DEFINING THE AFRICAN GREEN MONKEY (*CHLOROCEBUS AETHIOPS*): EXPRESSION BEHAVIOUR OF SELECTED LIPID METABOLISM GENES IN RESPONSE TO NIACIN

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DECLARATION

I declare that "**DEFINING THE African green monkey (*CHOROCEBUS AETHIOPS*): Expression behaviour of selected lipid metabolism genes in response to niacin"** 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.

Chesa Gift Chauke

Signature…… Date……

Dedicated to my daughter Nandi:

Success does not come to those who sit and wait.

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ABSTRACT

In this century most major medical advances have resulted in part from research on animals and non-human primates such as the African green monkey and therefore often serve as a critical link between basic research and human clinical application. Due to its close evolutionary relationship to humans, the African green monkey is known to be an excellent and most sought after models for studies of human cardiovascular disease (CVD). While the human genome project and some others related to model organisms are very well advanced or even complete, little sequence information has been acquired for the African green monkey. Given the importance of this species in biomedical research generally and CVD specifically, and the fundamental significance of sequence data, it is critical that this paucity of genome information concerning this specific animal model be addressed in order to better define the molecular basis and to further understand the mechanism of cholesterol metabolism in this species which will also contribute immensely to primatology.

There is a growing interest in the role of genetic polymorphisms in predicting susceptibility to disease and responsiveness to drug interventions. Since plasma lipid abnormalities are risk factors for coronary atherosclerosis, determination of these plasma lipid concentrations, especially for genes involved in lipid transport and metabolism may be influenced by genetic variations. In this study, the African green monkey was used as a model to evaluate the effect of niacin on plasma lipids and reverse cholesterol transport by examine gene expression and the influence of several polymorphisms found in genes that are involved in cholesterol metabolism in humans.

A survey of genetic variation spanning ten prioritised "candidate" genes was conducted, all of which are known to produce proteins that play key roles in the reverse cholesterol pathway (RCT), and in the homeostatic regulation of blood lipid profiles related to cardiovascular health and disease.

Reverse transcription polymerase chain reaction (RT-PCR) was used to evaluate mRNA expression of those "candidate" genes. Twenty two coincident single-nucleotide polymorphisms (cSNPs), reported to play a vital role in RCT, were genotyped within these genes. This study's findings implicate a subset of six of the twenty two genetic variants, spanning five "candidate" genes. To assess possible involvement of these prioritised "candidate" genes and their polymorphisms, biochemical analyses of known risk factors of coronary artery disease such as HDL-C and LDL-C were conducted. Eight healthy African green monkeys were entered in this study of which four were treated with niacin at an escalating dosage. Their mean lipid-lowering response following drug therapy was analysed, compared to those with the same genotype in a control group.

Niacin treatment was associated with a considerable reduction in LDL-Cholesterol, up-regulation of HDL synthesis, and increase of apo A-1 levels. Gene expression had minimal effect on niacin treatment, except CYP7A1 which was down-regulated at the same time when considerable change in HDL-C, LDL-C and apoA-1 levels was observed. The presence of CYP7A1:Asn233Ser polymorphism may have played a critical role in metabolising niacin and influencing the up-regulation of HDL-C synthesis in the African green monkey. Although cholesterol lowering alone may explain the anti-atherosclerotic effect of niacin on HDL-C, in this study, gene

expression data also shed some light in supporting the hypothesis that genetic variants may influence the expression of genes involved in RCT, which may also have played a role in the anti-atherosclerotic effect of the drug.

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ABBREVIATIONS

ABCA1	ATP binding membrane cassette transport protein A1
apoA-1	apolipoprotein A-1
apoCI	apolipoprotein CI
apoB	apolipoprotein B
apoCII	apolipoprotein CII
apoE	apolipoprotein E
ATP	adenosine triphosphate
BMI	body mass index
bp	base pair(s)
ºC	degrees Celsius
CAD	coronary artery disease
CE	cholesteryl esters
CETP	cholesteryl ester transfer protein
CHD	coronary heart disease
CVD	cardiovascular disease
CYP7A1	cholesterol 7 alpha-hydroxylase
CYP27A1	sterol 27-hydroxylase
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticular
FA	fatty acid
HDL-C	high density lipoprotein cholesterol
HL	hepatic lipase
HMG-CoA	3-hydroxymethyl-3-methylglutaryl coenzyme A
LCAT	lecithin-cholesterol acyltransferase
LDL-C	low density lipoprotein cholesterol
LDL-R	low density lipoprotein receptor
LPL	lipoprotein lipase
mRNA	messenger ribonucleic acid
ndHDL	nascent discoidal HDL
PCR	polymerase chain reaction

PLTP	phospholipid transfer protein
PPAR	peroxisome proliferator-activated receptor
RCT	reverse cholesterol transport
rpm	revolutions per minute
RT-PCR	reverse transcription-polymerase chain reaction
SNPs	single-nucleotide polymorphisms
SR-B1	scavanger receptor type B1
TBE	tris-borate-EDTA buffer
TC	total cholesterol
TE	tris-HCl buffer
TG	triglycerides
TGRL	triglyceride-rich lipoproteins
tRNA	total ribonucleic acid
µg	microgram
µL	microliter
µM	micromolar
VLDL	very low density lipoprotein cholesterol

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THE AFRICAN GREEN MONKEY (*CHLOROCEBUS AETHIOPS*): AN APPROPRIATE MODEL FOR ATHEROSCLEROSIS

1.1 INTRODUCTION

There are a number of approaches, in terms of basic systems, for studying and/or modelling the metabolic syndrome in order to improve our understanding of this condition. Using human subjects appear intuitively to be the most obvious choice but they have considerable limitations. This is due to the difficulty in controlling variables such as environment and diet, and by the slowness of lesion development. Other complicating factors arise from the area of ethics, considering the type of interventions typically required to study the pathophysiology and treatment of this condition. *In vitro* systems are another option in research, and often used as an alternative to animal models but they cannot mimic the complex interactions of various tissues involved in lipid metabolism and transport (Moghadasian et al., 2001). Therefore, animal models offer the best opportunities to study the metabolic syndrome including aetiology, pathophysiology and treatment. Variables can be well controlled, extensive biochemical, biomechanical and pathophysiological assays can be performed, and the necessary experimental interventions can be carried out. This way cause-and-effect association can be built.

Animal research has already provided much information about many aspects of human biology in health and disease, and has determined the potential benefit of many therapeutic interventions. To date, many animal species and models are

described in the research literature, however, choosing the appropriate model to answer a particular research question can be challenging.

Among the models of dyslipidemia and atherosclerosis, a number of wild-type, naturally defective, and genetically modified animals have been utilised. Due to the complexity as well as species specificity of lipid and lipoprotein metabolism, all these models come with their own limitations. Rodents have been extensively utilized in atherosclerosis studies since they are easy to handle and can be genetically manipulated. However, atherosclerosis is not a spontaneous disease for them and has to be induced because, unlike primates, they have high levels of high density lipoprotein (HDL) which plays a protective role through the process of reverse cholesterol transport (RCT) (Table 1.1). Rodents lack cholesteryl ester transfer protein (CETP) which plays a vital role in RCT by mediating the transfer of lipids between lipoproteins (Moghadasian et al., 2001).

Table 1.1: Advantages and disadvantages of certain animal species in atherosclerosis research (Moghadasian et al., 2001)

Animals	Advantages	Disadvantages
Mice	Well-defined genetically, easily bred, short generation time, inbred availability, easy handling and housing, availability of several transgenic lines	Highly resistant to atherogenesis, high HDL, no CETP, difficulties in frequent blood sampling and dissection of medium/ small-size vessels
Rabbits	Naturally LDL-receptor deficient strain, naturally hypertriglyceridemic strain, good size, easy to keep and handle, known to many investigators, good response to dietary cholesterol, availability of transgenic lines	Lesion locations less similar to those in humans, very high plasma cholesterol needed to induce atherosclerosis, hepatic lipase deficient, no spontaneous atherosclerosis, cholesterol storage syndrome on cholesterol feeding
Pigeons	Atherosclerosis susceptible strains, location, histology and progress of lesions similar to humans, low cost and easy to handling, sufficient size, good response to dietary cholesterol, short generation time, relatively long life span	Nonmammalian, lack of apo E, B_{48}, and chylomicron formation, viral infection seen associated with atherosclerosis, considerable changes in lipoprotein metabolism during egg-laying
Nonhuman primates	The closest species to humans, some species respond well to dietary cholesterol, spontaneous early stage atherosclerosis in some species	Variations in site of lesions, expensive and difficult to house and handle, limitations in availability, ethical concerns
Swine	Some physiological/anatomical similarities to humans, spontaneous atherosclerosis particularly in abdominal aorta, availability of miniature pigs, natural lipoprotein mutant strains	Require high cholesterol diet (4-5% w/w), less-known to investigators, very low baseline cholesterol level, difficulties in care and high maintenance cost
Dogs	Some physiological/anatomical similarities to humans, well-characterised lipoprotein profile	Atherosclerosis-resistant species, high HDL, expensive, poor response to dietary cholesterol, ethical concerns

Amongst the mammals used in the laboratory, Old World nonhuman primates are the closest living relatives of humans, both in evolutionary and genetic terms. Although Old World monkeys are separated evolutionarily from humans by more than 20 million years (Bullock et al., 1975), they have a close resemblance to man, and share many characteristics (Harris, 1970). Asian Macaca species such as rhesus monkeys and cynomolgus monkeys have been used extensively to study experimentally induced atherosclerosis (Moghadasian et al., 2001). In general, their responsiveness to dietary cholesterol is much exaggerated compared to humans (Pronczuk et al., 1991; Shamekh et al., 2011).

Amongst the African primates, a subspecies of the African green monkey, also known as the vervet monkey, has been recognised as a good model for the study of diet-

induced atherosclerosis (Bullock et al., 1975). The distribution and morphology of induced atherosclerotic lesions in this species is similar to those in humans, as is the degree of responsiveness to dietary cholesterol (Moghadasian et al., 2001).

Due to the close evolutionary relationship between humans and nonhuman primates, many basic features of genetics, development, physiology, and metabolism are shared (Shamekh et al., 2011). These various similarities at the levels of whole-body physiology and metabolism, organ function, cell structure, and even gene organization make nonhuman primates such as the African green monkey excellent, and most sought after models for studies of human cardiovascular disease (CVD) (Moghadasian et al., 2001). Relative scarcity and high cost are the two most important factors that prevent many researchers from having access to species of this order. However, notwithstanding their closeness to humans, validity for their use as models still has to be demonstrated in the different primates when there is a gap in background information, or in the case of using a species not previously applied. Spontaneous development of certain diseases and conditions (i.e. atherosclerosis), with similarities in the underlying pathophysiology and subsequent markers, as well as sharing of genes and gene expression is the most important validation. Extensive analysis of genome structure and function in this animal model could make immediate and significant contributions to the understanding of CVD. Therefore, a primate model of a human disease, such as atherosclerosis, is critical to the long-term success of biomedical research.

While the human genome project and some others related to model organisms are very well advanced or even complete, little sequence information has been acquired

for the African green monkey. Given the importance of this species in biomedical research generally and CVD specifically, and the fundamental significance of sequence data, it is critical that this paucity of genome information concerning this specific animal model be addressed.

Studies using animal models of atherosclerosis can help clarify the contribution(s) of the factors that alter both concentrations and compositions of plasma lipoproteins in atherogenesis. One of these is CETP, which modifies lipid composition in lipoprotein particles, and may play a role in atherogenicity (Quinet et al., 1991). In recent years; there has been an explosion in the number of *in vivo* and genetic studies that have largely been carried out using rodent models. As mentioned above, these represent the most common animal model for research in coronary heart disease (CHD), however, there are several physiological and developmental differences between rodents and humans reflective of their relatively ancient evolutionary divergence (approximately 65 to 75 million years ago) as compared to nonhuman primates (approximately 25 million years ago) (Moghadasian et al., 2001). Therefore, this study, sought to systematically examine the genetic basis of HDL-C of one of the African nonhuman primate, the vervet monkey, in order to better define the molecular genetics of this animal model, and to further understand the mechanism of cholesterol metabolism in this species. One of the principle goals was to identify genes influencing cardiovascular disease-related phenotypes during therapeutic intervention. An important preliminary step in meeting this goal was the identification of a genetic contribution to the variation observed in these phenotypes. Data generated in this study will enrich accumulating knowledge of the factors influencing CHD in primatology, and, importantly, will identify susceptibility genes of relevance to the

African green (vervet) monkey, thereby obviating the need to extrapolate data generated in humans and other primates such as chimpanzees and rhesus monkeys.

The study applied a number of original approaches to identify candidate susceptibility genes in a nonhuman primate model without available genome sequence data. Furthermore, the application of bioinformatics with molecular techniques such as real-time PCR represented a state of the art approach to use orthologous human reference sequence to identify for the first time in the African green monkey specific lipid metabolism candidate genes and coincident SNPs corresponding to ancestral polymorphisms present in both humans and nonhuman primates, and preserved in both lineages.

1.1.1 Use of the African green monkey as an animal model of atherosclerosis

The African green monkey (*Chlorocebus aethiops*) which is indigenous to Southern Africa is considered to be a most appropriate model for human physiology and for the study of cholesterol metabolism (Suckling et al., 1993). Over the past three decades, this species also been used in the area ophthalmology, virology and cardiovascular disease, amongst others (Suckling et al., 1993).

African green monkeys develop diet-induced atherosclerotic lesions which are topographically and morphologically similar to those of humans (Fincham et al., 1987). Intravascular metabolism of cholesterol appears to be also similar, with the presence of an active cholesteryl ester transfer system (Nichols et al., 1965). Humans have a well-characterized lecithin:cholesterol acyltransferase (LCAT) enzyme and the LCAT levels in the African green monkeys are similar and sensitive to dietary

perturbations (Carroll et al., 1981). Like humans, African green monkeys express only apolipoprotein B 100 (apoB 100) in the liver (Fernandez et al., 2008). A high cholesterol diet leads to the reduction in hepatic LDL receptor mRNA and downregulation of cholesterol 7-alpha-hydroxylase (CYP7A) activity (Fernandez et al., 2008). To date numerous researchers have studied the relation between dietary fat saturation and HDL levels in an attempt to understand the relation between diet and CHD. In spite of the comprehensive nature of the studies on cholesterol metabolism in this species, much remains to be learned. Furthermore, not much information is available on the genetics of the African green monkey. Therefore, the present study has been planned to define the molecular basis of lipoprotein metabolism of this species. This approach relies on the identification of genetic variants, which influence risk factors for the development of atherosclerosis.

The Primate Unit of the Medical Research Council has extensive experience using the African green monkey in atherosclerosis research. In order to produce high concentrations of LDL and associated atherosclerosis, this group has formulated a diet entirely which requires 3 to 4 years of feeding to induce advanced lesions with features of human atherosclerosis (Fincham et al., 1998). Since this diet does not modulate HDL, it is not useful when investigating this lipoprotein and lipid modulating compounds such as CETP inhibitors. Moreover, the African green monkey shows a satisfactory response with HDL-C increases to a variety of reference compounds used at this unit with inhibition of cholesterol biosynthesis by lowering LDL-C by more than 40%. This demonstrates that the African green monkey expresses lipoprotein (a) (Lp(a)) unlike rodents (Fincham et al., 1987; Benadé et al., 1997).

