-containing lipoproteins and atherosclerotic cardiovascular disease. *F1000Research,* 6.

SHAPIRO, M. D., TAVORI, H. & FAZIO, S. 2018. PCSK9: from basic science discoveries to clinical trials. *Circulation research,* 122**,** 1420-1438.

SHIMANO, H., HORTON, J. D., HAMMER, R. E., SHIMOMURA, I., BROWN, M. S. & GOLDSTEIN, J. L. 1996. Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. *The Journal of clinical investigation,* 98**,** 1575-1584.

SMUTS, C., KRUGER, M., VAN JAARSVELD, P., FINCHAM, J., SCHALL, R., VAN DER MERWE, K. & BENADÉ, A. 1992. The influence of fish oil supplementation on plasma lipoproteins and arterial lipids in vervet monkeys with established atherosclerosis. *Prostaglandins, leukotrienes and essential fatty acids,* 47**,** 129-138.

SONG, W.-L. & FITZGERALD, G. A. 2013. Niacin, an old drug with a new twist. *Journal of lipid research,* 54**,** 2586-2594.

STANCU, C. & SIMA, A. 2001. Statins: mechanism of action and effects. *Journal of cellular and molecular medicine,* 5**,** 378-387.

STARY, H. 1992. Composition and classification of human atherosclerotic lesions. *Virchows Archiv A,* 421**,** 277-290.

STEIN, E. A., MELLIS, S., YANCOPOULOS, G. D., STAHL, N., LOGAN, D., SMITH, W. B., LISBON, E., GUTIERREZ, M., WEBB, C. & WU, R. 2012. Effect of a monoclonal antibody to PCSK9 on LDL cholesterol. *New England Journal of Medicine,* 366**,** 1108-1118.

STEINBERG, D. 2011. *The Cholesterol Wars: The Skeptics vs the Preponderance of Evidence*, Elsevier.

STONE, N. J., ROBINSON, J. G., LICHTENSTEIN, A. H., MERZ, C. N. B., BLUM, C. B., ECKEL, R. H., GOLDBERG, A. C., GORDON, D., LEVY, D. & LLOYD-JONES, D. M. 2014. 2013 ACC/AHA guideline on the treatment of blood cholesterol to reduce atherosclerotic cardiovascular risk in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *Journal of the American College of Cardiology,* 63**,** 2889-2934.

STRAARUP, E. M., FISKER, N., HEDTJÄRN, M., LINDHOLM, M. W., ROSENBOHM, C., AARUP, V., HANSEN, H. F., ØRUM, H., HANSEN, J. B. R. & KOCH, T. 2010. Short locked nucleic acid antisense oligonucleotides potently reduce apolipoprotein B mRNA and serum cholesterol in mice and non-human primates. *Nucleic acids research,* 38**,** 7100-7111.

SUCKLING, K. E. & JACKSON, B. 1993. Animal models of human lipid metabolism. *Progress in lipid research,* 32**,** 1-24.

SUGANO, M., MAKINO, N., SAWADA, S., OTSUKA, S., WATANABE, M., OKAMOTO, H., KAMADA, M. & MIZUSHIMA, A. 1998. Effect of antisense oligonucleotides against cholesteryl ester transfer protein on the development of atherosclerosis in cholesterol-fed rabbits. *Journal of Biological Chemistry,* 273**,** 5033-5036.

SWENSON, T. L., HESLER, C. B., BROWN, M. L., QUINET, E., TROTTA, P., HASLANGER, M., GAETA, F., MARCEL, Y., MILNE, R. & TALL, A. 1989. Mechanism of cholesteryl ester transfer protein inhibition by a neutralizing monoclonal antibody and mapping of the monoclonal antibody epitope. *Journal of Biological Chemistry,* 264**,** 14318-14326.

SWIFT, L. L., ZHU, M.-Y., KAKKAD, B., JOVANOVSKA, A., NEELY, M. D., VALYI-NAGY, K., ROBERTS, R. L., ONG, D. E. & JEROME, W. G. 2003. Subcellular localization of microsomal triglyceride transfer protein. *Journal of lipid research,* 44**,** 1841-1849.

TABAS, I., WILLIAMS, K. J. & BORÉN, J. 2007. Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. *Circulation,* 116**,** 1832-1844.

TAI, M. H., CHEN, P. K., CHEN, P. Y., WU, M. J., HO, C. T. & YEN, J. H. 2014. Curcumin enhances cell-surface LDLR level and promotes LDL uptake through downregulation of PCSK9 gene expression in HepG2 cells. *Molecular nutrition & food research,* 58**,** 2133-2145.

TALAYERO, B. G. & SACKS, F. M. 2011. The role of triglycerides in atherosclerosis. *Current cardiology reports,* 13**,** 544.

TALL, A. R., JIANG, X.-C., LUO, Y. & SILVER, D. 2000. 1999 George Lyman Duff memorial lecture: lipid transfer proteins, HDL metabolism, and atherogenesis. *Arteriosclerosis, thrombosis, and vascular biology,* 20**,** 1185-1188.

TAVINTHARAN, S. & KASHYAP, M. L. 2001. The benefits of niacin in atherosclerosis. *Current atherosclerosis reports,* 3**,** 74-82.

TEH, E. M., DOLPHIN, P. J., BRECKENRIDGE, W. C. & TAN, M.-H. 1998. Human plasma CETP deficiency: identification of a novel mutation in exon 9 of the CETP gene in a Caucasian subject from North America. *Journal of lipid research,* 39**,** 442-456.

TOMKIN, G. H. & OWENS, D. 2012. LDL as a cause of atherosclerosis. *The Open Atherosclerosis & Thrombosis Journal,* 5**,** 13-21.

TOTH, P. P., THAKKER, K. M., JIANG, P. & PADLEY, R. J. 2012. Niacin extended-release/simvastatin combination therapy produces larger favorable changes in high-density lipoprotein particles than atorvastatin monotherapy. *Vascular health and risk management,* 8**,** 39.

TRAN, K., BORÉN, J., MACRI, J., WANG, Y., MCLEOD, R., AVRAMOGLU, R. K., ADELI, K. & YAO, Z. 1998. Functional analysis of disulfide linkages clustered within the amino terminus of human apolipoprotein B. *Journal of Biological Chemistry,* 273**,** 7244-7251.

TRAPANI, L. & PALLOTTINI, V. 2010. Age-related hypercholesterolemia and HMG-CoA reductase dysregulation: sex does matter (a gender perspective). *Current gerontology and geriatrics research,* 2010.

TRAPANI, L., SEGATTO, M., INCERPI, S. & PALLOTTINI, V. 2011. 3-Hydroxy-3-methylglutaryl coenzyme A reductase regulation by antioxidant compounds: new therapeutic tools for hypercholesterolemia? *Current molecular medicine,* 11**,** 790-797.

TRAPANI, L., SEGATTO, M. & PALLOTTINI, V. 2013. New compounds able to control hepatic cholesterol metabolism: Is it possible to avoid statin treatment in aged people? *World journal of hepatology,* 5**,** 676.

TRIALISTS, C. T. 2005. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90 056 participants in 14 randomised trials of statins. *The Lancet,* 366**,** 1267-1278.

TSAI, M. Y., LI, N., SHARRETT, A. R., SHEA, S., JACOBS JR, D. R., TRACY, R., ARNETT, D., ARENDS, V. & POST, W. 2009. Associations of genetic variants in ATP-binding cassette A1 and cholesteryl ester transfer protein and differences in lipoprotein subclasses in the multi-ethnic study of atherosclerosis. *Clinical chemistry,* 55**,** 481-488.

TUNSTALL-PEDOE, H., KUULASMAA, K., TOLONEN, H., DAVIDSON, M., MENDIS, S. & PROJECT, W. M. 2003. MONICA monograph and multimedia sourcebook: world's largest study of heart disease, stroke, risk factors, and population trends 1979-2002.

URBAN, D., PÖSS, J., BÖHM, M. & LAUFS, U. 2013. Targeting the proprotein convertase subtilisin/kexin type 9 for the treatment of dyslipidemia and atherosclerosis. *Journal of the American College of Cardiology,* 62**,** 1401-1408.

VAN CAPELLEVEEN, J. C., VAN DER VALK, F. M. & STROES, E. S. 2016. Current therapies for lowering lipoprotein (a). *Journal of lipid research,* 57**,** 1612-1618.

VAN ECK, M., YE, D., HILDEBRAND, R. B., KAR KRUIJT, J., DE HAAN, W., HOEKSTRA, M., RENSEN, P. C., EHNHOLM, C., JAUHIAINEN, M. & VAN BERKEL, T. J. 2007. Important role for bone marrow–derived cholesteryl ester transfer protein in lipoprotein cholesterol redistribution and atherosclerotic lesion development in LDL receptor knockout mice. *Circulation research,* 100**,** 678-685.

VARBO, A., BENN, M. & NORDESTGAARD, B. G. 2014. Remnant cholesterol as a cause of ischemic heart disease: evidence, definition, measurement, atherogenicity, high risk patients, and present and future treatment. *Pharmacology & therapeutics,* 141**,** 358-367.

VARBO, A., BENN, M., TYBJÆRG-HANSEN, A., JØRGENSEN, A. B., FRIKKE-SCHMIDT, R. & NORDESTGAARD, B. G. 2013a. Remnant cholesterol as a causal risk factor for ischemic heart disease. *Journal of the American College of Cardiology,* 61**,** 427-436.