The focus of atherosclerosis research is constantly evolving with early studies investigating the morphology, physiology and pathogenesis, and recent studies being more directed at the molecular and cellular mechanisms, as well as the preventive strategies. Numerous studies in humans, animals, and *in vitro*, are addressing the importance of RCT and cholesterol efflux in atherogenesis. It is very possible that augmentation of RCT and cholesterol efflux could be therapeutically useful. Potential major strategies include accelerating RCT and cholesterol efflux, which can be activated by increasing HDL and apolipoprotein A-I (apoA-I) levels, or by stimulating phospholipid transfer protein (PLTP) or CETP. Cholesterol efflux can be enhanced by facilitating pathways including ATP-binding membrane cassette transport protein A1 (ABCA1), scavenger receptor B1 (SR-B1), caveolin and sterol 27-hydroxylase (Cyp27A1) (Ohashi et al., 2005). However, uncertainties remain about the impact of RCT and cholesterol efflux on cardiovascular disease. Further exploration of modifiers of RCT and cholesterol efflux is warranted. Gaining insight into the entire picture of RCT and cholesterol efflux may enable us to develop more effective therapies for atherosclerosis in the future. Since HDL-based therapies have recently become the focus of attention, the African green monkey was used in this study to determine the effect of niacin on lipid metabolism at genetic level by the expression profile of the selected candidate genes of the RCT pathway.

1.1.2 Taxonomy and habitat

The African green monkey is a member of the subfamily *Cercopithecidea*, which includes the baboon and other Old World monkeys. Taxonomically they belong to the genus *Chlorocebus*. They are widely distributed throughout much of sub-Saharan Africa and inhibit diverse habitats, including semiarid Savannah, woodlands and

rainforest (Eley, 1992). In the wild, the African green monkeys are able to exploit patchily distributed plants and feed on fruits, leaves, insects, eggs, seafood, grasses, and seeds (Eley, 1989). Typically free-ranging, African green monkeys live in multi-male, multi-female groups of between 6 and 60 individuals per troop with a linear dominance hierarchy among the males and a matriarchial kin group relationship among the females (Eley, 1989). Each troop is led by an alpha male and reproduction is characterized by promiscuity, by either the alpha or subordinate mating with more than one female, just as females mate with more than one male. Therefore, inbreeding is a well-defined genetic consequence of this social system with an increase of homozygosity in the whole genome of this species (Charpentier et al., 2007). This situation would invariably be compounded in captivity due to small gene pools.

1.2 GENERAL BACKGROUND ON REVERSE CHOLESTEROL TRANSPORT AND CHOLESTEROL EFFLUX

Despite considerable progress in the development of new therapies to control atherosclerosis and its complications, coronary heart disease (CHD) remains the number one cause of death in the world. Atherosclerotic coronary artery disease (CAD) constitutes a major public health burden in developed and developing countries and by 2020 is predicted to be the single greatest cause of death worldwide (Murray et al., 1997). Although the sustained epidemic of HIV/AIDS causes more deaths in South Africa, the prevalence of CAD and other metabolic syndrome diseases such as type 2 diabetes is increasing dramatically and already account for more than a third of deaths in the population (Akinboboye et al., 2003; Sliwa et al., 2008 and Imoisili et al., 2009). Consistent with data from other parts of Africa suggesting a broadening pattern of cardiovascular disease involving a component of

greater burden imposed by atherosclerotic disease, it is essential that effective strategies for prevention or treatment are identified.

Preventing atherosclerosis holds the key to reduce the burden of cardiovascular disease, and a detailed understanding of the pathophysiology of atherosclerosis will facilitate the design of innovative therapeutic strategies for the management of dyslipidaemia and the prevention of morbid cardiovascular events.

Over the last few decades, our understanding of the basic mechanisms involved in atherosclerosis has progressed significantly. Cardiovascular disease (CVD) risk is influenced by several well-established risk factors, such as body mass index (BMI), an indicator of overweight and obesity, blood lipids, diabetes, and blood pressure (Morabia et al., 2003). These are intermediate phenotypes, having their own genetic and environmental determinants, including diet, nutrition, hormones, smoking, alcohol intake and physical activity (Bernstein et al., 2002). Studies in humans and mice indicate that both the type and quantity of blood-borne lipids are predictive of cardiovascular health or disease and that a relatively large number of proteins are involved directly and indirectly in the transport, maintenance and elimination of blood lipids, including high and low density lipoprotein cholesterol (HDL-C and LDL-C, respectively) (Fielding et al., 1995; Rader et al., 2000; Tall et al., 2000 and Glomset et al., 1973). Therefore, regulation of cholesterol levels is a complicated process, involving cholesterol uptake, biosynthesis, transport, metabolism, and secretion.

Since the pioneering work of John Gofman in the 1950s, our understanding of relative contributions of individual lipoproteins to overall cardiovascular risk has grown

substantially (Kapur et al., 2008) and modern medical therapy has resulted in a nearly 70% decrease in coronary heart disease (CHD)-related deaths (Choi et al., 2006). Preclinical research has gained further insight into the nature of HDL-C metabolism, specifically regarding the ability of HDL-C to promote reverse cholesterol transport (RCT).

RCT is a pathway that transports cholesterol from extrahepatic cells and tissues to the liver and intestine for excretion. By reducing the accumulation of cholesterol in the wall of arteries, RCT may prevent development of atherosclerosis. Cholesterol efflux, which is part of RCT, is a major process by which macrophages within the vessel wall secrete cholesterol outside cells (Khera et al.,2011). Other important factors include high-density lipoprotein (HDL), a subfraction of human plasma lipoproteins with apolipoprotein A-I (apoA-I.) as its principal apolipoprotein (Ohashi et al., 2005).

1.3 ROLE OF LIPIDS AND LIPOPROTEINS IN CHD

1.3.1 Lipid profile and CHD

Disorders in lipid (e.g., cholesterol and triglycerides) and lipoprotein metabolism are major established independent risk factors in the development and progression of atherosclerotic CHD (Laakso et al., 1993; Vergeer et al., 2010). Lipid-carrying proteins, termed as lipoproteins, are classified as 3 major classes, including very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL). VLDL carries mainly triglycerides, and cholesterol is carried mainly in LDL, and to a lesser extent in HDL particles. HDLs transport cholesterol from peripheral tissues to the liver for excretion and recycling. Several epidemiological studies have clearly shown that plasma cholesterol, triglycerides, and

LDL are positively correlated to the development of atherosclerotic CHD, whereas HDL is negatively correlated to CHD (Kanel et al., 1981; Brewer, 2011). Due to this differential correlation to CHD, LDL-cholesterol (LDL-C) is generally termed as "bad cholesterol" and HDL-cholesterol (HDL-C) as "good cholesterol".

1.3.2 Mechanistic role of lipoproteins in the pathogenesis of CHD

The classical view of atherosclerosis has changed considerably during the past decade. The original understanding of atherosclerosis processes include: a) the lipid-laden material builds up on the surface of a passive artery wall, b) the lipid build up or deposit (plaque) grows and eventually closes off an affected artery, c) the obstructed arteries limit blood supply to the target tissues, and d) the subsequent loss of viability of the blood-starved tissue (Laakso et al., 1993). These events result in several atherosclerotic cardiovascular disease complications including myocardial infarction such as, stroke and angina. Understanding the dynamic nature of vascular wall cells (versus previous notion as a passive carrier of blood) changed this original view considerably, and recent studies suggest that the changes in arterial wall cell interaction with blood components would lead to the initiation and progression of atherosclerosis. The current concepts suggest that the atherosclerotic vascular disease is characterized by: vascular endothelial activation and dysfunction, accumulation of fat-laden deposits within the arteries, monocyte-endothelial interaction and infiltration of monocytes, transformation of monocytes into lipid-laden foam cells, smooth muscle cell hypercellularity and intimal migration, dysregulated deposition and degradation of extracellular matrix proteins, and plaque rupture (Brewer, 2011).

The anti-atherogenic properties of HDL-C have been mainly attributed to the ability of apoprotein A-I containing HDL particles to initiate cholesterol efflux and facilitate the removal of excess cholesterol from peripheral tissues such as arteries and its delivery to the liver for removal through reverse cholesterol transport pathway (Kashyap, 1998; Khera et al., 2011). This highlighted the beneficial effects of HDL-C on atherosclerotic processes. The beneficial modulation of HDL particles by pharmacologic agents would therefore be of considerable importance in retarding or reversing atherosclerosis and CHD.

1.4 OVERVIEW OF RCT AND CHOLESTEROL EFFLUX

The sequence of events in RCT is described in Figure 1.1. ApoA-1 is first produced mainly by the liver, and released into the plasma. Circulating apoA-1 interacts with serum phospholipids and forms nascent discoidal HDL (ndHDL). Once the ndHDL is generated, it triggers cholesterol efflux in the macrophages and fibroblasts in the subendothelial space. Externalised cholesterol is absorbed by ndHDL, and subsequently is esterified by lecithin:cholesterol acyltransferase (LCAT). HDL particles are enriched with cholesteryl ester and become larger, resulting in HDL3 and HDL2. Phospholipid transfer protein (PLTP) is involved in this process by fusing two HDL3 into one HDL2 molecule. If HDL molecules are enriched with triglyceride, they are processed by the enzyme hepatic lipase (HL) and become smaller and denser. Cholesterol ester transfer protein (CETP) facilitates the equimolar exchange of cholesteryl esters from HDL for triglycerides in apoB100-containing lipoproteins. These cholesteryl esters are then delivered back to the liver via low-density-lipoprotein receptor (LDL-R), converted to bile salts, and eliminated through the gastrointestinal tract (Ohashi et al., 2005).

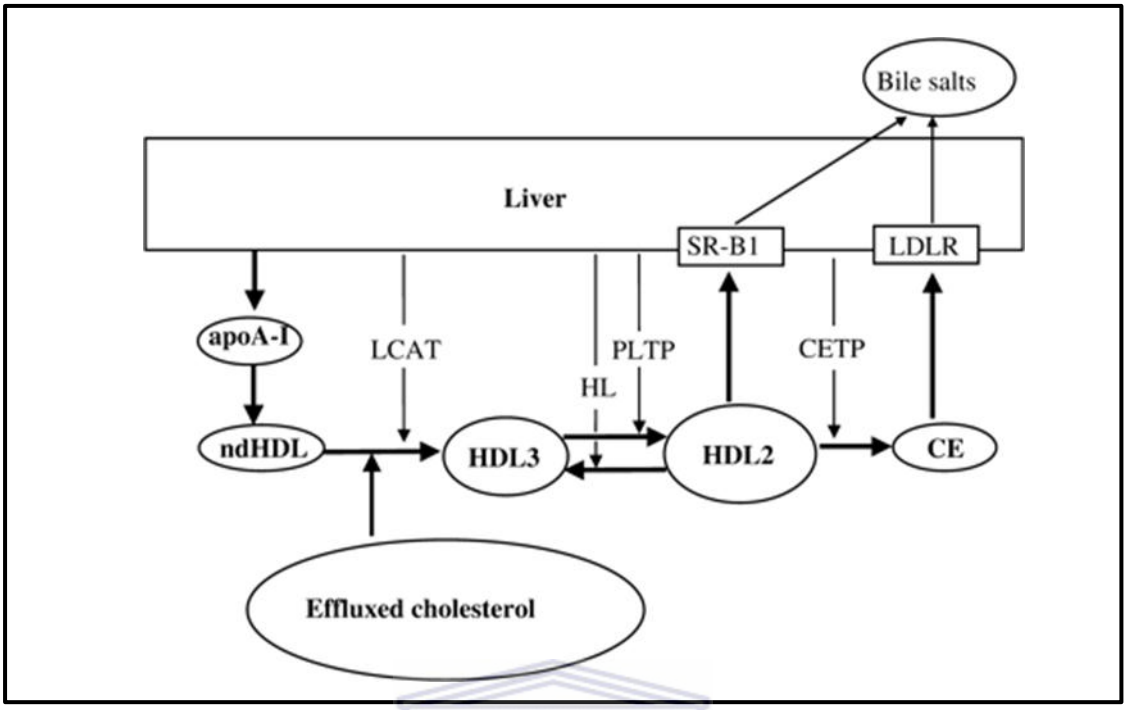

Figure 1.1: Reverse cholesterol transport pathway. Major constituents of RCT include acceptors such as high-density lipoprotein (HDL) and apolipoprotein A-I (apoA-I), and enzymes such as lecithin:cholesterol acyltransferase (LCAT), phospholipid transfer protein (PLTP), hepatic lipase (HL) and cholesterol ester transfer protein (CETP), which regulate cholesterol transport. Eventually, cholesterols in the HDL are delivered to the liver via scavenger receptor B1 (SR-B1), converted to bile salts and eliminated through the gastrointestinal tract. Cholesteryl esters (CE) could also be delivered to the liver via the low-density-lipoprotein receptor (LDLR). ndHDL, nascent discoidal high-density lipoprotein (Ohashi et al., 2005).

As acceptors such as apoA-1 and HDL approach macrophages in subintimal space, intracellular cholesterol can be released outside the cells for excretion, a process termed cholesterol efflux of macrophages (Figure 1.1). In this pathway, ATP-binding membrane cassette transport protein A1 (ABCA1) plays a major role in translocating cholesterol into the extracellular space (Oram et al., 2000). In addition to ABCA1, ABCG1, another member of the ABC transporter superfamily, is capable of mediating the active efflux of cholesterol and phospholipids mainly to lipid-rich acceptor particles in macrophages through the action of ABCA1 (Hu et al., 2010). This implies

a potential synergistic relationship between ABCA1 and ABCG1 in peripheral cholesterol export, where ABCA1 lipidates lipid-poor/free apoA-1 to generate nascent or pre*β*-HDL. These particles in turn serve as substrates for ABCG1-mediated cholesterol export (Gelissen et al., 2006; de Beer et al., 2011). It is not known whether ABCA1 and ABCG1 function dependently or independently in the process of macrophage cholesterol efflux and RCT in vivo. Together, ABCA1 and ABCG1 account for the major portion of the net cholesterol efflux from cholesterol-loaded macrophages to plasma lipoproteins in vivo (Tall et al., 2008). Four other factors are also known to be involved in this pathway. Scavenger receptor B1 (SR-B1) can induce cholesterol efflux by enabling HDL to bind to cells and recognise lipids within cholesterol-rich domains in the plasma membrane (William et al., 1999; de la Llera-Moya et al., 1999 and Hu et al., 2010). Caveolins have the capacity to bind cholesterol, and can transport cholesterol from the endoplasmic reticulum to the plasma membrane (Smart et al., 1996). Sterol 27-hydroxylase (CYP27A1) is also known as a contributor to cholesterol efflux (Escher et al., 2003). In addition to these pathways, cholesterol efflux can also occur via passive diffusion, in which cholesterol is desorbed down to the concentration gradient onto acceptor molecules (Kawano et al., 1993). Thus, RCT and cholesterol efflux constitute an efficient pathway by which excess cholesterol can be removed out of the body. Although extensive studies have been performed, RCT is a complicated process and its regulation mechanisms are largely unknown. Several key factors described above are involved in the RCT and cholesterol efflux, but the inter-relationship among these factors is not clear.

Experiments with transgenic animals suggest that disruption of one or more steps in RCT results in accelerated atherosclerosis, whereas overexpression of pivotal proteins

in RCT, such as apoA-1, PLTP, LCAT and SR-B1, exerts atheroprotective effects (von Eckardstein et al., 2000). The important lesson from these experimental approaches is that disruption of RCT and resulting atherosclerosis may occur in the presence of either decreased or increased HDL-C levels, depending on which step of RCT is dysfunctional.

1.4.1 Cholesterol efflux

A variety of evidence shows that RCT and cholesterol efflux play a major role in preventing atherosclerosis in humans. In fact, congenital impairment in genes involved in cholesterol efflux may augment atherosclerosis in some patients. On the other hand, acceleration of RCT and cholesterol efflux by increasing HDL or apoA-I levels may result in amelioration of atherosclerosis, suggesting a potential therapeutic tool for human atherosclerosis (Ohashi et al., 2005; Khera et al., 2011).

1.4.2 HDL levels and atherosclerosis

Several studies have shown that low levels of HDL2 and HDL3 are associated with increased progression of atherosclerosis and risk of cardiovascular disease (Miller et al., 1981; Sweetnam et al., 1994 and Ruotolo et al., 1998). Since HDL and apoA-1 are major receptors of cholesterol in the cholesterol efflux, increasing HDL levels may increase cholesterol efflux and RCT, contributing to reduced cardiovascular disease risks. Many attempts have been made to enhance HDL levels as anti-atherogenic therapy (Gordon et al., 1989 and Castelli et al., 1992). The prevention of cardiovascular disease is critically dependent on lipid-lowering, including 3-hydroxymethyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), cholesterol absorption inhibitors, bile acid resins, fibrates, and nicotinic acid

(nicin). Although these drugs are generally well tolerated, severe adverse effects can occur in a minority of patients. Significant progress has been made in the identification of common DNA sequence variations in genes influencing the pharmacokinetics and pharmacodynamics of most of these drugs and in disease-modifying genes relevant for coronary heart disease (CHD) (Schmitz et al., 2006).

Currently available lipid-modifying drug have generally modest effects on HDL-C levels. Statins are first-line drug therapy for treatment of elevated LDL-C as well as for most high-risk patients with low HDL-C, but raise HDL-C by only 5-10% (Yim et al., 2003). Fibrates, agonists of peroxisome proliferator-activated receptor (PPAR)-α, lower triglyceride levels very effectively and raise HDL-C by 5 -20% (Yim et al., 2003). Nicotinic acid (niacin) is the most effective HDL-raising drug currently available, with increases of up to 35% (Ohashi et al., 2005). Gemfibrozil therapy significantly reduced cardiovascular disease, with a modest increase in HDL levels (Frick et al., 1987). Niacin also lowers triglyceride levels, reduces LDL levels, and modestly lowers lipoprotein A [Lp(a)] levels (Canner et al., 1986 and Guyton et al., 2000). Several clinical trials have found niacin to be effective, alone or in combination with other drugs, in preventing coronary events, slowing atherosclerotic disease progression, and promoting lesion regression (Brown et al., 2001).

1.5 HDL AND REVERSE CHOLESTEROL TRANSPORT

1.5.1 Mechanisms

Reverse cholesterol transport describes the transfer of cholesterol from nonhepatic cells to the liver (von Eckardstein et al., 2000; 2001). Lipid-free apo A-I or lipid-poor pre–ß-HDL particles produced in the intestine or liver or shed during lipolysis of triglyceride-rich lipoproteins (TGRL) initiate efflux of phospholipids and cholesterol from cell membranes in a process facilitated by phospholipid transfer protein (PLTP). Cholesterol in these nascent discoidal HDL particles is then esterified by lecithin-cholesterol acyltransferase (LCAT). Cholesteryl esters readily move to the core of HDL particles, producing a steady gradient of free cholesterol and enabling HDL to accept cholesterol from various donors. The reciprocal exchange of cholesteryl ester for triglycerides mediated by CETP moves the bulk of the cholesteryl esters to lipoprotein remnant particles, which are subsequently cleared by the liver. At the same time, HDL becomes enriched with triglycerides, which are substrates for hepatic lipase (HL). The concerted action of CETP-mediated cholesteryl ester transfer and HL-mediated hydrolysis of triglycerides and phospholipids helps to form the smaller HDL particles that are the preferred binding partners for scavenger receptor type B1 (SR-B1), the major HDL receptor on hepatocytes. The binding of HDL with SR-B1 mediates the selective uptake of cholesteryl esters that have not undergone CETP-mediated transfer to apo B–containing particles (intermediate-density lipoprotein and low-density lipoprotein [LDL]). Lipid-free apolipoproteins or lipid-poor pre–ß-HDL are formed in reactions catalyzed by PLTP, CETP, and HL. Thus, as shown in Figure 1.2, reverse cholesterol transport can be envisioned as a cycle in which acceptors of cellular cholesterol (ie, apo A-I, pre–ß-HDL) are perpetually regenerated to undertake their function of inducing cholesterol efflux (Assmann et al., 2004).

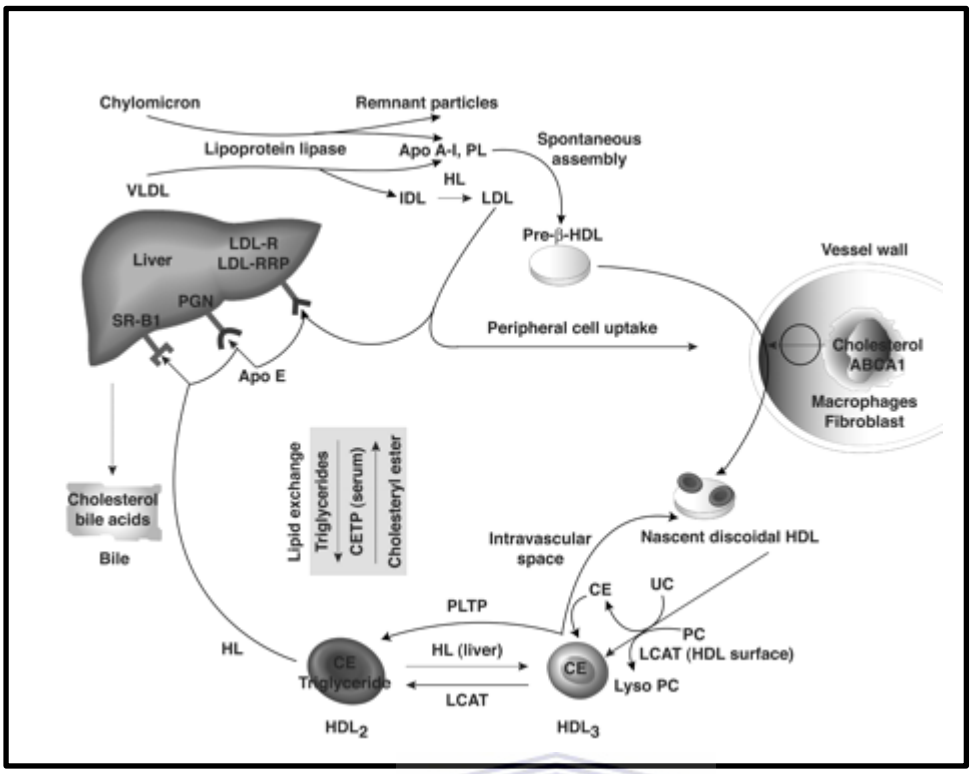

Figure 1.2: Pathways involved in the generation and conversion of HDL. ABC1 indicates adenosine triphosphate-binding cassette transporter 1; apo A-I, apolipoprotein A-I; apo E, apolipoprotein E; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; HDL, high-density lipoprotein; HL, hepatic lipase; IDL, intermediate-density lipoprotein; LCAT, lecithin-cholesterol acyltransferase; LDL, low-density lipoprotein; LDL-R, low-density lipoprotein receptor; LDL-RRP, low-density lipoprotein receptor-related protein; Lyso PC, lysophosphatidylcholine; PC, phosphatidylcholine; PGN, proteoglycans; PL, phospholipids; PLTP, phospholipid transfer protein; SR-B1, scavenger receptor B1; UC, unesterified cholesterol; and VLDL, very-low-density lipoprotein. Adapted from Toth PP (*Curr Atheroscler Rep*. 2003;5:386–393).

1.6 THERAPIES DIRECTED AT THE PROMOTION OF CHOLESTEROL EFFLUX AND RCT

The concept that the promotion of macrophage RCT could prevent progression or even induce regression of atherosclerosis is remarkably attractive. Data in animals suggest that atherosclerosis regression can be achieved through HDL-based interventions. A major area in HDL-based therapeutics involves the exploitation of the information to develop pharmacological approaches to enhance components of the RCT pathway. The actual mechanism by which HDL protects against atherosclerosis

is likely multifactorial and not yet fully elucidated; however, there are several proposed major atheroprotective mechanisms of HDL. An important concept is that simply raising HDL-C levels may not necessarily be the optimal target for the development of new therapies targeted toward HDL (Duffy et al., 2006). Some new therapeutic approaches are targeted toward promoting RCT, even if they do not rise HDL-C levels per se (Duffy et al., 2006). It could be that the function of HDL is more important than its concentration and those therapies that improve HDL "function," even if they do not increase HDL-C levels, may have important antiatherogenic and vascular protective effects (Navab et al., 2004).

1.6.1 Drug targets

A combination of genetic variations and environmental differences defines unique human characteristics, including individual responsiveness to drugs or susceptibilities to common diseases. Among several types of genetic variation, single-nucleotide polymorphisms (SNPs) are the most abundant throughout the genome. SNPs have lately received much attention in biomedical fields, because some of them can influence the quality and/or the quantity of particular gene products and thereby serve as markers of individual risk for adverse drug reactions or susceptibility to complex diseases (Ganji et al., 2003).

Proteins that transport drugs in the body play roles in absorption, distribution, and excretion of drugs. Hence, genetic variations in genes encoding transporters may cause individual differences in drug absorption, elimination and cellular uptake, thereby affecting drug response and toxicity (Iida et al., 2003).

1.6.1.1 Niacin as a lipid-regulating agent and its effect on atherosclerotic CHD

The use of niacin as a pharmacological agent has been reported as early as 1955 (Carlson, 2005), and currently it is a widely used agent in the treatment of dyslipidemia (Kamanna et al., 2008). In pharmacologic doses of 1-3 g/day, niacin reduces concentrations of total plasma cholesterol, apolipoprotein (apo) B, triglyceride, VLDL, LDL, and Lp(a), and increases HDL levels (Carlson, 2005). Niacin is the most potent available lipid-regulating agent to increase HDL levels. Because of these diverse effects on the lipid profile, niacin is considered as a broad-spectrum lipid-regulating agent. Several clinical trials (secondary prevention and angiographic studies) indicate that the treatment with niacin significantly reduces total mortality, coronary events, and retards the progression and induces regression of coronary atherosclerosis (Carlson, 2005). Although the use of niacin has in the past been associated with adverse effects (e.g., flushing and hepatic toxicity), recent studies utilizing newer formulations of niacin have shown a significant reduction in flushing with minimal to no hepatic toxicity, and comparable effects on plasma lipid profiles (Carlson, 2005; Kang et al., 2011). These newer formulations of niacin referred to as "niacin extended-release" have considerably renewed interest in the use of niacin as a broad-spectrum lipid-regulating agent and particularly for raising HDL.

1.6.1.2 Role of niacin in HDL metabolism

HDLs are a complex class of lipoproteins with hydrophobic core of cholesterol esters and triglycerides, and an outer hydrophilic layer of apolipoproteins, phospholipids and unesterified cholesterol. Apo AI and apo AII are the major proteins of HDL, accounting for approximately 70% and 20% of protein mass respectively (Yim et al., 2003). The liver and intestines are the major organs for synthesis and secretion of

HDL and its components. The plasma levels of HDL and its components are finely regulated by various synthetic and catabolic processes (Yim et al., 2003). Although niacin has long been used specifically to raise HDL, only recently studies are beginning to address the cellular mechanisms of action of niacin on HDL metabolism (Yim et al., 2003).

There are mainly two mechanisms by which niacin influences plasma lipids and the secretion of apo B bearing lipoproteins including VLDL particles in the liver. One mechanistic strategy to decrease elevated levels of lipids (e.g., triglycerides) in blood may be the inhibition of lipolysis in adipose tissue (Figure 1.3). Niacin, through hormone-sensitive lipase-mediated events, inhibits fatty acid mobilization from adipose tissue. Inhibition of fatty acid (FA) release from adipose tissue results in decreased availability of fatty acids for triglyceride (TG) synthesis (Ganji et al., 2003). Niacin-mediated inhibition of TG synthesis may decrease the lipidation of apo B and translocation through endoplasmic reticular (ER) membrane leading to increased intracellular apo B degradation. Increased hepatocyte apo B degradation by niacin would decrease the number of VLDL and their catabolic product, LDL particles, which explains the lower apo B and LDL concentrations observed clinically after niacin treatment (Ganji et al., 2003 and Kamanna et al., 2008). In-vitro studies in hepatocytes suggest that niacin inhibits the putative "HDL catabolism receptor" involving removal of HDL-apoA-I, but not the SR-BI receptor that mediates selective HDL-cholesterol ester removal (Ganji et al., 2003). These mechanisms of decreased HDL-apoA-I catabolism by niacin would increase HDL half-life and its concentration thereby augmenting cholesterol efflux and reverse cholesterol transport (RCT), and other HDL-related vascular beneficial effects. Increased residence time would also

allow HDL size to increase (HDL2 > HDL3) from peripheral tissue cholesterol uptake. Taken together, niacin, through these collaborative intracellular metabolic processes, favourably modulates LDL and HDL levels resulting in decreased atherosclerotic coronary artery disease (Ganji et al., 2003 and Kamanna et al., 2008).

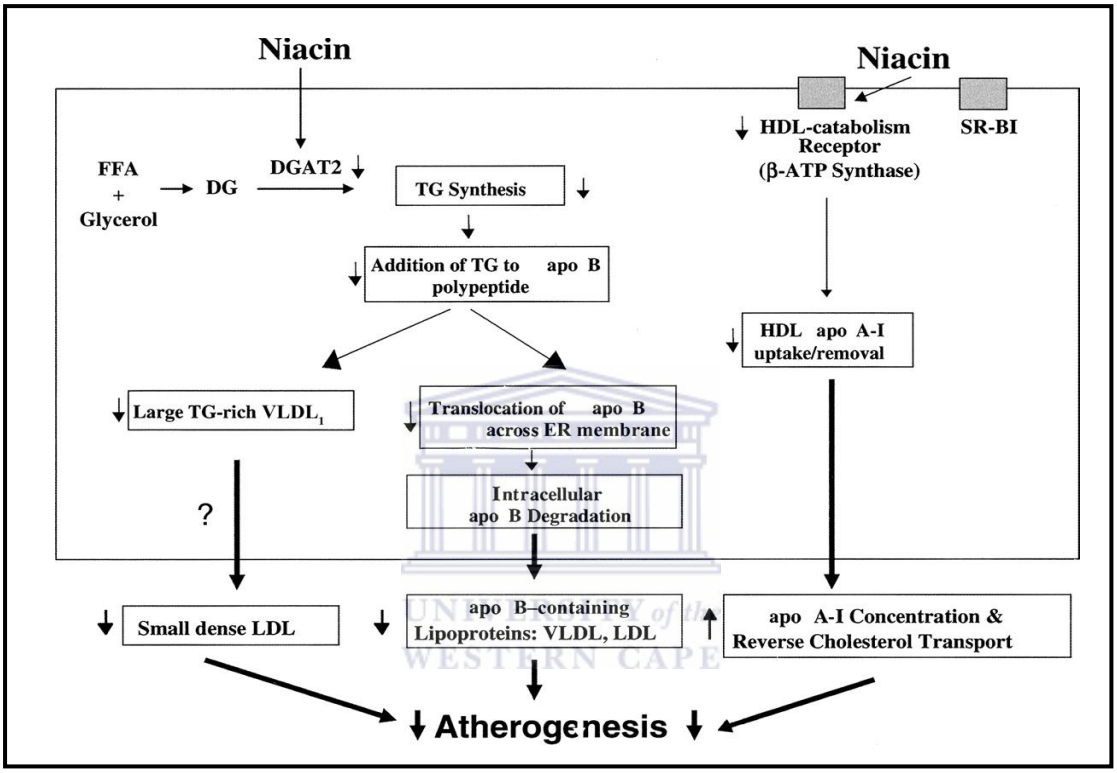

Figure 1.3: Overview of current concepts on mechanism of action of niacin on lipid and lipoprotein Metabolism (Kamanna et al., 2008).

1.7 GENE ACTIVITIES IN RCT AND CHOLESTEROL EFFLUX

There is interest in the role of genetic polymorphisms predicting susceptibility to disease and responsiveness to dietary and drug interventions. Small-scale studies have investigated the effects of polymorphisms on physiological or biological factors and have provided useful information on possible mechanistic links between variation at the gene level and risk factors for cardiovascular disease (CVD).