VARBO, A., BENN, M., TYBJÆRG-HANSEN, A. & NORDESTGAARD, B. G. 2013b. Elevated remnant cholesterol causes both low-grade inflammation and ischemic heart disease, whereas elevated low-density lipoprotein cholesterol causes ischemic heart disease without inflammation. *Circulation,* 128**,** 1298-1309.

VERGNES, L., CHIN, R. G., DE AGUIAR VALLIM, T., FONG, L. G., OSBORNE, T. F., YOUNG, S. G. & REUE, K. 2016. SREBP-2-deficient and hypomorphic mice reveal roles for SREBP-2 in embryonic development and SREBP-1c expression. *Journal of lipid research,* 57**,** 410-421.

VILAHUR, G., PADRO, T. & BADIMON, L. 2011. Atherosclerosis and thrombosis: insights from large animal models. *BioMed research international,* 2011.

VRABLIK, M., CESKA, R. & HORINEK, A. 2001. Major apolipoprotein B-100 mutations in lipoprotein metabolism and atherosclerosis. *Physiol Res,* 50**,** 337-43.

WALLACE, J. M., SCHWARZ, M., COWARD, P., HOUZE, J., SAWYER, J. K., KELLEY, K. L., CHAI, A. & RUDEL, L. L. 2005. Effects of peroxisome proliferator-activated receptor α/δ agonists on HDL-cholesterol in vervet monkeys. *Journal of lipid research,* 46**,** 1009-1016.

WANG, S., WANG, X., DENG, L., RASSART, E., MILNE, R. & TALL, A. 1993. Point mutagenesis of carboxyl-terminal amino acids of cholesteryl ester transfer protein. Opposite faces of an amphipathic helix important for cholesteryl ester transfer or for binding neutralizing antibody. *Journal of Biological Chemistry,* 268**,** 1955-1959.

WANG, Y., MCLEOD, R. S. & YAO, Z. 1997. Normal activity of microsomal triglyceride transfer protein is required for the oleate-induced secretion of very low density lipoproteins containing apolipoprotein B from McA-RH7777 cells. *Journal of Biological Chemistry,* 272**,** 12272-12278.

WEIGHT, M., BENADÉ, A., LOMBARD, C., FINCHAM, J., MARAIS, M., DANDO, B., SEIER, J. & KRITCHEVSKY, D. 1988. Low density lipoprotein kinetics in African Green monkeys showing variable cholesterolaemic responses to diets realistic for westernised people. *Atherosclerosis,* 73**,** 1-11.

WETTERAU, J. R., LIN, M. & JAMIL, H. 1997. Microsomal triglyceride transfer protein. *Biochimica et biophysica acta,* 1345**,** 136-150.

WHITFIELD, A. J., BARRETT, P. H. R., VAN BOCKXMEER, F. M. & BURNETT, J. R. 2004. Lipid disorders and mutations in the APOB gene. *Clinical chemistry,* 50**,** 1725-1732.

WINKEL, L. C., HOOGENDOORN, A., XING, R., WENTZEL, J. J. & VAN DER HEIDEN, K. 2015. Animal models of surgically manipulated flow velocities to study shear stress-induced atherosclerosis. *Atherosclerosis,* 241**,** 100-110.

WOJTCZAK, A. & SKRETKOWICZ, J. 2008. Genetic determinants in ischemic heart disease. *Acta Pol Pharm,* 65**,** 607-610.

WU, Z.-H. & ZHAO, S.-P. 2009. Niacin promotes cholesterol efflux through stimulation of the PPARγ-LXRα-ABCA1 pathway in 3T3-L1 adipocytes. *Pharmacology,* 84**,** 282-287.

WYNCHANK, S., FINCHAM, J., KLOPPER, J., WASSERMAN, H. & WEIGHT, M. 1989. Biodistribution of 1311-radiolabelled plasma low density lipoprotein in hyperlipidaemic Vervet monkeys. *Atherosclerosis,* 80**,** 159-167.

XU, M., YUAN, G. & WEI, F. 2010. Effect of atorvastatin in patients with chronic heart failure–insights from randomized clinical trials. *Archives of medical science: AMS,* 6**,** 866.

YAMASHITA, S., HIRANO, K.-I., SAKAI, N. & MATSUZAWA, Y. 2000. Molecular biology and pathophysiological aspects of plasma cholesteryl ester transfer protein. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids,* 1529**,** 257-275.

YUSUF, S., REDDY, S., ÔUNPUU, S. & ANAND, S. 2001a. Global burden of cardiovascular diseases: part I: general considerations, the epidemiologic transition, risk factors, and impact of urbanization. *Circulation,* 104**,** 2746-2753.

YUSUF, S., REDDY, S., ÔUNPUU, S. & ANAND, S. 2001b. Global burden of cardiovascular diseases: Part II: variations in cardiovascular disease by specific ethnic groups and geographic regions and prevention strategies. *Circulation,* 104**,** 2855-2864.

ZAID, A., ROUBTSOVA, A., ESSALMANI, R., MARCINKIEWICZ, J., CHAMBERLAND, A., HAMELIN, J., TREMBLAY, M., JACQUES, H., JIN, W. & DAVIGNON, J. 2008. Proprotein convertase subtilisin/kexin type 9 (PCSK9): hepatocyte-specific low-density lipoprotein receptor degradation and critical role in mouse liver regeneration. *Hepatology,* 48**,** 646-654.

ZEMAN, M., VECKA, M., PERLÍK, F., HROMÁDKA, R., STAŇKOVÁ, B., TVRZICKÁ, E. & ŽÁK, A. 2015. Niacin in the treatment of hyperlipidemias in light of new clinical trials: has niacin lost its place? *Medical science monitor: international medical journal of experimental and clinical research,* 21**,** 2156.

ZHANG, D.-W., LAGACE, T. A., GARUTI, R., ZHAO, Z., MCDONALD, M., HORTON, J. D., COHEN, J. C. & HOBBS, H. H. 2007. Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling and increases degradation. *Journal of Biological Chemistry,* 282**,** 18602-18612.

ZHAO, Z., TUAKLI-WOSORNU, Y., LAGACE, T. A., KINCH, L., GRISHIN, N. V., HORTON, J. D., COHEN, J. C. & HOBBS, H. H. 2006. Molecular characterization of loss-of-function mutations in PCSK9 and identification of a compound heterozygote. *The American Journal of Human Genetics,* 79**,** 514-523.

ZHOU, L., LI, C., GAO, L. & WANG, A. 2015. High-density lipoprotein synthesis and metabolism. *Molecular medicine reports,* 12**,** 4015-4021.

CHAPTER SIX:
APPENDICES

6.1 Appendix A: The letter of approval from ECRA.

ETHICS COMMITTEE FOR RESEARCH ON ANIMALS (ECRA)

Animal Ethics Approval Certificate

APPROVAL PERIOD: October 2018 – February 2019

Decision of the Animal Ethics Committee for the use of living vertebrates for research, diagnostic procedures and product development

PROJECT NUMBER:	Ref 11.18			
PROJECT TITLE:	**The impact of Niacin on PCSK9 levels in Vervet monkeys (Chlorocebus aethiops)**			
PROJECT LEADER:	Mr Thobile Ngqaneka			
DIVISION:	PUDAC, SAMRC, Cape Town			
CATEGORY:	NCD/CVD			
SPECIES OF ANIMAL:	**Vervet monkeys (Chlorocebus aethiops)**			
NUMBER OF ANIMALS:	16	Male//4-5 kg// >3-5 years,	Females//3-4 kg// > 3-5 years	
NOT APPROVED:	n/a			
APPROVED:	9 November 2018			

PLEASE NOTE: Should the number or species of animal(s) required, or the experimental procedure(s) change, please submit a written request to the ECRA for approval before commencing with the experiment.

L MAREE

Dr L Maree DATE: 9 November 2018

CHAIRPERSON : ETHICS COMMITTEE FOR RESEARCH ON ANIMALS

6.2 Appendix B: The selection and biochemical parameters of the monkeys.

Table B1: Animal selection based on lipogram levels

Groups	Gender	Animal ID	Cholesterol (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)	TG (mmol/l)	Non-HDL-C (mmol/l)	Cholesterol Ratio
Control	Females	401	3.2	1.6	1.4	0.56	1.6	2
		412	3.7	2.1	1.4	0.88	1.6	1.8
		416	3.5	2.1	1.3	0.64	1.4	1.7
		418	3.1	2	1	0.44	1.1	1.6
	Males	394	3.3	1.9	1.3	0.29	1.4	1.7
		413	2.4	1.7	0.6	0.36	0.7	1.4
		391	3	1.6	1.2	0.38	1.4	1.9
		398	2.8	1.6	1	0.44	1.2	1.8
		Mean	**3.5875**	**1.8375**	**1.35**	**0.95125**	**1.75**	**1.9625**
		SD	**0.203101**	**0.199553**	**0.119523**	**0.422118**	**0.177281**	**0.206588**
Niacin	Females	399	3.8	2.1	1.4	0.41	1.7	1.8
		402	4.5	2.8	1.5	0.48	1.7	1.6
		410	5	2.6	2.2	0.65	2.4	1.9
		415	5.7	2.1	3.3	0.59	3.6	2.7
	Males	389	2.4	1.7	0.6	0.43	0.7	1.4
		397	3.4	2.1	1.4	0.49	1.3	1.6
		406	3.9	2.3	1.5	0.35	1.6	1.7
		1	3.1	2.1	0.8	0.29	1	1.5
		Mean	**3.975**	**2.225**	**1.5875**	**0.46125**	**1.75**	**1.775**
		SD	**1.06335**	**0.341216**	**0.844203**	**0.118736**	**0.905539**	**0.406202**

6.3 Appendix C: The treatment period and food consumption

Table C1: Treatment periods.

Period	Weeks	Start	End
Baseline	Week (1 - 2)	02.01.19	16.01.19
Treatment	Week 3-14	16.01.19	10.04.19
Washout	Week 15-18	11.04.19	08.05.19

Table C2: Food consumption for the 30g bolus in mean ± SD %.

Group	Baseline (T0)	T1	T 2	T3	T4	T5
Niacin	100	100	100	100	100	100
Control	100	100	100	100	100	100

Table C3: Food consumption for the 70g bolus in mean ± SD %.

Group	Baseline (T0)	T1	T 2	T3	T4	T5
Niacin	100	100	100	100	100	100
Control	100	100	100	100	100	100

6.4 Appendix D: The niacin treatment interval

Baseline (T0)

Groups	Animal ID	Cholesterol (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)	TG (mmol/l)	Non-HDL-C (mmol/l)	Cholesterol Ratio	Weight (Kg)
Control	401	3.7	1.7	1.5	0.82	2	2.2	5.66
	412	3.4	1.9	1.2	1.22	1.5	1.8	3.97
	416	3.9	2	1.6	0.88	1.9	2	3.11
	418	3.3	1.7	1.1	1.06	1.6	1.9	3.47
	394	3.6	2	1.4	0.38	1.6	1.8	4.28
	413	2.6	1.7	0.7	0.32	0.9	1.5	5.9
	391	2.9	1.4	0.9	0.79	1.5	2.1	6.11
	398	2.7	1.4	1	0.64	1.3	1.9	5.1
	Mean	**3.26**	**1.73**	**1.18**	**0.76**	**1.54**	**1.90**	**4.70**
	SD	**0.45**	**0.22**	**0.29**	**0.29**	**0.32**	**0.20**	**1.08**
Niacin	399	4.2	2	1.8	0.83	2.2	2.1	3.7
	402	3.4	1.7	1.3	0.74	1.7	2	3.08
	410	5	2.1	2.3	0.52	2.9	2.4	4.14
	415	5.6	2.2	3	0.83	3.4	2.5	4.36
	389	3.3	1.7	1.2	0.81	1.6	1.9	5.33
	397	3.3	1.6	1.2	1.33	1.7	2.1	6.09
	406	4	1.9	1.8	0.57	2.1	2.1	4.91
	1	3.6	2	1.3	0.29	1.6	1.8	5.84
	Mean	**4.05**	**1.9**	**1.74**	**0.74**	**2.15**	**2.11**	**4.68**
	SD	**0.80**	**0.2**	**0.60**	**0.28**	**0.63**	**0.22**	**0.98**

Time point (T1)

Groups	Animal ID	Cholesterol (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)	TG (mmol/l)	Non-HDL-C (mmol/l)	Cholesterol Ratio	Weight (Kg)
Control	401	3.2	1.6	1.4	0.56	1.6	2	5.66
	412	3.7	2.1	1.4	0.88	1.6	1.8	4.08
	416	3.5	2.1	1.3	0.64	1.4	1.7	3.35
	418	3.1	2	1	0.44	1.1	1.6	3.57
	394	3.3	1.9	1.3	0.29	1.4	1.7	4.51
	413	2.4	1.7	0.6	0.36	0.7	1.4	6.06
	391	3	1.6	1.2	0.38	1.4	1.9	6.01
	398	2.8	1.6	1	0.44	1.2	1.8	5.19
	Mean	**3.13**	**1.83**	**1.15**	**0.50**	**1.3**	**1.74**	**4.80**
	SD	**0.38**	**0.21**	**0.25**	**0.18**	**0.28**	**0.17**	**1.01**
Niacin	399	3.8	2.1	1.4	0.41	1.7	1.8	3.59
	402	4.5	2.8	1.5	0.48	1.7	1.6	3.08
	410	5	2.6	2.2	0.65	2.4	1.9	4.03
	415	5.7	2.1	3.3	0.59	3.6	2.7	3.91
	389	2.4	1.7	0.6	0.43	0.7	1.4	5.63
	397	3.4	2.1	1.4	0.49	1.3	1.6	6.31
	406	3.9	2.3	1.5	0.35	1.6	1.7	5.1
	1	3.1	2.1	0.8	0.29	1	1.5	6.01
	Mean	**3.98**	**2.23**	**1.59**	**0.46**	**1.75**	**1.78**	**4.71**
	SD	**0.99**	**0.32**	**0.79**	**0.11**	**0.85**	**0.38**	**1.13**

Time point 2 (T_2)

Groups	Animal ID	Cholesterol (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)	TG (mmol/l)	Non-HDL-C (mmol/l)	Cholesterol Ratio	Weight (Kg)
Control	401	3.7	1.8	1.6	0.43	1.9	2.1	5.53
	412	4	2.2	1.4	0.77	1.8	1.8	4.1
	416	3.4	2.1	1.2	0.39	1.3	1.6	3.29
	418	2.9	1.6	1	0.7	1.3	1.8	3.51
	394	3.5	1.9	1.3	0.3	1.6	1.8	4.4
	413	2.8	1.7	0.8	0.38	1.1	1.6	5.92
	391	3.2	1.5	1.4	0.23	1.7	2.1	5.73
	398	2.8	1.5	1	0.39	1.3	1.9	5.01
	Mean	**3.29**	**1.79**	**1.21**	**0.45**	**1.5**	**1.84**	**4.69**
	SD	**0.41**	**0.25**	**0.25**	**0.18**	**0.27**	**0.18**	**0.95**
Niacin	399	4.2	2.2	1.9	0.6	2	1.9	3.42
	402	3.9	2.6	1	0.93	1.3	1.5	3.1
	410	4.9	2.4	2.5	0.78	2.5	2	3.95
	415	5.8	2	3.9	0.44	3.8	2.9	3.58
	389	2.8	2	0.8	0.46	0.8	1.4	5.32
	397	3.6	2.2	1.3	0.88	1.4	1.6	6.19
	406	3.8	2.2	1.5	0.47	1.6	1.7	4.99
	1	3.4	2.3	1	0.22	1.1	1.5	5.9
	Mean	**4.05**	**2.24**	**1.74**	**0.60**	**1.81**	**1.81**	**4.56**
	SD	**0.87**	**0.19**	**0.97**	**0.23**	**0.90**	**0.45**	**1.12**

Time point 3 (T_3)

Groups	Animal ID	Cholesterol (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)	TG (mmol/l)	Non-HDL-C (mmol/l)	Cholesterol Ratio	Weight (Kg)
Control	401	3.5	1.9	1.6	0.73	1.6	1.8	5.39
	412	4	2.3	1.5	1.16	1.7	1.7	4.21
	416	3.8	2.2	1.4	0.86	1.6	1.7	3.35
	418	3.5	1.8	1.5	0.85	1.7	1.9	3.5
	394	3.7	2	1.2	0.43	1.7	1.9	4.53
	413	2.7	1.9	0.8	0.29	0.8	1.4	6.08
	391	3	1.5	0.9	0.43	1.5	2	5.89
	398	3.2	1.9	1.1	0.55	1.3	1.7	5.14
	Mean	**3.43**	**1.94**	**1.25**	**0.66**	**1.49**	**1.76**	**4.76**
	SD	**0.41**	**0.23**	**0.28**	**0.27**	**0.29**	**0.17**	**0.97**
Niacin	399	4.1	2.5	1.5	0.38	1.6	1.6	3.35
	402	5	3.2	1.6	0.57	1.8	1.6	3.16
	410	4.5	3.1	1.5	0.51	1.4	1.5	3.97
	415	4.7	2.5	2.2	0.61	2.2	1.9	3.59
	389	2.8	2	0.6	0.43	0.8	1.4	5.56
	397	3.9	2.5	1.2	0.97	1.4	1.6	6.4
	406	3.7	2.3	1.5	0.44	1.4	1.6	5.27
	1	3.5	2.4	0.9	0.56	1.1	1.5	6.04
	Mean	**4.03**	**2.56**	**1.38**	**0.56**	**1.46**	**1.59**	**4.67**
	SD	**0.66**	**0.37**	**0.45**	**0.17**	**0.40**	**0.14**	**1.21**

Time point 4 (T_4)

Groups	Animal ID	Cholesterol (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)	TG (mmol/l)	Non-HDL-C (mmol/l)	Cholesterol Ratio	Weight (Kg)
Control	401	3.5	1.8	1.7	0.36	1.7	1.9	5.29
	412	3.3	2	1.2	0.7	1.3	1.7	4.27
	416	3.1	1.8	1.1	0.75	1.3	1.7	3.48
	418	3.3	1.7	1.4	0.82	1.6	1.9	3.52
	394	3.4	2.1	1.2	0.27	1.3	1.6	4.58
	413	2.7	1.8	0.8	0.32	0.9	1.5	6.1
	391	2.8	1.5	1.1	0.28	1.3	1.9	6.18
	398	2.8	1.8	0.9	0.34	1	1.6	5.21
	Mean	**3.11**	**1.81**	**1.18**	**0.48**	**1.3**	**1.73**	**4.83**
	SD	**0.29**	**0.17**	**0.26**	**0.22**	**0.25**	**0.15**	**0.98**
Niacin	399	3.7	2.5	1.3	0.44	1.2	1.5	3.43
	402	4.4	2.7	1.7	0.41	1.7	1.6	3.37
	410	4.6	2.9	1.6	0.48	1.7	1.6	4.17
	415	4.4	2.1	2.1	0.27	2.3	2.1	3.69
	389	2.4	1.9	0.5	0.4	0.5	1.3	5.58
	397	3.8	2.5	1.3	1.01	1.3	1.5	6.54
	406	3.3	2.2	1.2	0.25	1.1	1.5	5.37
	1	3.6	2.6	1.1	0.19	1	1.4	6.08
	Mean	**3.78**	**2.43**	**1.35**	**0.43**	**1.35**	**1.56**	**4.78**
	SD	**0.67**	**0.31**	**0.44**	**0.24**	**0.51**	**0.22**	**1.18**