Plasma measures of lipid metabolism are well-established risk factors of CAD, the leading cause of death worldwide (Klos et al., 2006). Increased risk of CAD has been associated with elevated TG, TC, and LDL-C, and decreased HDL-C (Austin et al., 1998; Castelli et al., 1992, Gordon et al, 1989 and Davignon et al., 1996). CAD has also been associated with low plasma apoA-I and high apoB (Brunzell et al., 1984). These lipids and apolipoproteins are regulated by complex metabolic and cellular processes, which, in turn, are influenced by both genetic and environmental factors (Sing et al., 2003).

The hypothesis that high-density lipoprotein cholesterol (HDL-C) is protective towards atherosclerosis was first proposed by Barr et al. (1951). The protective action of HDL may reflect the role of HDL particles in the reverse cholesterol transport (RCT) pathway. Indeed, HDL particles are involved in the uptake of cholesterol from peripheral tissues and its transport back to the liver for excretion. The molecular regulation of HDL metabolism and RCT is complex. RCT is the process by which cholesterol is effluxed from peripheral tissues onto acceptor particles, primarily HDL, in the plasma for uptake by the liver. HDLs consist of cholesterol, phospholipids, triglycerides, and apolipoproteins (Tall, 1990). Lipid-poor apoA-I promotes efflux of cholesterol and phospholipids through interaction with ABCA1. The enzyme LCAT converts unesterified cholesterol to cholesteryl ester (CE) within the HDL particle. HDL CE is subsequently returned to the liver by 3 distinct pathways. In the first pathway, the SR-B1 mediates selective uptake of HDL cholesteryl esters by the liver. In the second pathway, CETP transfers cholesteryl ester from HDL to apoB-containing lipoproteins in exchange for triglycerides. Finally, there is a pathway that leads to uptake of holo-HDL particles and degradation of HDL-associated proteins

such as apoA-I. In part, this pathway may involve the formation of large HDL, enriched with apoE (Tall et al., 2000).

RCT involves numerous lipid transfer proteins, enzymes, apolipoproteins, and membrane-bound receptors (Fielding et al., 1995; Khera et al., 2011). The genes encoding these proteins, as well as genes encoding proteins that regulate their transcription, are candidates for influencing variation in plasma levels of apoA-I, apoB, HDL-C, LDL-C, TC, and TG. Based on a model RCT pathway (von Eckardstein et al., 2001; Ren et al., 2006), to date, a set of 54 genes involved in RCT for evaluating the impact of genetic variation on variation in plasma lipid and lipoporotein levels have been identified by Klos et al., (2006). CETP, lipoprotein lipase (LPL), HL and apolipoprotein E (apoE) are key players in the metabolism of the major plasma lipoproteins and are potential candidates for the plasma modulation of LDL particle size. Disturbances in these systems are integral components of life-threatening diseases. Enhancement of cholesterol efflux and RCT is considered an important target for anti-atherosclerotic drug therapy (von Eckardstein et al., 2000).

CETP mediates the transfer of hydrophobic lipids between VLDL, LDL and HDL. Genetic CETP deficiency is associated with markedly elevated concentrations of HDL cholesterol and a polydisperse LDL size distribution pattern (Yamashita et al., 2000). The first common functional polymorphism in the CETP gene promoter (-629C/A) was described by Dachet et al., 2000. The CETP-629C/A polymorphism is in strong linkage disequilibrium with the extensively studied TaqIB polymorphism, recently reported to be associated with decreased CHD risk (B2 allele) (Ordovas et al., 2000).

LPL hydrolyses triglycerides of chylomicrons and VLDL and mediates the cellular binding and uptake of lipoproteins. The LPL 447X allele has been associated with a beneficial lipid profile and a lower risk of CHD in most studies. HL plays a pivotal role in the metabolism of LDL and HDL. Four common polymorphisms in the HL promoter are in complete linkage disequilibrium. Recently, the -514T allele was also shown to decrease the transcriptional activity of the HL gene in vitro (Deeb et al., 2000).The common polymorphism in the apoE gene (e2/e3/e4) has been extensively studied, and the apoE e4 allele has been reported to be associated with decreased LDL particle size, and increased risk of CHD (Wilson et al., 1996).

ABCA1 is a member of the ATP-binding cassette transporter family, which plays a most important role in apoA-I-mediated cholesterol efflux from peripheral cells, the first step in reverse cholesterol transport (Yamakawa-Kobayashi et al., 2004). ABCA1 has been identified to be a transporter that effluxes excess cellular cholesterol to poorly lipidated apoA-1, thus playing a pivotal role in the reverse cholesterol transport process (Brewer et al., 2004). Regulation of ABCA1 transporter gene expression plays a key role in determining intracellular cholesterol levels (Brewer et al., 2004), therefore, ABCA1 is an attractive candidate gene for the modulation of plasma HDL cholesterol concentration (Wang et al., 2000). Common variants of this gene may be a genetic factor for atherosclerosis (Kyriakou et al., 2005).

1.7.1 Effects of mutations and intervention on HDL metabolism

There is interest in the role of genetic polymorphisms in predicting the susceptibility to disease and responsiveness to dietary and drug interventions (Vergani et al., 2006). Small-scale studies have looked at the effects of polymorphisms on physiological or

biochemical factors and have provided useful information on possible mechanistic links between variation at the gene level and risk factors for cardiovascular disease (CVD) (Freeman et al., 2003). For the purpose of this study, 10 "candidate" genes known to be involved in RCT and HDL metabolism were identified and investigated in the African green monkey. Each gene is described below.

Since the African green monkey is closely related to humans, coincident single nucleotide polymorphisms (cSNPs) occurring at the same locus in both humans and other nonhuman primates were prioritised for this study (Table 1.2). In the absence of the African green monkey genome sequence, the rhesus macaque (*Macaca mulatta*) genome was used for comparison with the humans. Since rhesus macaque and African green monkey belong to the same family (Cercopithecidae) and are closely related; an assumption was made that both species are likely to share the same cSNPs. Shared ancestral polymorphisms are polymorphic sites that originated in the ancestral species and have survived genetic drift in both the human and other species. The molecular mechanism underlying this variation remains unknown. Hodgkinson et al. (2009) discovered that a surprising number of human polymorphic sites are also polymorphic in nonhuman primates. These cSNPs not only occur significantly more frequently than expected under independence but also cannot be easily explained by natural selection (Hodgkinson et al. 2009; Hodgkinson and Eyre-Walker 2010).

Table 1.2: List of prioritised coincident SNPs

Coincident SNPs				
Genes	SNPs	Human	Rhesus	Baboon
CETP	I405V	X	X	X
	Ala373Pro	X	X	X
ABCA1	Ile883Met	X	X	X
	E1172D	X	X	X
	V771M	X	X	X
	V825I	X	X	X
	R219K	X	X	X
LCAT	Ser232Thr	X	X	X
	LCATu3	X	X	X
SR-B1	A350A	X	X	X
	G2S	X	X	X
apoA-1	*MspI*	X	X	X
	G-75A	X	X	X
apoB	T71I	X	X	X
	4311S	X	X	X
apoCI	*HpaI*	X	X	X
apoCII	Lue96Arg	X	X	X
	62 A>C	X	X	X
apoE	Cys112Arg	X	X	X
	Arg158Cys	X	X	X
CYP7A1	Asn233Ser	X	X	X
	A-278C	X	X	X

1.7.1.1 Cholesteryl ester transfer protein

CETP modifies the lipid composition of the plasma by transferring triglycerides and cholesterol esters between lipoproteins, thereby decreasing plasma HDL-C concentrations and increasing the proportion of lipids present in the atherogenic LDL-C and VLDL fractions. Increased risk for atherosclerosis with increased CETP activity has been shown in CETP transgenic mouse models, and there is inconclusive evidence of a similar risk in humans. (Carlquist et al., 2003).

It has long been theorized that the atheroprotective effect of HDL is primarily due to its role in RCT. RCT is a complex pathway, involving transport proteins, modifying enzymes, and cell surface receptors. One of the enzymes with a key role in RCT is CETP, which promotes the exchange of cholesteryl esters from HDL to the apoB-containing lipoproteins, thus, providing an avenue for the uptake of cholesteryl esters by hepatic receptors (Bruce et al., 1998). Several common mutations, or polymorphisms, have been identified in the CETP gene (Figure 1.4). Polymorphisms identified in the coding sequence of the CETP gene include A373P, I405V, and R451G. Two of these variants, Ala373Pro and R451G, are associated with increased CETP activity and reduced HDL-C levels, whereas the I405V variant is associated with reduced CETP mass and increased HDL-C levels (Bruce et al., 1998). Among the most widely studied CETP variants is *Taq*IB, a silent base change affecting the 277th nucleotide in the first intron of the CETP gene (Boekholdt et al., 2004).

In this study, the more common I405V polymorphism was selected since it is associated with vascular disease. In the homozygous form for the rarest allele (V/V genotype) the I405V polymorphism is associated with a reduction in CETP activity, and with changes in the levels of HDL-C and the composition of HDL and LDL (Okumura et al., 2002, Barzilai et al., 2003, Boekholdt et al., 2004 and Brousseau et al., 2004).

CETP deficiency in man is associated with raised HDL-C up to 5 mmol/l (Jukema et al., 2004). Several pharmaceutical companies have developed CETP inhibitors, such as torcetrapib and JTT-705 (Brousseau et al., 2004). Administration of a high-dose

torcetrapib and JIT-705 to humans decreased CETP activity significantly and increased HDL-C by 34 to 106% (Jukema et al., 2004).

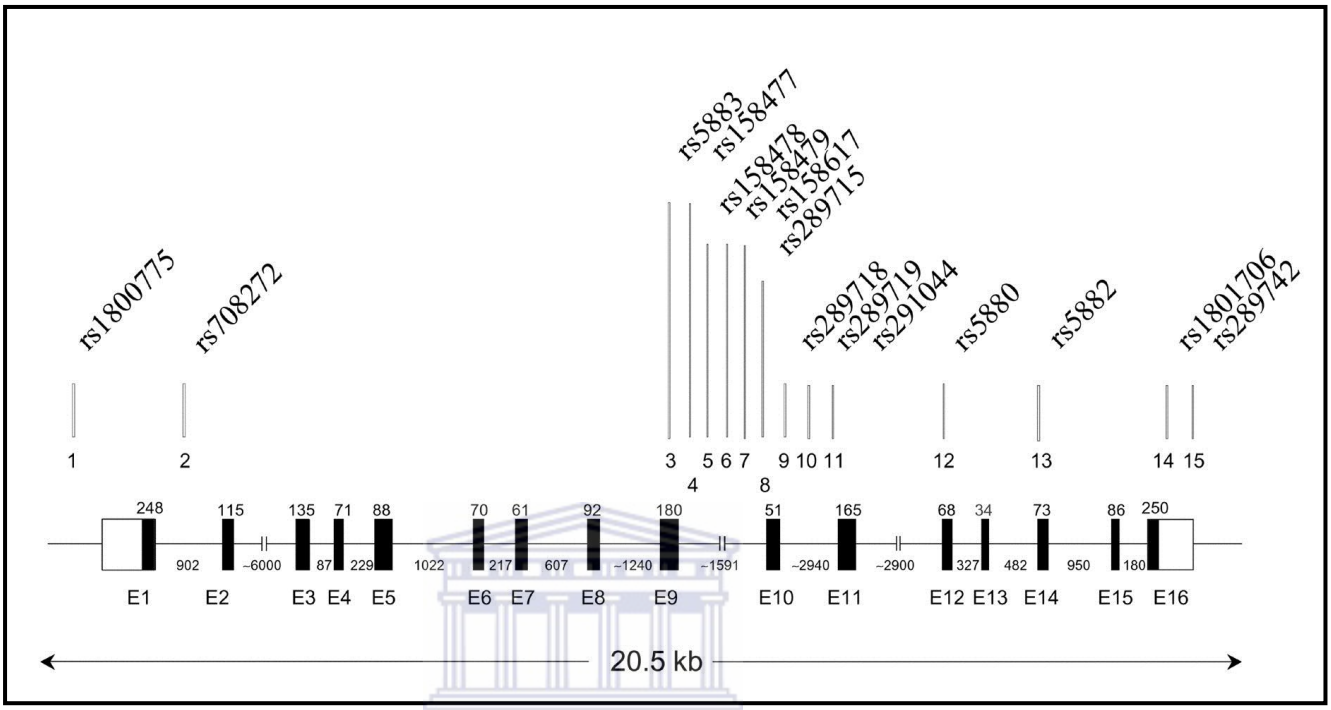

Figure 1.4: Structure of the CETP gene and the locations of SNPs tested (Bansal et al., 2002).

1.7.1.2 ATP binding cassette transporter Al

ABCA1 is a member of the ATP-binding cassette transporter family, which plays a most important role in apoAI-mediated cholesterol efflux from peripheral cells, the first step in RCT (Yamakawa-Kobayashi et al., 2004). ABCA1 has been identified to be a transporter that effluxes excess cellular cholesterol to poorly lapidated apoA-1, thus playing a pivotal role in the reverse cholesterol transport process (Brewer et al., 2004). Regulation of ABCA1 transporter gene expression plays a key role in determining intracellular cholesterol levels (Brewer et al., 2004), therefore, ABCA1 is an attractive candidate gene for the determination of plasma HDL cholesterol

concentration (Wang et al., 2000). Common variants of this gene may be a genetic factor for atherosclerosis (Kyriakou et al., 2005).

Twelve SNPs were identified in coding regions of the gene, six of which resulted in amino acid substitutions (Frikke-Schmidt et al., 2004). R219K, V771M, V825I, I883M, E1172D, and R1587K all substitute similar amino acids. R219K and R1587K are located in the two major extracellular loops of the ABCA1 protein, important for the interaction with apoA-I and for cholesterol efflux. V771M, V825I, I883M, and E1172D are located in the middle part of the protein corresponding to the fifth and sixth transmembrane α-helix, the seventh hydrophobic segment (H7), and the first regulatory segment (R1), respectively (Figure 1.5). The amino acid residues affected by these six non-synonymous SNPs are situated in highly conserved (V771, V825, E1172, R1587) or less-conserved (R219, I883) areas of ABCA1, and are either completely conserved between species (V771, E1172) or vary between 2 (R219, V825, R1587) or more (I883) similar amino acids (Figure 1.5). All six non-synonymous SNPs are common and have been reported previously (Frikke-Schmidt et al., 2004). In this study, five SNPs of ABCA1 were chosen based on their function and location within the gene (R219K, E1172D, V771M, V825I and I883M).

Complete ABCA1 deficiency is known as Tangier's disease. This condition leads to premature atherosclerosis, because compromised cholesterol efflux from peripheral cells to apoA-I results in the accumulation of cholesteryl esters in peripheral tissue (Jukema et al., 2004). Since upregulation of ABCA1 in animals has produced positive effects by reducing the risk of CAD (Zwart et al., 2002), several pharmaceutical companies are working on the development of ABCA1 agonists. However, negative

outcomes on lesion development associated with the effect of ABCA1 have also reported (Joyce et al., 2002; Van Eck et al., 2006).