Time point 5 (T_5)

Groups	Animal ID	Cholesterol (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)	TG (mmol/l)	Non-HDL-C (mmol/l)	Cholesterol Ratio	Weight (Kg)
Control	401	3.3	1.8	1.4	0.76	1.5	1.8	5.29
	412	3.4	1.8	1.2	1.61	1.8	1.9	4.33
	416	3.2	1.8	1.1	1.16	1.4	1.8	3.51
	418	3.4	1.8	1.2	1.56	1.6	1.9	3.6
	394	3.5	2.1	1.3	0.36	1.4	1.7	4.56
	413	2.6	1.7	0.6	0.31	0.9	1.5	6.07
	391	2.7	1.5	1	0.76	1.2	1.8	5.9
	398	3.2	1.7	1.2	0.53	1.5	1.9	5.09
	Mean	**3.16**	**1.78**	**1.13**	**0.88**	**1.41**	**1.79**	**4.79**
	SD	**0.31**	**0.16**	**0.23**	**0.48**	**0.25**	**0.13**	**0.90**
Niacin	399	3.8	2	1.6	1.03	1.8	1.9	3.66
	402	3	1.7	0.9	0.55	1.3	1.8	3.38
	410	4.7	2.6	1.9	1.02	2.1	1.8	4.25
	415	4.7	2.3	2.3	0.85	2.4	2	3.8
	389	3.1	1.9	0.9	0.77	1.2	1.6	5.41
	397	3.8	2.1	1.6	0.64	1.7	1.8	6.35
	406	3.5	2	1.3	0.21	1.5	1.8	4.97
	1	3.2	2.1	0.9	0.26	1.1	1.5	5.93
	Mean	**3.73**	**2.09**	**1.43**	**0.67**	**1.64**	**1.78**	**4.72**
	SD	**0.63**	**0.25**	**0.49**	**0.29**	**0.42**	**0.15**	**1.04**

6.5 Appendix E: Biochemical parameters and the P-values

Total cholesterol

	Animal ID	T0	T1	T2	T3	T4	T5
Control	**401**	3.7	3.2	3.7	3.5	3.5	3.3
	412	3.4	3.7	4	4	3.3	3.4
	416	3.9	3.5	3.4	3.8	3.1	3.2
	418	3.3	3.1	2.9	3.5	3.3	3.4
	394	3.6	3.3	3.5	3.7	3.4	3.5
	413	2.6	2.4	2.8	2.7	2.7	2.6
	391	2.9	3	3.2	3	2.8	2.7
	398	2.7	2.8	2.8	3.2	2.8	3.2
	Mean	**3.26**	**3.13**	**3.29**	**3.43**	**3.11**	**3.16**
	SD	**0.45**	**0.38**	**0.41**	**0.41**	**0.29**	**0.31**
Niacin	**399**	4.2	3.8	4.2	4.1	3.7	3.8
	402	3.4	4.5	3.9	5	4.4	3
	410	5	5	4.9	4.5	4.6	4.7
	415	5.6	5.7	5.8	4.7	4.4	4.7
	389	3.3	2.4	2.8	2.8	2.4	3.1
	397	3.3	3.4	3.6	3.9	3.8	3.8
	406	4	3.9	3.8	3.7	3.3	3.5
	1	3.6	3.1	3.4	3.5	3.6	3.2
	Mean	**4.05**	**3.98**	**4.05**	**4.03**	**3.78**	**3.73**
	SD	**0.80**	**0.99**	**0.87**	**0.66**	**0.67**	**0.63**
	P-value	**0.04***	**0.05***	**0.06**	**0.06**	**0.03***	**0.05***

* (p < 0, 05)

LDL-C

	Animal ID	T0	T1	T2	T3	T4	T5
Control	**401**	1.5	1.4	1.6	1.6	1.7	1.4
	412	1.2	1.4	1.4	1.5	1.2	1.2
	416	1.6	1.3	1.2	1.4	1.1	1.1
	418	1.1	1	1	1.5	1.4	1.2
	394	1.4	1.3	1.3	1.2	1.2	1.3
	413	0.7	0.6	0.8	0.8	0.8	0.6
	391	0.9	1.2	1.4	0.9	1.1	1
	398	1	1	1	1.1	0.9	1.2
	Mean	**1.18**	**1.15**	**1.21**	**1.25**	**1.18**	**1.13**
	SD	**0.29**	**0.25**	**0.25**	**0.28**	**0.26**	**0.23**
Niacin	**399**	1.8	1.4	1.9	1.5	1.3	1.6
	402	1.3	1.5	1	1.6	1.7	0.9
	410	2.3	2.2	2.5	1.5	1.6	1.9
	415	3	3.3	3.9	2.2	2.1	2.3
	389	1.2	0.6	0.8	0.6	0.5	0.9
	397	1.2	1.4	1.3	1.2	1.3	1.6
	406	1.8	1.5	1.5	1.5	1.2	1.3
	1	1.3	0.8	1	0.9	1.1	0.9
	Mean	**1.74**	**1.59**	**1.74**	**1.38**	**1.35**	**1.43**
	SD	**0.59**	**0.65**	**0.77**	**0.43**	**0.42**	**0.46**
	P-value	**0.04***	**0.18**	**0.19**	**0.54**	**0.38**	**0.16**

* (p < 0, 05)

HDL-C

	Animal ID	T0	T1	T2	T3	T4	T5
Control	401	1.7	1.6	1.8	1.9	1.8	1.8
	412	1.9	2.1	2.2	2.3	2	1.8
	416	2	2.1	2.1	2.2	1.8	1.8
	418	1.7	2	1.6	1.8	1.7	1.8
	394	2	1.9	1.9	2	2.1	2.1
	413	1.7	1.7	1.7	1.9	1.8	1.7
	391	1.4	1.6	1.5	1.5	1.5	1.5
	398	1.4	1.6	1.5	1.9	1.8	1.7
	Mean	**1.73**	**1.83**	**1.79**	**1.94**	**1.81**	**1.78**
	SD	**0.22**	**0.21**	**0.25**	**0.23**	**0.17**	**0.16**
Niacin	399	2	2.1	2.2	2.5	2.5	2
	402	1.7	2.8	2.6	3.2	2.7	1.7
	410	2.1	2.6	2.4	3.1	2.9	2.6
	415	2.2	2.1	2	2.5	2.1	2.3
	389	1.7	1.7	2	2	1.9	1.9
	397	1.6	2.1	2.2	2.5	2.5	2.1
	406	1.9	2.3	2.2	2.3	2.2	2
	1	2	2.1	2.3	2.4	2.6	2.1
	Mean	**1.9**	**2.23**	**2.24**	**2.56**	**2.43**	**2.1**
	SD	**0.2**	**0.32**	**0.19**	**0.37**	**0.31**	**0.25**
	P-value	**0.14**	**0.02**	**0.00***	**0.00***	**0.00****	**0.01**

* (p < 0, 05). ** (p < 0, 005).

TG

	Animal ID	T0	T1	T2	T3	T4	T5
Control	**401**	0.82	0.56	0.43	0.73	0.36	0.76
	412	1.22	0.88	0.77	1.16	0.7	1.61
	416	0.88	0.64	0.39	0.86	0.75	1.16
	418	1.06	0.44	0.7	0.85	0.82	1.56
	394	0.38	0.29	0.3	0.43	0.27	0.36
	413	0.32	0.36	0.38	0.29	0.32	0.31
	391	0.79	0.38	0.23	0.43	0.28	0.76
	398	0.64	0.44	0.39	0.55	0.34	0.53
	Mean	**0.76**	**0.5**	**0.45**	**0.66**	**0.48**	**0.88**
	SD	**0.29**	**0.18**	**0.18**	**0.27**	**0.22**	**0.48**
Niacin	**399**	0.83	0.41	0.6	0.38	0.44	1.03
	402	0.74	0.48	0.93	0.57	0.41	0.55
	410	0.52	0.65	0.78	0.51	0.48	1.02
	415	0.83	0.59	0.44	0.61	0.27	0.85
	389	0.81	0.43	0.46	0.43	0.4	0.77
	397	1.33	0.49	0.88	0.97	1.01	0.64
	406	0.57	0.35	0.47	0.44	0.25	0.21
	1	0.29	0.29	0.22	0.56	0.19	0.26
	Mean	**0.74**	**0.46**	**0.6**	**0.56**	**0.43**	**0.67**
	SD	**0.28**	**0.11**	**0.23**	**0.17**	**0.24**	**0.29**
	P-value	**0.88**	**0.64**	**0.20**	**0.41**	**0.70**	**0.33**

6.6 Appendix F: Clinical observations.

LOG SHEET SAMPLE

Table F1: Check list: behaviour
0 = absent, not observed, not displayed + = weak, poor, minimal
++ = normal, average, moderate +++ = excessive, strong

Date	Exp. No.	Monkey No.	alert	fearful	aggressive	confused	depressed

Table F 2: Check list: motor function and activity
0 = absent, not observed, not displayed + = weak, poor, minimal
++ = normal, average, moderate +++ = excessive, strong

Date	Exp. No.	posture	coordination	locomotion	active	Use of exercise cage

Table F3: Check list: physical
0 = absent, not observed, not displayed + = weak, poor, minimal
++ = normal, average, moderate +++ = excessive, strong, copious
disc. = discoloured d = discharge s = soft w = watery

Date	Exp. No.	Monkey No.	coat	feces	urine	eyes	nose	ears	genitals	rectal

6.7 Appendix H: Contains supplementary data for genotyping.

Table H1: The designed primer pairs for *PCSK9*, *CETP*, *LDLR*, *APOB-100* and *SREBP-2*

Gene	Exon	Forward sequence	Reverse sequence	Product size (bp)	Annealing temperature (^{0}c)
PCSK9	1	CCACACCCTAGACGGTTTCC	AGATCGTGCCAAGCGAAGAG	659	60
	2	TGGGTCCGCATTTGGTAACT	GACATTGCGGACCCTTGAGT	749	61
	3	GGGACAAACCGAATTGCCAG	GGCAGAGCAAATGGATTCAGC	579	61
	4-5	CTAAAATGAGCCGACCAGCG	TCAAGTCTGCACTCCTTGGG	1192	66
	6	GCTGGGGTTGGCTAGTTTGA	GCTCAAGTGGTCAGTCATGC	1137	66
	7	ACCCGAGGGACATTCAGAGA	ACAGACCCTGACTGCTAAAGG	926	52
	8-9	CCTGGCCGATAAGACAGCAA	CCCATCCAGCACCCATTGAA	1105	66
	10	TGACGAGGGTGCTTGAGTTG	TTAGGCAAAGCACCTGCCAT	553	62
	11	CCCTGACCATCTGTTTGGCT	TGGATCTGAAGCAAGCGTGA	623	68
LDLR	1	ACTGGAGTGGGAATCAGAGC	CGTGCCATTACCCTACAAGTCT	581	64
	2	TCTTGGGTGCCTTCCTTGTG	TTTCCAGCCGCCATCATCAA	533	66
	4	ACTCAACCCAAAATAAGGACAGGA	TCGAAATCCACTTCAACACCT	779	60
	5	CACAGGGACCAACGAATGCT	TAACAGATGGGCCGCAAACA	217	58
	6	GGTCACGTCATGGTTGAGGA	ATATGCAGTCATGCCCCTGG	488	64
	9	CAGCAGGGCTATTTCCCAAGC	CAGTTTGGGCTTGTCCCAGAG	329	64
	10	TCAGGCTCACACATGGTTGG	CACGACCAGTTTTCCGCATT	388	60
SREBP-2	1	GGTTGTCGGGTGTCATGGG	TCCACTCCGCTGTTAAGGC	902	64
	2	TCCTGTCCTGTTGTCCTGGG	ACCTGCCATTCCTGAAGACGA	749	60

	3	CACTTGCCGTCCAGAAAGGA	CAACCAGTGCCTCCCTGAATC	570	-
	4	GTGTCTGGCTTTCCTGAGTCTT	AGACCCTCCCATACCTACTCAG	925	-
	5	AGGTCTGTGTTGAGGTTGCT	TTCACTTACCTTGAGCCTGGG	393	-
	6-7	GTGCTAGGGCTCTGCTCTTT	GTTTTTCTCCAGGCTAGGCCC	583	64
	8	TGGGGTGGGTGTAGACAATG	CCCTATCAGAAGCAAGCAGGT	968	64
	9	AGGCAAGCCCAAGTTCAGTC	TCAATGCTGTGAGTTGCCCC	1106	64
	10	AGGGATTCTTCCTGGGGGAT	TAGGGTGCAGAACTGGCAAAT	558	58
	11	TTGATCGTGCTGGAGAGAGC	GCTAGGTAAGTGCTCAGGGG	301	60
	12	CCGAGGGGAAAGTACAGCAG	TGGCTCCAAGGTATTCTCACC	581	-
	13	GTAGTTCTGGGCTTTGGTGGA	ACTTGGTCTAGGAGGAAAGGC	457	-
	14	CATGAGGTGGGCCTTGTGTA	ACTGATGTGATGAGGGGCTG	598	62
	15	GTGCTGGTCTATTCCCCTCC	GTTGTGCAGAGCAGGACACT	519	
	16	CAGACGTGATGGTGAAGCCT	GGGACTCTGGGAGCCTAAGA	665	60
	18-19	TACGGGACAACAGAGGTTGC	TTTCTGCCCTGCCACCTATC	1189	-
CETP	1	TGAGGGGAGACAAGGAGAAGT	TCTGTGAAAAGACCCCTGGC	384	55
	2	TAAACTGAGAGGGAGGCCCA	AGAGGGGCTGAGAGGTTGAT	502	67
	3-5	CCTGGATGACCCCCAACATC	GGGCTTGGAACAGTGCTTTG	915	68
	6-7	GACACACAGCTCACCACTGA	TAGGGCAGCAGATGGCTTTC	1006	68
	8	GGGCTCTACCTACCCAGACA	ATCAGGAATGCAGAGGAGCG	429	60
	9	AAGTGCCATGCTCTGAACCA	CGCGAGTTTCCGAGTTTCCT	475	-
	10	CTTAGGTCGTTTCCAGGGCA	GCTCCATGCCAAACACCCTA	507	63

	11	CCATTTCTGAGGGCAAGGACT	GCAAGAGACAACCAGTGCCG	361	66
	12	CTAACCCCAAAGCCACAGGT	CATGCCTCAAGGGGAAGGAG	377	64
	13	ATCTCAAGAGCTGCCCCAAA	CCCAAAAGACATGGCAGGGA	340	60
	14	ATGAATGCTCGTCTAGGCCG	GGCGTGCAATGACACCAGTA	322	60
	15-16	GTCCAAAGGGGTCTCAGCAT	GGTGATACACATCCCTTCCCC	683	-
APOB-100	2	AAGGGTGAAACCTGTGCCTG	TTGGGACAGAACAGAAGGGC	339	62
	3	AGTCACGGAATTAAGGCGCT	AGTTCTGGGATGTTCTGCGT	624	64
	4	AAAGGGCCAGCAATACGACT	CAGCAATTCCCTGACCCACA	485	98
	5	ACTCTGTTTGCACTCACCCA	GAGACTGGCAACAGATCCCG	1177	-
	6	TCCCTCCCAAGTACCCAAGG	AAAGGTGCCCACTAGCTTGAA	311	62
	7	GAAGACACAGGGACCGATCA	GCCTGGAACAGAGCACTTGA	254	66
	8	TGGGCAGGACTTGGGTAGAT	ATAGAGGTAGCCAGAACACCAG	323	69
	9	CCCTTTTCCCCTGTGCTGAT	CTTCTGGAGAGCACTTGCGA	636	62.5
	10	TCGCTACAGATCCACAGTCC	GGAGACGCACAGAAGTGCAAG	529	66
	12	CTGCGGGTAATCTCAGTCTTT	TCCCCAGTACCTTCCAAATCC	331	59
	13-14	AACAGGCATTCCACAGGCAT	GAGTCATGGTAGGAAGTGCC	1048	58
	15	GTCCCAAAGCCAAGGAAGCA	TCCCAGCAGGTCTGGTTGAT	633	66
	16	AGCAGACAGGTCCCACATTG	TCAGCAAAGGTAGAGGAAGGT	572	64
	18	TTTGCCCGGAGGTGAGAAAG	TGTGTAGCCTGGTGAAATCCTG	383	66.5
	20	CCCTTGCCTTGGGTTCTTGT	AGACTGTGAGCAGAGATGAAGC	346	66

21	TTCCTAGCCCAGAAGGACTGT	AAACTCACACTGACCTCGCA	1373	52
22-23	CTGCGAGGTCAGTGTGAGTT	TTTGGATACCTTCCTGCACCC	901	-
24	ACAGTTCTTTGCCTCGTGGT	TGACAGGGTTGGCATTCTCA	590	64
25-26	GAGTGGCTTCGGGAATGAGG	GAGCCTGCTATGAACTAGCCC	725	-

6.8Appendix I: Depicts supplementary gene expression data.

Table I1: The RNA integrity based on the Nanodrop and Bio-analyzer results.

Groups	Animal ID	T0		T1		T2		T3		T4		T5	
Control		C (ng/µ)	RIN	C (ng/µ)	RIN	C (ng/µ)	RIN	C (ng/µ)	RIN	C (ng/µ)	RIN	C (ng/µ)	RIN
	391	54.4	7.8	53.8	8.6	23.9	8.0	75.8	9.1	75.8	9.1	40.3	9.0
	394	51.5	8.2	88.5	8.1	102.5	7.1	95.7	8.7	95.7	8.7	150.8	9.2
	398	14.0	7.6	23.9	7.4	17.2	7.6	133.2	8.7	133.2	8.7	38.6	9.2
	401	41.1	8.5	1.36	7.0	24.9	8.3	59.9	8.8	59.9	8.8	31.6	9.2
	412	70.1	8.0	119.7	8.0	179.5	8.1	57.5	9.5	57.5	9.5	65.4	9.3
	413	73.1	7.8	31.2	8.2	13.1	7.0	89.0	9.3	89.0	9.3	75.0	9.7
	416	18.3	8.1	36.1	9.0	77.4	7.2	51.1	9.2	51.1	9.2	60.3	9.7
	418	50.2	7.8	38.1	8.3	88.3	7.4	42.1	9.0	0.0	0.0	131.6	9.8
Niacin	1	95.4	7.3	116.5	8.1	54.1	7.7	77.7	8.9	75.9	8.8	112.6	9.0
	389	19.2	7.5	27.9	7.9	37.7	7.0	109.9	8.6	34.9	8.6	66.4	8.9
	397	73.4	8.2	114.9	8.1	169.7	9.0	142.3	9.2	105	9.1	84.1	9.2
	399	6.8	5.6	16.0	7.6	10.7	7.0	15.5	8.7	9.8	8.7	27.8	9.3
	402	36.0	7.8	50.2	8.1	103.0	9.1	86.2	9	64.4	9.3	52.2	8.8
	406	19.5	7.2	19.0	7.8	30.6	6.9	57.1	8.7	66.4	9.3	148.1	9.3
	410	39.7	7.6	17.3	7.7	28.4	7.1	38.0	9.2	63.4	9.0	79.5	9.3
	415	41.0	7.6	36.4	8.3	41.5	7.2	61.1	8.7	44.0	9.5	33.5	9.6

6.9 Appendix J: Shows raw data of gene expression.

Table J1a: *PCSK9* mRNA expression in (a.u).