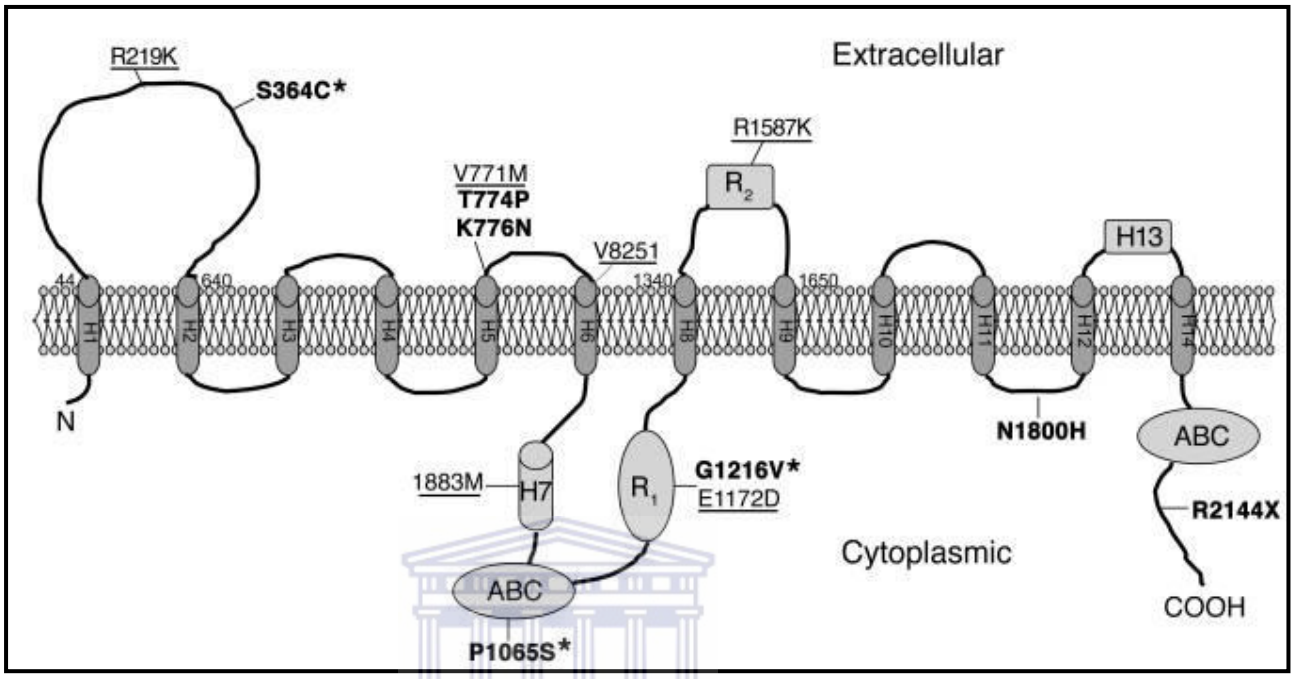

Figure 1.5: Topological model of ABCA1 (Frikke-Schmidt et al., 2004).

1.7.1.3 Apolipoprotein A-1

ApoA-I is the major protein constituent of HDL particles and plays a central role in lipid metabolism and CHD risk (Segrest et al., 2000 and Stein et al., 1999). Several epidemiological studies have reported that HDL-C and apoA-I are inversely related to the incidence and severity of CAD and can independently predict the risk of CAD (Stampfer et al., 1991; van der Steeg et al., 2008). Complete apoA-I deficiency results in low or absent HDL and is associated with premature atherosclerosis (Funke et al., 1997). Partial deficiencies decrease HDL and increase the risk for CAD. ApoA-I promotes cholesterol efflux from tissues to the liver for excretion. Its anti-atherogenic effects are based on its acceptor function for ABCAl-mediated free cholesterol efflux from the peripheral cell. It is a cofactor for LCAT which is responsible for the

formation of most plasma cholesteryl esters (Fielding et al., 1995). ApoA-1 is therefore an important component of reverse cholesterol transport.

The gene for apoA-I is known to be in a cluster with apoC-III and apoA-IV on chromosome 11 (Karathanasis, 1985) and several studies have reported an association between DNA polymorphisms of this gene cluster and differences in the levels of HDL-C and apoA-I in CAD patients and healthy individuals (Ordovas et al., 1991). A common G-to-A transition located 75 base pairs (bp) upstream from the transcription start site of the apoA-I gene has been described and studied extensively (Pagani et al., 1990). This substitution destroys an *Msp* I cutting site and can be easily detected with the polymerase chain reaction (PCR). In three independent studies, the *A* allele has been associated with higher levels of both HDL-C and apoA-I (Pagani et al., 1990). It has been reported that individuals carrying the *A* allele show higher concentrations of apoA-I and HDL cholesterol than those with the *G/G* wild type (Pagani et al., 1990). In this study, we attempt to identify the role of two polymorphisms (this one included) in the African green monkey by modulating HDL metabolism with niacin.

It is interesting to note that niacin extended-release treatment significantly improves apoA-1 levels and lowers the number of atherogenic LDL particles, VLDL and chylomicron particles (Insull et al., 2010).

A topic of growing interest is the apoA-I_{Milano} mutation (Franceschini et al., 1980). Carriers of this mutation have very low HDL-C levels, but surprisingly exhibit a decreased risk of CAD. Its positive effects regarding atherosclerosis and the fact that apoA-I has a long elimination half-life make it a promising candidate for therapeutic

applications (Jukema et al., 2004). The apoA-I_{Milano} gene possesses structural differences that impart a loss in lipid-binding capacity and accelerated catabolism, which is the mechanism underlying a more efficient uptake and removal of tissue lipids (Futterman et al., 2005). The identification of a naturally occurring variant of apoA-I (the apoA-IM) led to the finding that this HDL/apolipoprotein variant was antiatherogenic. Its effect is associated with a rapid increase in cholesterol efflux–promoting capacity, mobilization of free cholesterol from tissues, and reduction of lipid and macrophage content within the vascular plaque (Futterman et al., 2005).

1.7.1.4 Lecithin-cholesterol acyltransferase

LCAT is one of the key enzymes controlling cholesterol homeostasis and transport and has a pivotal role in HDL-C maturation and remodeling (Asztalos et al., 2007). LCAT synthesizes the majority of cholesteryl esters in plasma by transferring a fatty acid from lecithin (phosphatidyl choline) to the 3-hydroxyl group of cholesterol. Although it accentuates RCT from plasma to liver and induces cholesterol degradation, the debate about LCAT's role in RCT and protection against atherosclerosis is ongoing (Brown et al., 2010). In the absence of LCAT, RCT can still continue. The main factors that maintain the cholesterol homeostasis within macrophages are preβ HDL and ABCA1 transporter. Free cholesterol can also be taken up via SR-BI in the liver (Rader, 2009). Although patients with LCAT deficiency do not show progressive atherosclerosis, a decrease in LCAT activity is associated with an increase in carotid artery intima media thickness (Hovingh et al., 2005). Increased LCAT activity is thought to be atheroprotective, but there are a limited number of studies, and the measurement of LCAT activity is still not

standardized. Additional data on LCAT's genetic and enzymatic properties should help to understand the role of LCAT in normal HDL-C function.

Mutations in the LCAT gene can cause complete LCAT deficiency, also known as familial LCAT deficiency or a strong decrease in LCAT activity known as Fish-eye disease (Jukema et al., 2004). Both conditions are rare, and characterised by extensive corneal opacity and very low HDL-C. Although HDL-C is low in these patients, premature atherosclerosis is not a prominent characteristic in these conditions. Animal studies show that the expression rate of LCAT affects sensitivity to atherosclerosis. As the LCAT gene is a relatively small gene, gene therapy might be possible in future, but clinical applications still have to be developed (Seguret-Mace et al., 1996).

1.7.1.5 Scavenger receptor class B type I

SR-B1 is a cell surface glycoprotein that plays an integral part in cholesterol homeostasis (Connelly et al., 2004). Identified at first as a HDL receptor, SR-B1 is primarily expressed in organs that require free cholesterol, such as the liver and steroidogenic tissues. The receptor binds with the highest affinity to HDL; however, it also binds less efficiently with apoB-containing lipoproteins (Calvo et al., 1997). Upon binding to the lipoprotein particles, SR-B1 delivers cholesterol into the cells via a selective uptake process. Thereafter, the cholesterol-depleted lipoproteins are recycled back into the circulation. Additionally, SR-B1 stimulates cholesterol efflux out of the cell thereby promoting the clearance of cholesterol from the periphery as well as the secretion of biliary cholesterol (Connelly et al., 2004). By regulating the bidirectional flux of cholesterol between lipoproteins and cells, SR-B1 is a key regulator in reverse cholesterol transport.

The influence of SR-B1 on plasma HDL concentrations has been demonstrated in mouse models where hepatic overexpression of the receptor resulted in lower HDL-cholesterol, lower apoA-I, and increased biliary cholesterol (Ji et al., 1999). These changes are a manifestation of the hindered hepatic uptake of cholesterol ester from HDL. Overexpression of SR-BI in mice causes increased HDL-C excretion in bile, resulting in a decrease in HDL-C and atherosclerosis (Silver et al., 2001). SR-BI knockout mice are characterised by raised plasma HDL levels and an increased progression of atherosclerosis (Rigotti et al., 1997). This observation suggests that uninhibited reverse transport of cholesterol might be of greater importance than elevating HDL-C per se! In addition to selective cholesterol uptake, there is evidence of SR-B1 involvement in metabolism of apoB-containing lipoproteins. Through the regulation of plasma cholesterol, SR-B1 appears to be protective against the development of atherosclerotic lesions in animal models (Kozarsky et al., 1997).

In humans, investigations of sequence variants or polymorphisms in the SR-B1 gene have shed light on the influence of this receptor on plasma lipid concentrations. The SR-B1 gene has been mapped to human 12q24.31 and encodes a 509 amino acid protein (Van Eck et al., 2005). Three common single nucleotide polymorphisms (SNP) in exon 1 (p.Gly2Ser), exon 8 (c.1119C>T), and intron 5 (c.795+54C>T) have been associated with lipid parameters in epidemiological studies (Morabia et al., 2003). The c.1119C>T polymorphism also known as A350A was also identified in this study and has been associated with higher HDL-C and lower LDL-C concentrations, particularly in men (Morabia et al., 2003). In light of prior reports of the effect of c.1119C>T on fasted lipid concentration, it was hypothesized that this

polymorphism would be associated with an anti-atherogenic profile. This study investigated the association between c.1119C>T and niacin drug therapy response.

Currently, human deficiencies of SR-BI have not been described. Epidemiological studies have shown that SR-BI variants are associated with HDL-C and LDL-C concentrations. There seems to be a connection to triglycerides and obesity as well (Osgood et al., 2003).

1.7.1.6 Cholesterol 7-alpha-hydroxylase

CYP7A1 catalyzes the first reaction in the cholesterol catabolic pathway in the liver (Nakamoto et al., 2006). This pathway converts cholesterol to bile acids, which is the primary mechanism for the removal of cholesterol from the body. The CYP7A1 catalytic reaction is the rate-limiting step and the major site for regulating homeostasis of cholesterol and bile acids. The gene encoding CYP7A1 was mapped to chromosome 8q11 (Cohen et al., 1992). The CYP7A1 gene spans about 10 kb and contains 6 exons, 5 introns, one 5'-UTR, and one 3'-UTR (Nakamoto et al., 2006).

In mice and rats the synthesis of bile acids through this pathway is under feed-forward regulation by cholesterol via a transcriptional mechanism involving the nuclear receptor known as the liver x receptor a (LXRa; NR1H3) (Agellon, et al., 2002). LXRa normally binds to a direct repeat of the hexameric hormone response element separated by four nucleotides as a heterodimer with retinoid x receptor (RXR; NR2B1) and is activated by oxysterols (Gbaguidi et al., 2004). The human and nonhuman primate CYP7A1 gene, unlike the rat and murine Cyp7a1 genes, is not

stimulated by oxysterols because the CYP7A1 gene promoter does not interact with RXR:LXRa (Gbaguidi et al., 2004).

Genetic variations in the CYP7A1 gene associated to disorders of cholesterol and bile acid metabolism have been studied extensively in different laboratories, and have been associated with metabolic disorders of cholesterol and bile acids, including hypercholesterolemia, hypertriglyceridemia, arteriosclerosis, and gallstone disease (Nakamoto et al., 2006). The information has indicated that genetic variations in the CYP7A1 gene have high impact on human cholesterol metabolic regulation and human health.

1.7.1.7 Apolipoprotein C-II

ApoC-II is a 79-amino acid residue protein that has a crucial role in lipoprotein metabolism as a cofactor for lipoprotein lipase (LPL), which catalyzes the lipolysis of triglycerides in plasma chylomicrons and VLDL (Chen et al., 2007). ApoC-II is synthesized primarily by the liver. The apoC-II gene has been mapped on chromosome 19 in a gene cluster containing the apoE/C-I/C-I'/C-IV/C-II gene and spans 45 kb of chromosomal region (Chen et al., 2007). The 0.55-kb intergenic region between apoC-II and apoC-IV genes is a strong cell type-specific promoter (Vorgia et al., 1998). The exact function of apoC-IV is unknown, although it appears to play a role in lipid metabolism. Overall, the association of lipid related traits with variants in the apoE/C1/C4/C2 gene cluster has been fairly inconsistent, with the exception of the apoE gene variants. Such inconsistency is possibly related to the close linkage of these genes on chromosome 19q (Chen et al., 2007).

In this study, two single nucleotide polymorphisms were identified and investigated to determine if they have any influence in the regulation and function of the reverse cholesterol transport pathway in the presence of niacin therapy. The distribution of apoC-II on plasma lipoprotein is known to continually change between high density lipoproteins and triglyceride-rich lipoproteins as a result of the secretion, metabolic conversion, and catabolism of plasma lipoprotein (Fojo et al., 1984).

1.7.1.8 Apolipoprotein C-I

ApoC-I is a constituent of triglyceride-rich lipoproteins and high density lipoproteins the importance of which in plasma lipoprotein metabolism is increasingly evident (Xu et al., 1999). The apoC-I gene lies in a gene cluster containing apoE and apoC-II on chromosome 19 (Gao et al., 2002). Its function is to displace apoE from triglycerides-rich emulsions and lipoproteins (Xu et al., 1999). ApoC-I decreases binging of VLDL, a model of poor-lipolysis lipoprotein remnants, to a remnant receptor, the low density lipoprotein receptor-related protein (LRP) (Xu et al., 1999). The presence of the *Hpa I* in the ApoC-I promoter has a significant effect on apoC-I transcription. This polymorphism has been associated with lipoprotein metabolism disorder (Gao et al., 2002). Based on this information, this particular polymorphism was genotyped in the African Green monkey in this study to assess if it might have any involvement in niacin therapy response.

1.7.1.9 Apolipoprotein B

ApoB is an essential structural protein component of LDL and VLDL particles, which act as transporters for triglyceride and cholesterol between the liver and peripheral tissues and plays an important role in the maintenance of cholesterol homeostasis in

all mammals (Chan *et al*. 2000). Owing to the importance of lipoproteins in lipid metabolism, even small changes in the structure or function of the protein may have a large impact on plasma lipid levels, and thereby on the risk of CVD (Bentzen et al., 2002). ApoB circulates in two distinct forms (apoB100 and apoB48), encoded by a single gene composed of 29 exons localized on chromosome 2 (Kane, 1983).

Polymorphisms in apoB may associate with increased or decreased levels of apolipoprotein B and LDL-C (Humphries, 1988). Several polymorphisms in the apoB gene have been described (Humphries, 1988), some of which have an effect on the levels of cholesterol and triglyceride. The most frequently investigated are T2488T (*Xba*I), E4154K (*Eco*RI), R3611Q (*Msp*I), N4311S and an insertion/deletion in the signal peptide (AA12ins/del) (Figure 1.6). Various studies have shown an association between some of these polymorphisms and lipid or lipoprotein levels (Hansen et al., 1993; Moreel et al., 1992; Xu et al., 1990). In this study, two apoB polymorphisms (T71I and N4311S) were investigated. T71I and N4311S are known to be associated with lower plasma levels of LDL cholesterol. These sequence variants most likely lower LDL cholesterol levels by either interfering with lipidation of nascent apolipoprotein B by reducing the production of LDL from VLDL or accelerating LDL clearance by the LDL receptor (Benn et al., 2008).