	PCSK9	T0		T1		T2		T3		T4		T5	
		CT	GOI-HKG	CT	GOI-HKG	CT	GOI-HKG	CT	GOI-HKG	CT	GOI-HKG	CT	GOI-HKG
Control	391	34.44	11.36	31.39	9.25	34.36	10.26	33.93	8.55	33.93	8.55	30.21	7.58
	394	30.46	10.53	35.82	12.85	35.84	8.72	36.47	12.81	36.47	12.81	35.08	5.92
	398	30.72	9.19	29.62	8.19	30.06	7.39	33.70	4.84	33.70	4.84	30.28	8.61
	401	33.20	12.50	34.79	8.88	0.00	0.00	39.08	11.89	39.08	11.89	30.36	7.89
	412	35.77	14.27	32.92	6.30	36.02	8.23	37.40	13.55	37.40	13.55	0.00	0.00
	413	36.46	13.44	30.04	7.60	29.96	5.83	35.56	8.07	35.56	8.07	37.02	8.85
	416	29.64	7.99	28.92	7.72	37.53	11.33	36.64	8.75	36.64	8.75	39.72	11.01
	418	31.21	10.04	29.74	9.63	37.00	12.48	26.54	6.78	26.54	6.78	38.21	10.62
	Mean	**32.74**	**11.16**	**31.65**	**8.80**	**34.40**	**8.03**	**34.92**	**9.40**	**34.92**	**9.40**	**34.41**	**8.64**
	SD	**2.60**	**2.15**	**2.58**	**1.95**	**3.16**	**3.89**	**3.82**	**3.06**	**3.82**	**3.06**	**4.10**	**1.76**
Niacin	1	36.66	13.52	35.88	6.88	35.86	6.37	33.76	9.83	37.06	9.12	36.83	8.36
	389	30.25	8.87	29.28	8.25	29.35	7.72	34.26	6.01	29.49	7.43	0.00	0.00
	397	36.51	15.46	36.42	12.38	33.84	7.70	34.67	10.15	37.50	9.49	0.00	0.00
	399	31.25	8.46	29.45	7.71	34.06	11.28	28.19	5.67	29.67	6.52	29.57	6.76
	402	29.93	9.21	36.58	11.04	37.76	8.54	34.10	7.15	35.17	7.11	35.85	8.47
	406	29.71	8.82	29.41	7.71	27.75	5.88	37.53	9.44	35.87	8.75	36.63	9.47
	410	30.46	9.25	29.81	8.17	31.77	10.04	27.19	3.75	33.89	7.95	39.70	11.84
	415	29.24	9.34	29.22	8.69	0.00	0.00	36.16	10.30	29.10	7.90	30.95	8.90
	Mean	**31.75**	**10.37**	**32.01**	**8.86**	**32.91**	**8.22**	**33.23**	**7.79**	**33.47**	**8.03**	**34.92**	**8.97**
	SD	**3.04**	**2.61**	**3.56**	**1.87**	**3.54**	**1.92**	**3.65**	**2.48**	**3.53**	**1.02**	**3.87**	**1.67**
	P-values	**0.52**		**0.96**		**0.91**		**0.27**		**0.25**		**0.74**	

CT: cycle threshold, GOI: gene of interest, HKG: house-keeping gene

Table J1b: *LDLR* mRNA expression in (a.u).

Groups	LDLR	T0		T1		T2		T3		T4		T5	
		CT	GOI-HKG	CT	GOI-HKG	CT	GOI-HKG	CT	GOI-HKG	CT	GOI-HKG	CT	GOI-HKG
Control	391	26.27	3.18	26.52	4.38	27.93	3.83	27.66	2.28	27.66	2.28	26.24	3.62
	394	24.79	4.86	27.60	4.63	29.84	2.71	26.95	3.29	26.95	3.29	31.51	2.34
	398	26.65	5.12	26.15	4.72	26.93	4.25	30.38	1.51	30.38	1.51	25.95	4.29
	401	25.82	5.12	30.77	4.87	0.00	0.00	29.58	2.38	29.58	2.38	27.02	4.55
	412	25.90	4.39	29.60	2.98	28.91	1.12	30.09	6.23	30.09	6.23	29.89	2.42
	413	27.07	4.05	27.62	5.18	29.11	4.98	29.44	1.96	29.44	1.96	30.67	2.50
	416	26.48	4.82	26.18	4.98	29.34	3.14	30.06	2.18	30.06	2.18	31.46	2.76
	418	25.93	4.76	25.59	5.48	28.27	3.75	25.34	5.58	25.34	5.58	30.77	3.18
	Mean	**26.11**	**4.54**	**27.50**	**4.65**	**28.62**	**2.97**	**28.69**	**3.17**	**28.69**	**3.17**	**29.19**	**3.21**
	SD	**0.68**	**0.65**	**1.83**	**0.75**	**0.98**	**1.66**	**1.83**	**1.77**	**1.83**	**1.77**	**2.38**	**0.86**
Niacin	1	27.20	4.06	31.93	2.93	31.63	2.14	26.99	3.07	30.80	2.86	31.00	2.52
	389	26.51	5.13	25.81	4.78	25.72	4.09	30.46	2.21	25.00	2.94	29.42	2.71
	397	25.33	4.28	27.66	3.62	27.65	1.51	25.76	1.24	28.69	0.68	28.60	2.34
	399	27.79	5.00	26.96	5.22	27.45	4.67	26.69	4.18	26.67	3.52	26.76	3.96
	402	26.09	5.37	29.27	3.73	32.81	3.58	29.15	2.21	31.33	3.28	31.54	4.15
	406	26.26	5.36	26.29	4.60	26.26	4.40	30.61	2.52	28.83	1.71	31.27	4.11
	410	26.24	5.04	26.51	4.88	26.11	4.38	24.89	1.45	28.10	2.16	30.45	2.59
	415	24.91	5.01	25.75	5.22	0.00	0.00	29.32	3.46	24.30	3.10	27.09	5.04
	Mean	**26.29**	**4.91**	**27.52**	**4.37**	**28.23**	**3.10**	**27.98**	**2.54**	**27.97**	**2.53**	**29.52**	**3.43**
	SD	**0.93**	**0.48**	**2.12**	**0.84**	**2.83**	**1.69**	**2.18**	**0.99**	**2.52**	**0.95**	**1.87**	**1.01**
	P-values	**0.22**		**0.50**		**0.89**		**0.39**		**0.38**		**0.65**	

CT: cycle threshold, GOI: gene of interest, HKG: house-keeping gene

Table J1c: *SREBP-2* mRNA expression in (a.u).

Groups	SREBP-2	T0		T1		T2		T3		T4		T5	
		CT	GOI-HKG	CT	GOI-HKG	CT	GOI-HKG	CT	GOI-HKG	CT	GOI-HKG	CT	GOI-HKG
Control	391	24.15	1.07	24.15	2.00	25.38	1.28	27.98	2.60	27.98	2.60	23.87	1.25
	394	22.71	2.77	24.48	1.51	26.66	-0.47	24.44	0.78	24.44	0.78	30.12	0.95
	398	24.47	2.93	23.79	2.36	24.37	1.70	27.74	-1.12	27.74	-1.12	23.45	1.78
	401	23.48	2.78	26.97	1.07	0.00	0.00	26.71	-0.49	26.71	-0.49	23.83	1.36
	412	23.53	2.02	26.67	0.05	26.45	-1.34	26.81	2.96	26.81	2.96	28.04	0.56
	413	24.62	1.61	24.59	2.15	25.86	1.73	26.96	-0.53	26.96	-0.53	29.01	0.84
	416	23.95	2.30	23.35	2.15	26.03	-0.17	27.18	-0.71	27.18	-0.71	29.63	0.92
	418	23.53	2.36	21.94	1.83	24.50	-0.03	22.88	3.12	22.88	3.12	28.45	0.87
	Mean	**23.81**	**2.23**	**24.49**	**1.64**	**25.61**	**0.34**	**26.34**	**0.83**	**26.34**	**0.83**	**27.05**	**1.07**
	SD	**0.62**	**0.64**	**1.66**	**0.76**	**0.90**	**1.11**	**1.76**	**1.80**	**1.76**	**1.80**	**2.83**	**0.38**
Niacin	1	24.53	1.40	28.97	-0.02	28.19	-1.29	24.39	0.47	27.66	-0.27	29.77	1.29
	389	24.26	2.88	23.30	2.27	23.21	1.57	25.57	-2.68	23.03	0.97	28.18	1.47
	397	23.02	1.98	25.18	1.14	25.36	-0.78	24.23	-0.29	26.40	-1.62	27.95	1.69
	399	25.45	2.65	23.77	2.04	24.03	1.25	23.89	1.37	23.47	0.32	24.01	1.20
	402	23.34	2.62	25.64	0.10	28.52	-0.70	26.07	-0.88	27.28	-0.78	29.07	1.68
	406	24.14	3.25	24.17	2.47	23.65	1.79	27.70	-0.39	26.77	-0.35	29.15	1.99
	410	23.66	2.45	23.64	2.00	23.39	1.66	22.47	-0.97	25.38	-0.56	28.61	0.75
	415	23.02	3.13	22.81	2.29	0.00	0.00	26.47	0.61	22.48	1.28	23.90	1.85
	Mean	**23.93**	**2.54**	**24.69**	**1.54**	**25.19**	**0.50**	**25.10**	**-0.34**	**25.31**	**-0.13**	**27.58**	**1.49**
	SD	**0.83**	**0.61**	**1.97**	**1.01**	**2.27**	**1.36**	**1.67**	**1.24**	**2.05**	**0.95**	**2.31**	**0.40**
	P-values	**0.33**		**0.82**		**0.80**		**0.15**		**0.21**		**0.05**	

CT: cycle threshold, GOI: gene of interest, HKG: house-keeping gene

Table J1d: *CETP* mRNA expression in (a.u).