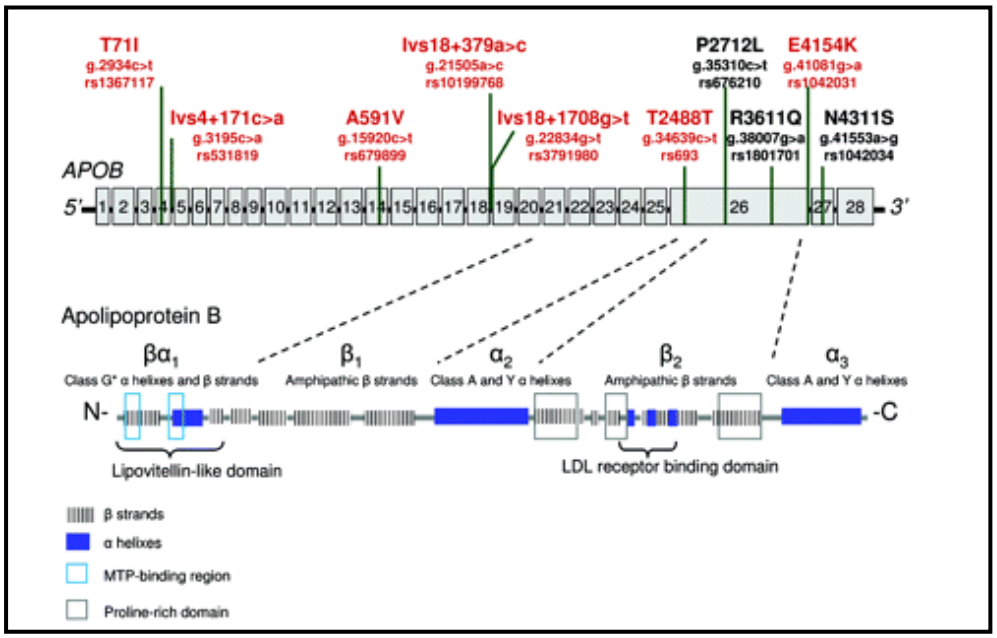

Figure 1.6: Location of the 10 SNPs relative to the amino acid sequence and the structural and functional domains of apolipoprotein B. T71I located in domains crucial for lipidation of the nascent apolipoprotein B; N4311S in domain known to or suspected of regulating binding to the LDL receptor (Benn et al., 2008).

1.7.1.10 Apolipoprotein E

ApoE is an exchangeable protein that plays an important role in lipid metabolism, especially in the removal of atherogenic remnants of triglyceride-rich lipoproteins (Hixson et al., 1990). The human apoE gene spans 3.7 kb including four exons and is located on chromosome 19 (Hixson et al., 1990).Three common alleles of apoE encoding isoforms e2, e3 and e4 have been identified (Hixson et al., 1990). The isoforms are characterized by the presence of amino acid Cys (apoe2 and apoe3) or Arg (apoe4) at position 112 of the mature apoE polypeptide chain and Cys (apoe2) or Arg (apoe3 and apoe4) at position 158 (Koch et al., 2002). Apoe3 is the most common of these isoforms, and is distinguished by cysteine at position 112 (112cys) and arginine at position 158 (158arg) in the receptor-binding region of apoE (Koch et al., 2002). The presence of apoE allele e4 is known to be associated with the

pathogenesis of peripheral and coronary artery disease and the e2 allele is known to be protective from atherosclerosis.

The variability of amino acids 112 and 158 is based on SNPs present at nucleotide position 334 and 472, respectively, of the apoE gene (Koch et al., 2002). The SNPs are known as 334T/C and 472C/T and both were investigated in this study.

1.8 RESEARCH OBJECTIVES

The broad focus of this study was to determine the effect of an HDL cholesterol-raising agent, niacin, on the lipid metabolism or RCT pathway by genetic strategies in case-control studies in African green monkeys, since no data are available in this area for this species.

The choice of this RCT system is also informed by the fact that currently, there is a focus on HDL-based therapies to further reduce atherothrombotic vascular diseases.

Even though the African green monkey has already provided invaluable insight in cardiovascular disease studies, and has been pivotal in defining the cellular events in the initiation and development of lesions in atherosclerosis research, the underlying molecular dynamics have not been fully explored in this species. The main objective of this project was to make an original contribution to primatology by defining the molecular genetics of the African green monkey in relation to CHD. This is the first study of a controlled pharmacological intervention linked to genetic determinants of lipid metabolism in the African green monkey.

At present, there is very little data in GenBank for nonhuman primates, and almost none for the African green monkey. As discussed above, nonhuman primates including the African green monkey are closely related to humans both in evolutionary and genetic terms. Therefore, shared ancestral polymorphisms known as coincident SNPs (cSNPs) were prioritized for this study, since they are polymorphic sites that originated in the ancestral species and have survived genetic drift in both species.

The specific objectives of the study included the following:

- To use an integrated combination of clinical, molecular biological and bioinformatics strategies to identify orthologous human candidate genes for lipid metabolism in African green monkeys:
    1. To choose candidate genes which have been reported in literature to be linked to lipid metabolism in humans.
    2. To choose (cSNPs) within recognized candidate genes.
- To assess the levels of HDL-C, LDL-C, TC, TG and lipoproteins after lipid-modulating niacin administration.
- To determine the effect of niacin on lipid metabolism at the genetic level by the expression profile of the selected candidate genes
- To assess the possible involvement of these genes in cholesterol metabolism pathway or reverse cholesterol transport pathway in case:control studies in a colony of African green monkeys after administration of niacin.

1.8.1 Hypothesis

The genetic mechanisms and dynamics that control reverse cholesterol transport in the African green monkeys (AGM) are similar to those found in other nonhuman primates and humans, and make the AGM therefore a useful model for human cardiovascular disease.

1.9 OVERVIEW

The layout of this dissertation contains an introduction, experimental procedures, results, discussion and conclusion in the next two chapters to follow. The final chapter consists of a general discussion and conclusion to sum-up the findings. Additional information on experimental design and data acquisition is included in the Appendix section.

EFFECTS OF SHORT-TERM TREATMENT WITH NIACIN AS MONOTHERAPY ON PLASMA LIPOPROTEIN IN A NONHUMAN PRIMATE MODEL OF ATHEROSCLEROSIS

2.3 INTRODUCTION

In this century many major medical advances have resulted in part from research on animals including nonhuman primates such as the African green monkey (*Chlorocebus aethiops*). These, therefore, often serve as a critical link between basic research and human clinical application (Suckling et al., 1993). In this context, the establishment of suitable animal models has proven to be invaluable for the study of human cardiovascular disease.

The African green monkey has been an excellent model for the study of cholesterol metabolism (Weight et al., 1988 and Suckling et al., 1993). Since elevated dietary cholesterol levels induce atherosclerosis in this primate (Bullock et al., 1975), and many aspects of dietary cholesterol metabolism have been investigated. In spite of the comprehensive nature of these studies, much of the molecular basis remains to be understood and defined in this species.

In this first study, the effects of niacin (Sigma) were investigated in the African green monkey to evaluate the effect of this compound on plasma lipids and reverse cholesterol transport pathway. This is in preparation for the second study which is investigating the genetic dynamics as a result of this intervention. Since the objective of this first part, was to increase HDL-C level through therapeutic intervention; niacin

was selected since it is known to be the most potent HDL cholesterol-raising agent currently used (Rader 2003; Muller et al., 2007; Kamanna et al., 2008).

Slow-release niacin favourably affects all lipids and lipoproteins (Mckenney, 2004). Niacin also enhances the transcription of peroxisome proliferator-activated receptor γ (PPARγ) and ATP binding cassette transporter A1 (ABCA1), the latter is a key transporter for cellular cholesterol into apolipoprotein A-1 containing HDL particles (Rubic et al., 2004). These effects of niacin cause a decrease in LDL and TG, and an increase in HDL, which are changes that have demonstrated to reduce the complications of atherosclerotic disease. High-density lipoprotein (HDL)-based therapies have recently become the focus of attention (Shah et al., 2005) which is shifting from just lowering of the lipid 'concentrations' to an emphasis on vascular biology, pharmacogenetics and a greater understanding of the importance of HDL cholesterol.

A reduced level of HDL-C is an important cardiovascular risk factor (Gordon et al., 1989). Moreover, HDLs exert various potentially antiatherogenic properties. As a consequence, therapeutic modifications of HDL-C levels have attracted considerable interest. Drugs increasing HDL-C are sought for antiatherogenic therapies, and drugs decreasing HDL-C are suspected to increase cardiovascular risk (von Eckardstein et al., 2001).

HDL is involved in RCT and an important antiatherogenic function of HDL, namely, the HDL-mediated efflux of cholesterol from non-hepatic cells and its subsequent delivery to the liver and steroidogenic organs (von Eckardstein et al., 2001).

Enhancements of cholesterol efflux and of RCT are considered important targets for antiatherosclerotic drug therapy. Levels and composition of HDL subclasses in plasma are regulated by many factors, including apolipoproteins, lipolytic enzymes, lipid transfer proteins, receptors, and cellular transporters (von Eckardstein et al., 2001). These factors have become important targets in the development of effective strategies to prevent vascular/atherosclerotic disease.

Via its RCT mechanism, HDL holds the promise of not only halting progression of atherosclerosis but also including a true regression of atherosclerotic lesions. A number of agents are available that increase HDL cholesterol levels. Statins are the most potent LDL cholesterol-lowering agents and produce modest increases in HDL cholesterol, although their effects vary. Niacin is the most potent HDL cholesterol-raising agent currently used and slow-release niacins have reduced the side effects and have made this class of drugs more tolerable (Rader, 2003). Other agents that modify HDL cholesterol include fibrates, glitazones, omega-3 fatty acids and estrogen. The precise mechanisms through which these classes of agents increase HDL cholesterol levels generally are not well defined.

2.4 MATERIALS AND METHODS

The study was approved by the Ethics Committee of the MRC (REF 11/07). The project was carried out in the Primate Unit of the Technology and Innovation Directorate, MRC, Parow Valley. Selected subjects were healthy adult female monkeys with normal plasma HDL. All individuals were housed singly during the study but had regular access to exercise cages and environmental enrichment. All individuals were identified with numbers in ink tattoo.

2.2.1 Management of animals and environment

The African green monkeys used in this study were bred and maintained in the Primate Unit of the MRC under identical housing conditions according to the South African National Standard for the Care and Use of Animals for Scientific Purposes (The SANS 10386:2008). The closed indoor environment was maintained at a temperature of 24 – 26ºC, 45% humidity, 15 – 20 air changes/hour and a photoperiod of 12h.

The monkeys received a diet of pre-cooked maize meal mixed with a vitamin and mineral concentrate, egg powder, bean flour and sunflower oil in the morning. The diet was further supplemented with vitamin C and vitamin D3 and each monkey received 66g (dry weight) of the diet per day. The diet was mixed with water to make a very stiff porridge that can be formed into balls which the monkeys can grasp and handle. At lunch the monkeys received fruit (apples, oranges, mandarins) and in the evening maize meal without the vitamins but containing milled seeds. The diet supplies 2412 kJ/day (range of recommendations for primates is 1380 - 2510), 12% energy from protein, 20% energy from fat and 75% energy from carbohydrates. Water was available *ad lib* via an automatic watering device.

2.2.2 Formulations and administration of compounds

Niacin was supplied in a powder form. An exact amount of niacin based on each animal's body weight was weighed and mixed in a 30g portion of maintenance diet.

2.2.3 Selection criteria and treatment

Selection of animals included in this study was based on genotyping of selected cSNPs. Genotypes were obtained from 25 monkeys using polymerase chain reaction amplification of genomic DNA and sequencing. However, due to the small gene pool of the MRC Primate colony genotype variations were not obtained. Eight healthy adult females were therefore selected for this study. The subjects were divided into 2 groups as indicated in Table 2.1. Four females were assigned to a treatment and the other four to a control (placebo) group. The control group received maintenance diet throughout the study. The treatment group received an exact amount of niacin based on each animal's body weight which was mixed into a 30 g portion of food (Table 2.2). The compound was administered once per day at escalating doses for three consecutive periods of two weeks, starting at 35 mg/kg. Based upon the effects on total cholesterol, HDL-cholesterol and triglycerides as well as clinical examination, the dose could be increased to 75 then to 100 mg/kg for the next two-week periods of treatment, or maintained at 35 or 75 mg/kg (Table 2.2). Each treated animal received niacin daily throughout the treatment period of four months, which was followed by two weeks of washout.

Table 2.1: Group selection and treatment allocation

Group	Treatment	Doses mg/kg	n	Treatment time
1	Control	-	4	7:00 am
2	Niacin	35mg/kg.d 75mg/kg.d 100mg/kg.d	4	7:00 am

Table 2.2: Treatments to determine efficacy

Treatment	Identification no. of monkey	Bodyweight (kg) at first baseline	Total dose (mg)	Frequency of treatment
Group 1 Control	108 243 215 97	3.55 3.84 3.68 3.50	N/A	once/day at 7H00 am
Group 2 Niacin 35mg/kg, 70mg/kg, 100mg/kg	795 322 339 77	3.56 3.02 3.29 3.30	124.6, 249.2, 356 105.7, 211.4, 302 115.2, 230.3, 329 115.5, 231, 330	once/day at 7H00 am

2.5.4 Duration of treatment

The study started on 21/01/08 and was completed on 23/06/08. The duration of the treatment was four months with a 4-week washout period (Table 2.3).

Table 2.3: Treatment periods

Period	Weeks	Dates
Baseline	-1 and -2	21.01.08 – 04.02.08
Treatment	1-16	04.02.08– 26.05.08
Washout	17-20	27.05.08 – 23.06.08

2.2.5 Blood sampling

All blood was obtained via femoral venipuncture after Ketamine anaesthesia at 5mg/kg bodyweight four hours after compound administration. Blood was collected in EDTA-containing tubes after overnight fasting according to the schedule provided in Table 2.4. On each day of sampling, overnight-fasted monkeys received the total dose of the compound in small food balls and blood was taken 4 hours after compound administration. The rest of the food was given after the blood collection.

For plasma isolation, blood was collected in EDTA-containing tubes. Plasma was isolated at 4°C (1400 x g, 10 minutes) and maintained at -80°C for biochemical analysis. Additionally, for gene expression analysis in chapter 3, more blood was collected in PAXgene Blood RNA Tubes (BRT) and stored at room temperature for 2 hours before being stored at -80°C.

Table 2.4: Blood sampling schedule and analyses

Sampling	Day	Time of treatment	Volume	Analysis
Baseline 1	-14	4h post-dose	2ml	TC, HDL, LDL, Trigs, Apo A
Week 2 (treatment)	Day 14	4h post-dose	2ml	TC, HDL, LDL, Trigs, Apo A
Week 4 (treatment)	Day 28	4h post-dose	2ml	TC, HDL, LDL, Trigs, Apo A
Week 6 (treatment)	Day 42	4h post-dose	2ml	TC, HDL, LDL, Trigs, Apo A
Week 8 (treatment)	Day 54	4h post-dose	2ml	TC, HDL, LDL, Trigs, Apo A
Week 10 (treatment)	Day 68	4h post-dose	4ml	TC, HDL, LDL, Trigs, Apo A
Week 12 (treatment)	Day 82	4h post-dose	2ml	TC, HDL, LDL, Trigs, Apo A
Week 14 (treatment)	Day 96	4h post-dose	2ml	TC, HDL, LDL, Trigs, Apo A
Week 16 (treatment)	Day 110	4h post-dose	2ml	TC, HDL, LDL, Trigs, Apo A
Week 18	Day 124	Washout	2ml	TC, HDL, LDL, Trigs, Apo A

2.2.6 Observations

2.2.6.1 Clinical examination

Each animal was observed once a day, at approximately the same time twice/week for the recording of clinical signs **.** Niacin is known to cause severe side effects, therefore special monitoring was considered for this study. Food intake was measured daily.

2.2.6.2 Body weights

The body weight of each animal was recorded at each blood sampling.