Groups	CETP	T0		T1		T2		T3		T4		T5	
		CT	GOI-HKG	CT	GOI-HKG	CT	GOI-HKG	CT	GOI-HKG	CT	GOI-HKG	CT	GOI-HKG
Control	391	27.22	4.13	27.33	5.18	28.49	4.40	35.00	9.62	35.00	9.62	35.07	12.45
	394	25.50	5.56	27.32	4.35	30.25	3.12	39.04	15.38	39.04	15.38	31.74	2.58
	398	26.94	5.40	26.70	5.27	27.43	4.76	29.38	0.51	29.38	0.51	35.58	13.92
	401	25.71	5.01	29.84	3.93	0.00	0.00	28.84	1.64	28.84	1.64	34.80	12.33
	412	26.02	4.51	30.18	3.56	29.82	2.02	30.08	6.22	30.08	6.22	31.29	3.81
	413	27.64	4.63	27.52	5.09	29.11	4.98	36.54	9.05	36.54	9.05	32.00	3.84
	416	0.00	0.00	25.18	3.98	29.54	3.34	38.85	10.96	38.85	10.96	37.62	8.91
	418	0.00	0.00	24.66	4.55	27.77	3.24	34.66	14.90	34.66	14.90	30.02	2.43
	Mean	**26.50**	**4.87**	**27.34**	**4.49**	**28.92**	**3.23**	**34.05**	**8.54**	**34.05**	**8.54**	**33.52**	**7.53**
	SD	**0.88**	**4.13**	**1.95**	**0.64**	**1.06**	**1.63**	**4.14**	**5.50**	**4.14**	**5.50**	**2.61**	**4.90**
Niacin	1	28.12	4.98	32.52	3.53	31.92	2.43	36.32	12.40	31.64	3.70	32.25	3.77
	389	25.91	4.53	24.25	3.22	24.80	3.17	29.37	1.12	25.29	3.23	38.74	12.03
	397	25.59	4.54	27.45	3.41	28.12	1.98	25.48	0.97	30.31	2.29	30.10	3.84
	399	28.16	5.37	25.85	4.11	26.58	3.80	34.88	12.36	26.99	3.84	35.84	13.04
	402	26.03	5.31	28.97	3.43	31.87	2.65	37.82	10.87	30.89	2.83	31.39	4.00
	406	26.51	5.61	25.18	3.49	25.18	3.32	29.66	1.57	29.10	1.98	30.16	3.00
	410	26.85	5.64	25.78	4.14	25.67	3.94	30.83	7.39	28.98	3.04	39.18	11.32
	415	25.57	5.68	24.51	3.99	0.00	0.00	39.19	13.34	24.90	3.69	35.45	13.40
	Mean	**26.59**	**5.21**	**26.81**	**3.66**	**27.73**	**3.04**	**32.94**	**7.50**	**28.51**	**3.08**	**34.14**	**8.05**
	SD	**1.05**	**0.47**	**2.78**	**0.36**	**3.04**	**1.27**	**4.81**	**5.50**	**2.54**	**0.68**	**3.67**	**4.75**
	P-values	**0.25**		**0.01**		**0.45**		**0.71**		**0.01**		**0.83**	

CT: cycle threshold, GOI: gene of interest, HKG: house-keeping gene

Table J1e: *APOB-100* mRNA expression in (a.u).

Groups	APOB-100	T0		T1		T2		T3		T4		T5	
		CT	GOI-HKG	CT	GOI-HKG	CT	GOI-HKG	CT	GOI-HKG	CT	GOI-HKG	CT	GOI-HKG
Control	391	36.93	13.85	34.03	11.88	37.58	13.48	36.68	11.30	36.68	11.30	35.22	12.59
	394	33.06	13.12	37.76	14.79	35.86	8.74	34.62	10.97	34.62	10.97	34.85	5.69
	398	32.20	10.67	32.21	10.78	33.10	10.42	33.78	4.91	33.78	4.91	39.54	17.88
	401	34.66	13.96	32.96	7.05	0.00	0.00	36.84	9.64	36.84	9.64	34.01	11.54
	412	37.36	15.86	27.53	0.91	32.95	5.16	37.60	13.74	37.60	13.74	35.27	7.79
	413	34.67	11.66	32.63	10.20	26.08	1.95	33.87	6.38	33.87	6.38	0.00	0.00
	416	0.00	0.00	29.98	8.78	37.42	11.22	30.81	2.92	30.81	2.92	30.81	2.10
	418	0.00	0.00	31.79	11.68	36.80	12.28	37.60	17.84	37.60	17.84	35.50	7.91
	Mean	**34.81**	**13.18**	**32.36**	**9.51**	**34.26**	**7.91**	**35.22**	**9.71**	**35.22**	**9.71**	**35.03**	**9.36**
	SD	**2.05**	**1.84**	**2.97**	**4.16**	**4.08**	**4.98**	**2.38**	**4.88**	**2.38**	**4.88**	**2.57**	**5.14**
Niacin	1	33.31	11.93	36.40	7.40	29.80	0.32	37.28	13.36	30.04	2.10	33.80	5.33
	389	35.88	14.83	31.94	10.91	32.47	10.84	35.44	7.19	29.87	7.81	35.57	8.86
	397	34.70	11.90	35.23	11.19	36.74	10.60	35.19	10.67	38.69	10.67	34.92	8.67
	399	31.88	11.16	33.55	11.82	31.10	8.33	36.65	14.13	29.74	6.59	35.47	12.67
	402	31.13	10.23	35.25	9.71	34.93	5.71	33.20	6.25	36.80	8.75	33.20	5.81
	406	33.03	11.82	30.91	9.22	32.24	10.38	34.45	6.37	36.78	9.66	34.67	7.51
	410	30.68	10.79	31.82	10.18	31.49	9.76	33.95	10.51	27.96	2.02	33.02	5.16
	415	0.00	0.00	30.76	10.23	0.00	0.00	36.69	10.84	31.79	10.59	35.09	13.04
	Mean	**32.94**	**11.81**	**33.23**	**10.08**	**32.68**	**7.99**	**35.36**	**9.92**	**32.71**	**7.27**	**34.47**	**8.38**
	SD	**1.89**	**1.48**	**2.18**	**1.36**	**2.38**	**4.53**	**1.45**	**3.04**	**4.08**	**3.50**	**1.00**	**3.10**
	P-values	**0.16**		**0.72**		**0.71**		**0.92**		**0.27**		**0.66**	

CT: cycle threshold, GOI: gene of interest, HKG: house-keeping gene

6.10 Appendix K: The impact of *PCSK9* mutations in affected animals.

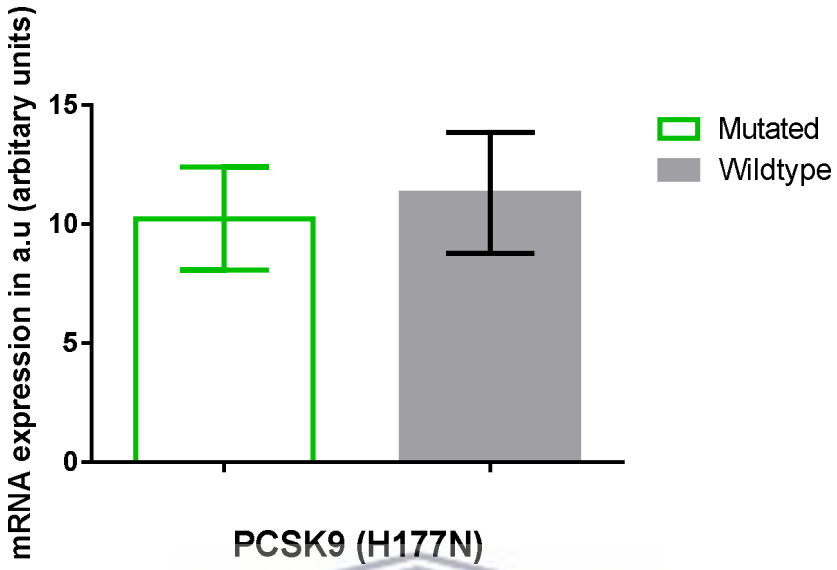


Figure K1a: *PCSK9* (H177N) gene expression of the mutated animals compared with the wild-type animals. The data were expressed as mean ± SD and mRNA expression in a.u. (arbitrary units).

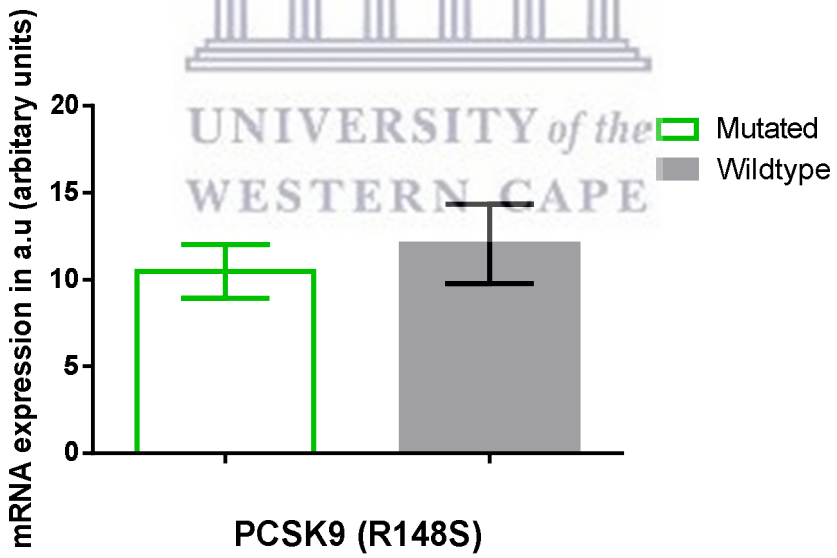


Figure K1b: *PCSK9* (R148S) gene expression of the wild-type individuals compared with the mutated individuals. The data were expressed as mean ± SD and mRNA expression in a.u. (arbitrary units).

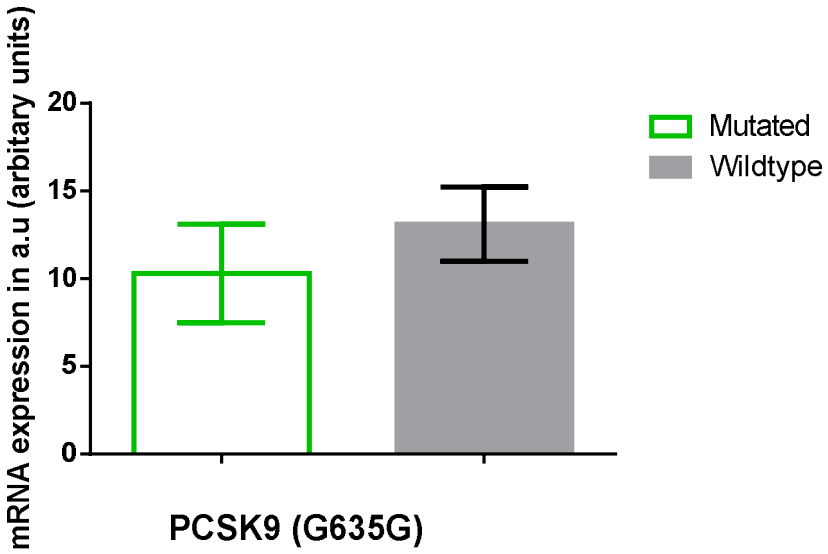


Figure K1c: *PCSK9* (G635G) gene expression of the mutated animals compared with the wild-type animals. The data were expressed as mean ± SD and mRNA expression in a.u. (arbitrary units).

6.11 Appendix L: The impact of *LDLR* variant in affected animals.

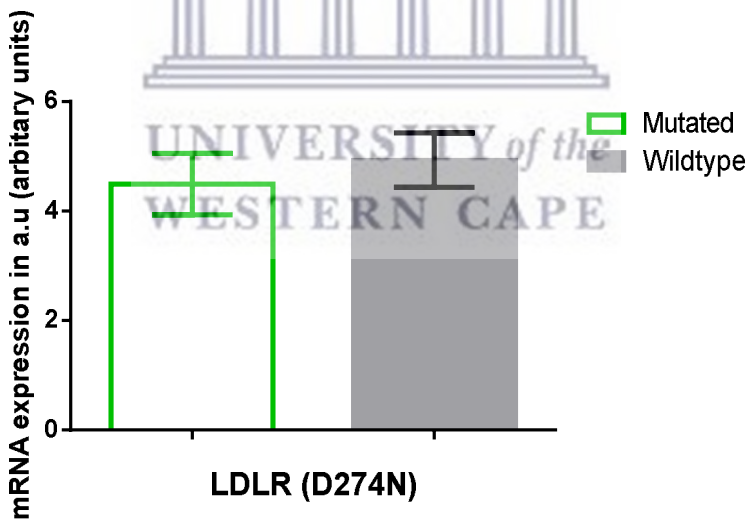
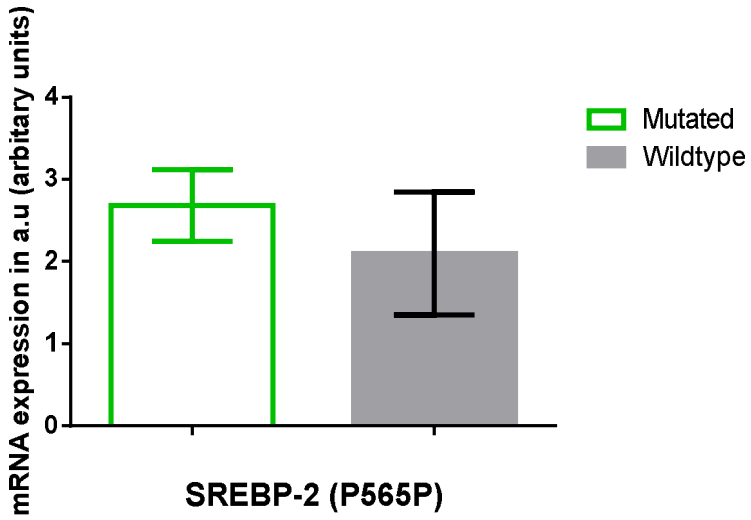


Figure L1: The gene expression of *LDLR* (D274N*)* analyzed by comparing the treated group with the control group. The data were expressed as mean ± SD and mRNA expression in a.u. (arbitrary units).

6.12 Appendix M: The impact of *SREBP-2* mutation in affected animals.



Figures M1: Depicting *SREBP-2* (P565P) expression patterns with regards to mutated and wild-type subjects. The data were expressed as mean ± SD and mRNA expression in a.u. (arbitrary units).

6.13 Appendix N: The impact of *CETP* variant in affected animals.

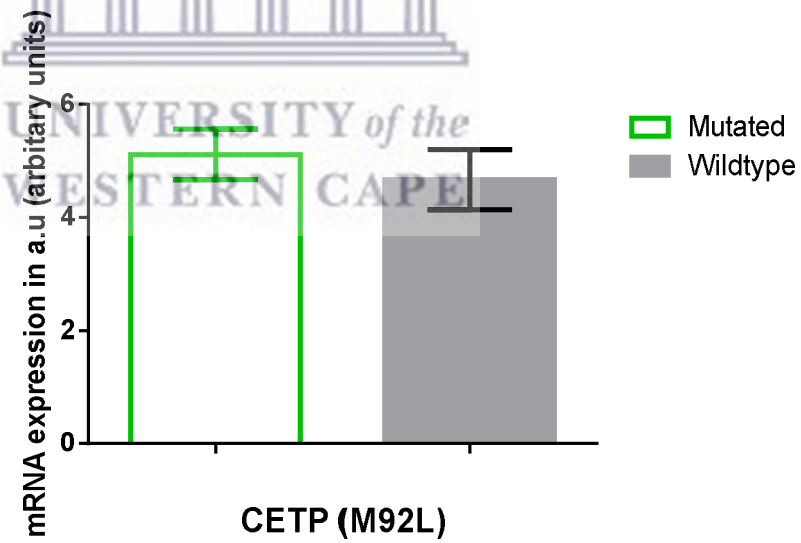


Figure N1: Detailing the mRNA expression of *CETP* gene in animals harbouring M92L variant and those without it. The data were expressed as mean ± SD and mRNA expression in a.u. (arbitrary units).

6.14 Appendix O: The impact of *APOB-100* mutations in affected animals.

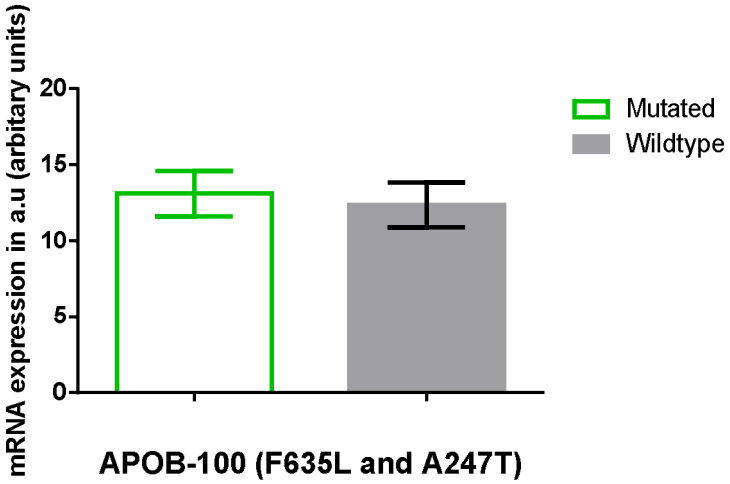


Figure O1: The gene expression of *APOB-100* (F635L and A247T) analyzed by comparing the treated group with the control group. The data were expressed as mean ± SD and mRNA expression in a.u. (arbitrary units).