2.2.6.3 Clinical observations

Clinical observations were recorded twice/week according to three categories (Appendix I):

- Physical: condition of coat, faeces and urine; discharge from eyes, ears, nose genitals or rectum; any other symptoms or lesions.
- Behaviour: alert, fearful, aggressive, confused, depressed, vocalization.
- Motor function and activity: posture, coordination, locomotion and activity level.

2.2.7 Biochemical analysis

Biochemical analysis of known risk factors of coronary artery disease such as HDL-C and LDL-C were conducted for each time point of the two weeks interval throughout the study. Levels of HDL-C, LDL-C, TC, TG and apoA-1 were measured at baseline and after every two weeks for four months up to the washout period. Biochemical analysis was conducted by PathCare on SYNCHRON LX Systems Manual, 2000 (Appendix I).

2.2.8 Statistical Analysis

The lipid profile data presented are the mean ± SEM. The primary end point of the study was a reduction in the LDL-Cholesterol concentration, and an increase in HDL-Cholesterol between the baseline and the end of the treatment phase. Multiple comparison analysis was used to determine statistical significance at each time point. ANOVA was used to compare baseline levels and changes from baseline between the

niacin and control groups. Statistical significance was calculated by using the Student *t* test, and a value of $P < 0.05$ was considered significant. Under the assumption of an α of 5%, 8 African green monkeys were required to show a 39% reduction in LDL-Cholesterol and 30% increase in HDL-Cholesterol with a power of 80%.

2.6 RESULTS

Baseline characteristics of the eight African green monkeys enrolled in this study are listed in Table 2.2. All four treated monkeys received a maximum dose of 100 mg/kg.

2.3.2 Efficacy

2.3.2.1 **Total Cholesterol**

Total plasma cholesterol increased by 31% from week 4 when the dosage was increased from 70 mg/kg to 100 mg/kg and decreased by 18% from week 10 to week 16, while the control decreased by 9% from week 4 to week 16. Differences between the two groups were statistically significant at week 8 (p = 0.01) and week 10 (p = 0.02) (Figure 2.1). The level of total cholesterol in the control group remained relatively constant throughout the study as compared to the experimental group, which increased during treatment, declined from week 10 and returned back to baseline after washout period (Figure 2.1).

2.3.1.2 HDL-Cholesterol

HDL increased in Group 1 (niacin) from baseline to week 6 by 122% and declined thereafter by 28% to the cessation of treatment with the greatest decrease of 37% from week 16 to the end of washout period in week 20. The HDL remained higher than that

of the control Group 2 which only increased by 30% from baseline to the end of the study. Differences between the two groups were statistically significant at week 6 ($p = 0.02$) and week 8 ($p = 0.01$) (Figure 2.2). The level of HDL-C in the control group remained relatively constant throughout the study as compared to the experimental group, which increased during treatment and returned back to baseline after the washout period at week 16 (Figure 2.2). The strongest increase in HDL-C occurred early during treatment from baseline (2.18 ± 0.92 mmol/L) to week 6 (4.85 ± 0.82 mmol/L). Niacin appeared to have increased HDL-C, but did not sustain the same effect after week 6 when the dose was maintained at 100 mg/kg and this lead to a decline in HDL-C synthesis (Figure 2.2).

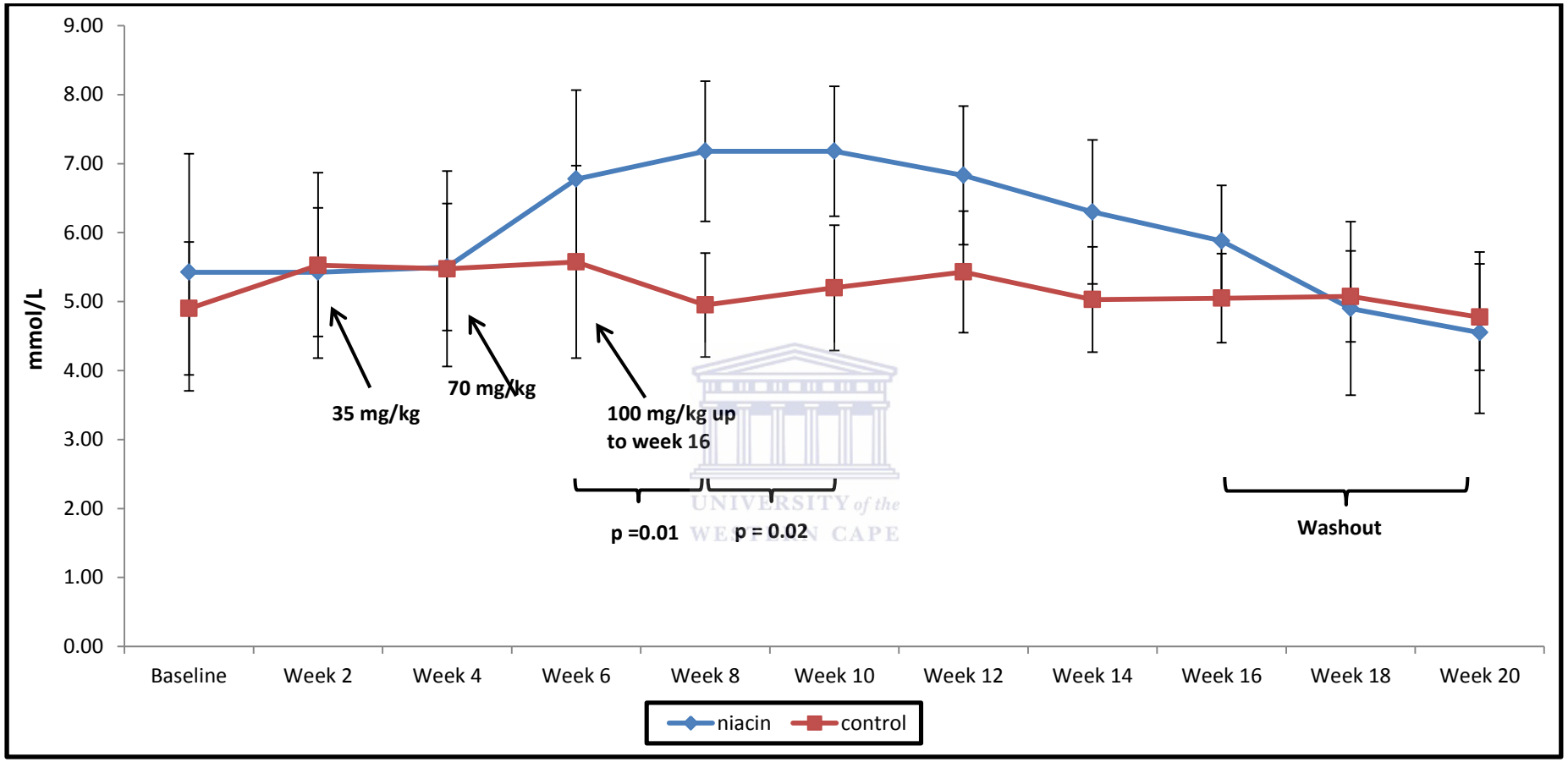

Figure 2.1: Total plasma cholesterol. Treatment schedule was as follows: The treatment group received niacin at 35 mg/kg/d (week 2 to week 3), 70 mg/kg/d (week 4 to week 5), 100 mg/kg/d (week 6 to end of week 16). The control group received the vehicle during the entire study period. Differences between the two groups were statistically significant at week 8 (p = 0.01) and week 10 (p = 0.02).

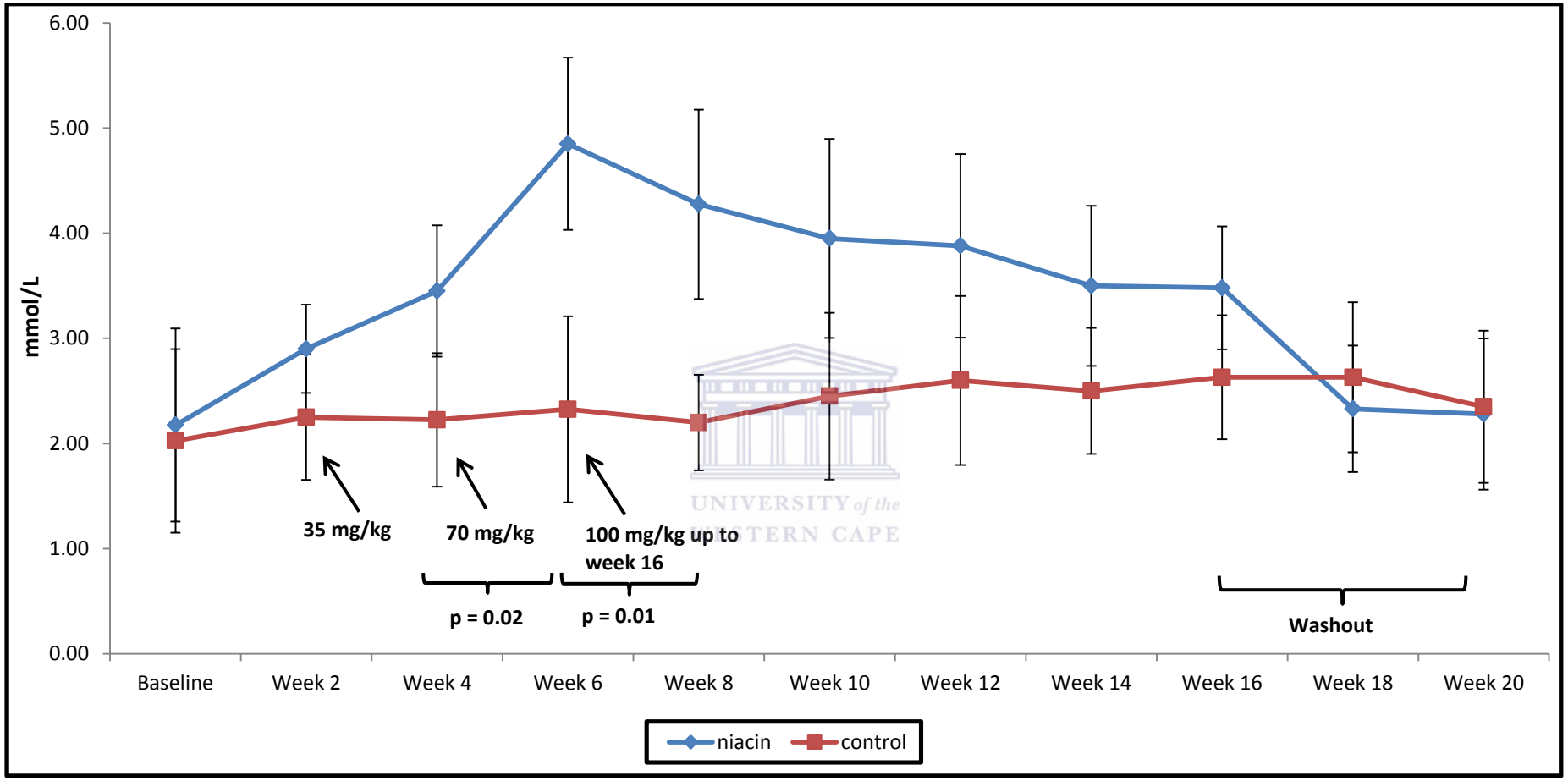

Figure 2.2: HDL-Cholesterol. The treatment schedule was as follows: Treatment group received niacin at 35 mg/kg/d (week 2 to week 3), 70 mg/kg/d (week 4 to week 5), 100 mg/kg/d (week 6 to end of week 16). The control group received the vehicle during the entire study period. Differences between the two groups were statistically significant at week 6 (p = 0.02) and week 8 (p = 0.01).

2.3.1.3 LDL-Cholesterol

LDL decreased in Group 1 (niacin) from baseline to the end of the treatment in week 16 by 39% and in the control Group 2, by 17%. It remained higher than in Group 1 throughout (Figure 2.3). Differences between the two groups were statistically significant at week 6 ($p = 0.04$) (Figure 2.3). The strongest decline in LDL-C occurred early during treatment from baseline (2.55 ± 0.55 mmol/L) to week 4 (1.28 ± 0.46 mmol/L) by 53%. The level of LDL-C, increased thereafter to 2.35 ± 0.52 at week 10 and to 1.58 ± 0.33 at the end of treatment (Figure 2.3).

2.3.1.4 Triglycerides

In Group 1, Triglycerides decreased by 32% from baseline to week 6, increased by 298% to week 10, and then decreased again by 56% to the end of treatment in week 16. Overall, Triglycerides increased by 20% from baseline to the cessation of treatment in Group 1 (niacin). The control displayed a similar pattern with Triglycerides decreasing by 30% from baseline to week 6, increased by 120% to week 8, then declined by 54% to week 16 towards the end of treatment. Triglycerides decreased by 26% in the control group overall (Figure 2.4). Statistically significant differences were not observed between the two groups.

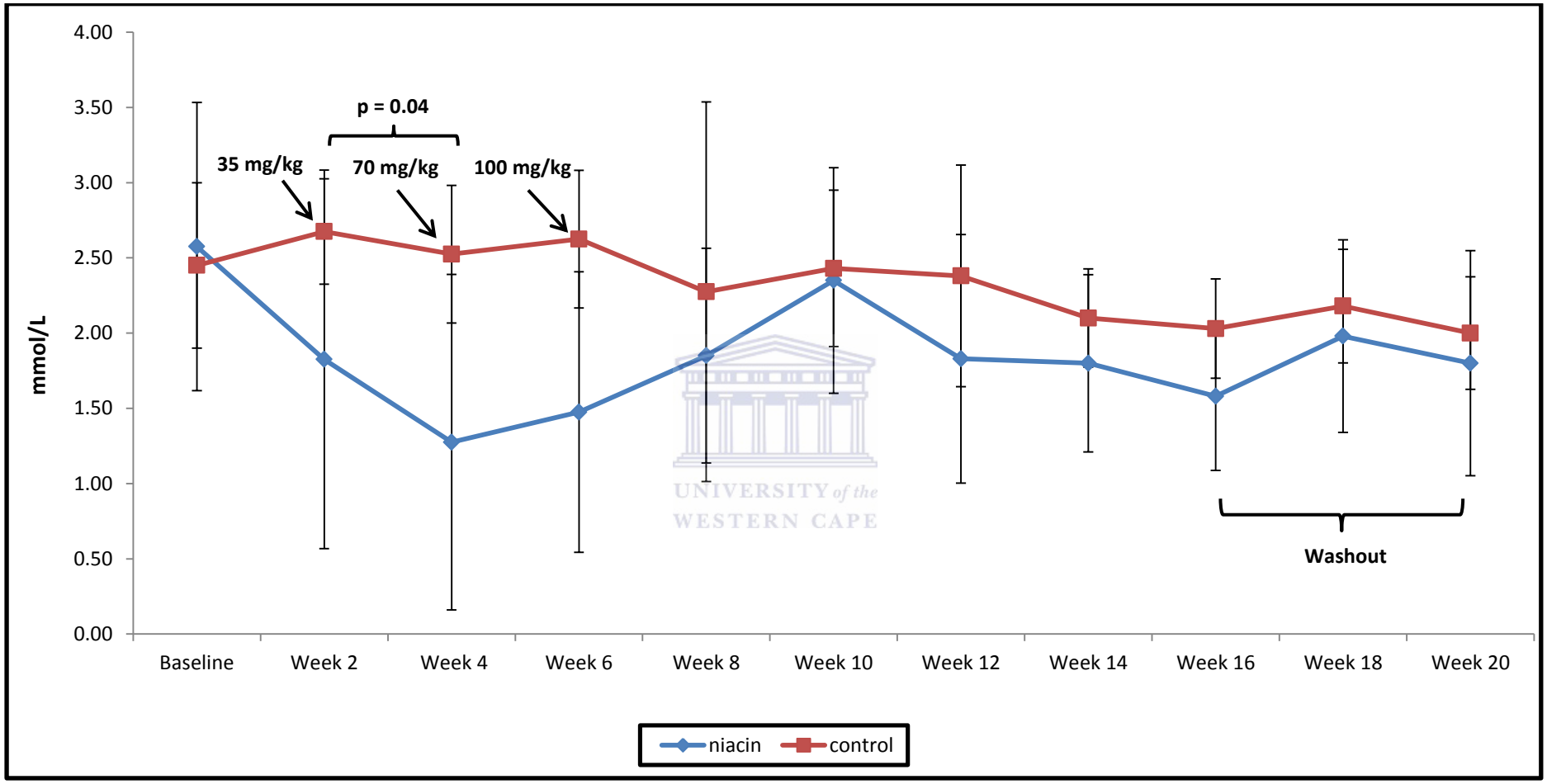

Figure 2.3: LDL-Cholesterol. Treatment schedule was as follows: The treatment group received niacin at 35 mg/kg/d (week 2 to week 3), 70 mg/kg/d (week 4 to week 5), 100 mg/kg/d (week 6 to end of week 16). The control group received the vehicle during the entire study period. Differences between the two groups were statistically significant at week 4 (p = 0.04).

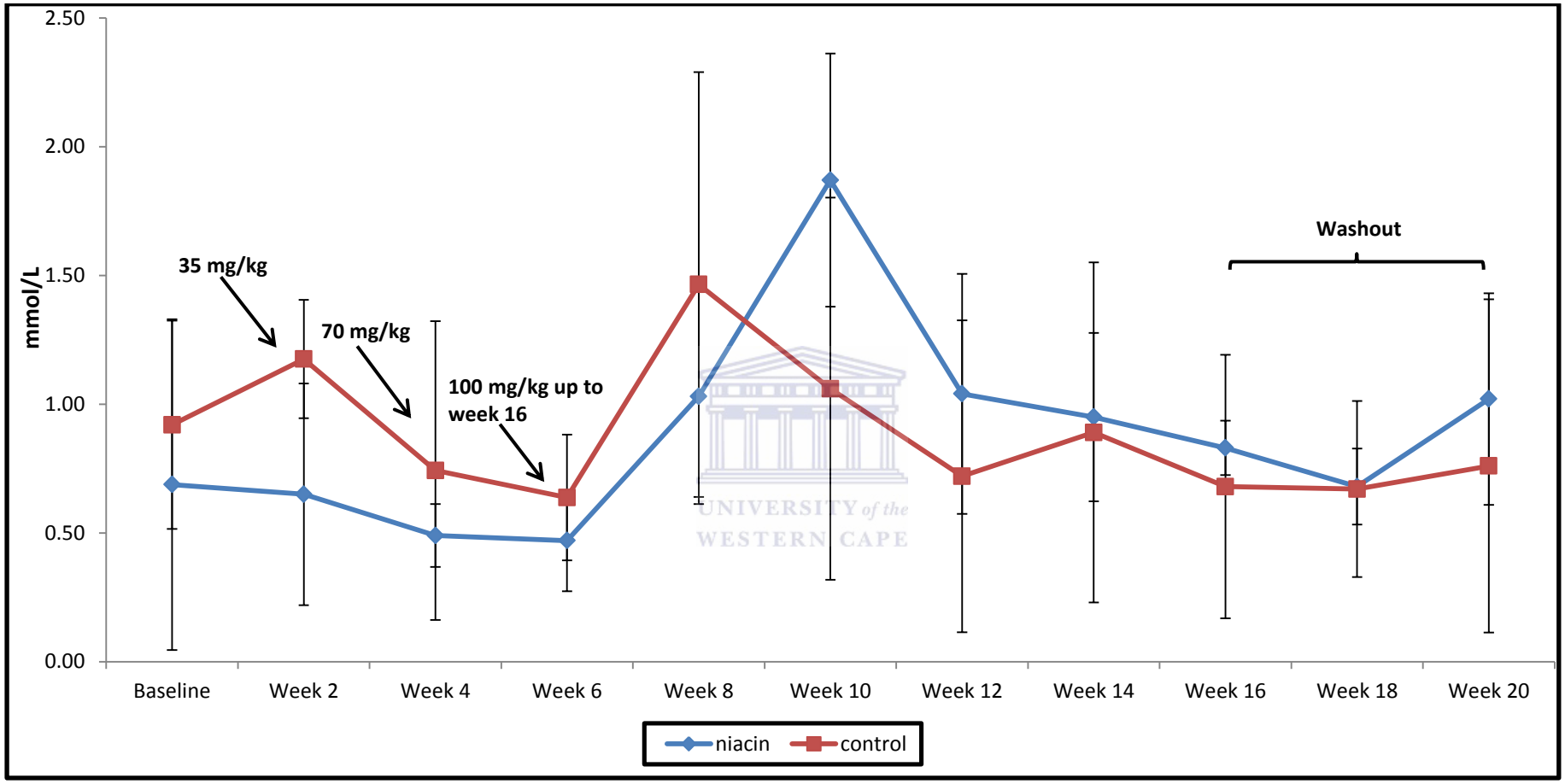

Figure 2.4: Triglycerides. Treatment schedule was as follows: The treatment group received niacin at 35 mg/kg/d (week 2 to week 3), 70 mg/kg/d (week 4 to week 5), 100 mg/kg/d (week 6 to end of week 16). The control group received the vehicle during the entire study period.

2.3.1.5 ApoA-I

ApoA-I increased in groups, 1 and 2 from baseline to the end of treatment in week 16 by 26% and 30% respectively. It remained higher in the treated than the control group throughout the treatment period (Figure 2.5). Differences between the two groups were statistically significant at week 4 ($p = 0.01$), week 8 ($p = 0.00$), week 10 ($p = 0.01$), week 12 ($p = 0.01$) and week 14 ($p = 0.00$) (Figure 2.5). The level of apoA-1 in the control group remained relatively constant throughout the study as compared to the experimental group, which increased during treatment phase (Figure 2.5). The strongest increase in apoA-I occurred early during treatment from baseline (1.99 ± 0.37 gl/L) to week 14 (3.10 ± 0.27 gl/L) by 50%, but declined from week 14, two weeks before the washout. Niacin appeared to have increased apoA-I, however the same effect was not sustained throughout the treatment period (Figure 2.5).

2.3.1.6 Bodyweight

There were no treatment related changes (Figure 2.6).

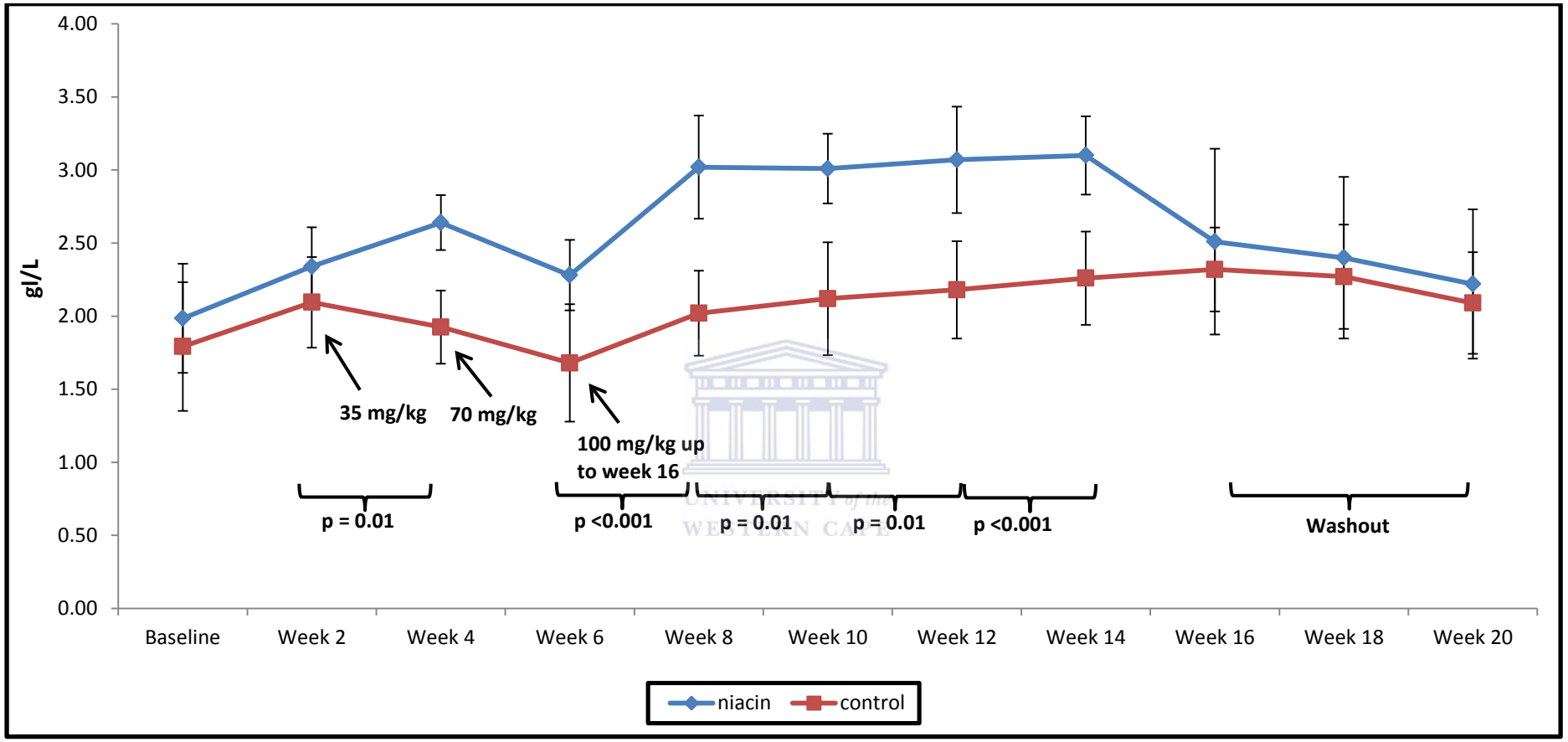

Figure 2.5: ApoA-1. Treatment schedule was as follows: The treatment group received niacin at 35 mg/kg/d (week 2 to week 3), 70 mg/kg/d (week 4 to week 5), 100 mg/kg/d (week 6 to end of week 16). The control group received the vehicle during the entire study period. Differences between the two groups were statistically significant at week 4 ($p = 0.01$), week 8 ($p < 0.001$), week 10 ($p = 0.01$), week 12 ($p = 0.01$) and week 14 ($p < 0.001$).

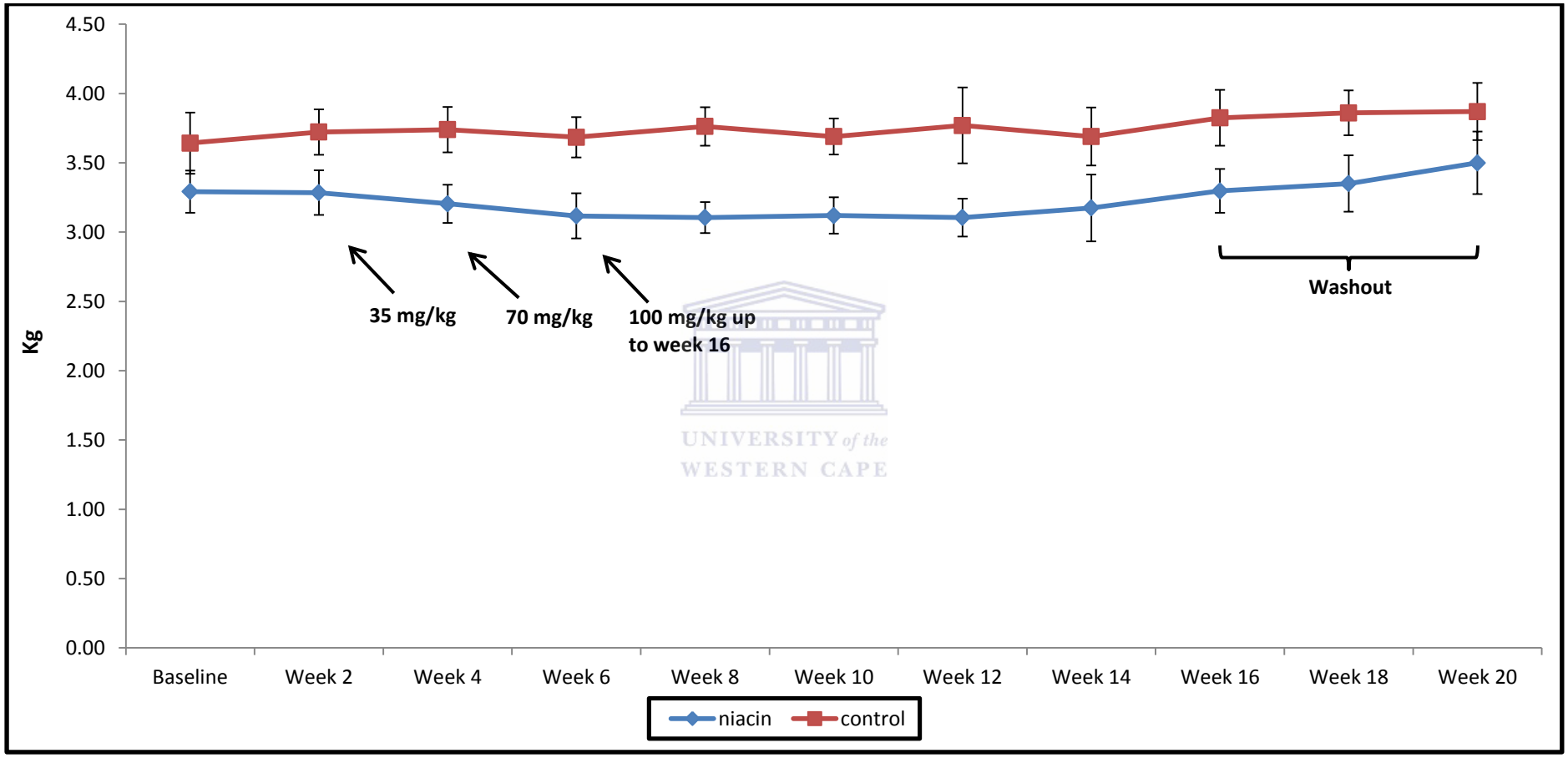

Figure 2.6: Bodyweight. Treatment schedule was as follows: The treatment group received niacin at 35 mg/kg/d (week 2 to week 3), 70 mg/kg/d (week 4 to week 5), 100 mg/kg/d (week 6 to end of week 16). The control group received the vehicle during the entire study period.

2.3.1.7 Summary

The level of total cholesterol, HDL-C, LDL-C and apoA-I in the control group (Figure 2.7A) remained relatively constant throughout the study as compared to the experimental group (Figure 2.7B), which changed during niacin treatment. Before the washout period, HDL-C, LDL-C and apoA-I concentrations in the treated group were 3.61 ± 0.20, 1.84 ± 0.22 mmol/L and 2.66. ± 0.21 gl/L, respectively (Table 2.5). HDL-C and apoA-I levels increased whereas LDL-C decreased during the treatment period ($P < 0.05$ for all) (Figure 2.8). Mean of all values were calculated and statistically significant differences between treated and control groups were obtained except for triglycerides (Table 2.5, Figure 2.8).

Table 2.5: Total Cholesterol, HDL-C, LDL-C, apoA-I and triglyceride concentrations at the end of treatment

	Control	Niacin	P-value
T Chol (mmol/L; mean ± SEM)	5.24 ± 0.74	6.28 ± 0.26	0.001
HDL-C(mmol/L; mean ± SEM)	2.36 ± 0.77	3.61 ± 0.20	0.001
LDL-C(mmol/L; mean ± SEM)	2.39 ± 0.41	1.84 ± 0.22	0.003
Trig(mmol/L; mean ± SEM)	0.92 ± 0.42	0.89 ± 0.27	0.850
apoA-I(gl/L; mean ± SEM)	2.04 ± 0.41	2.66 ± 0.21	0.001

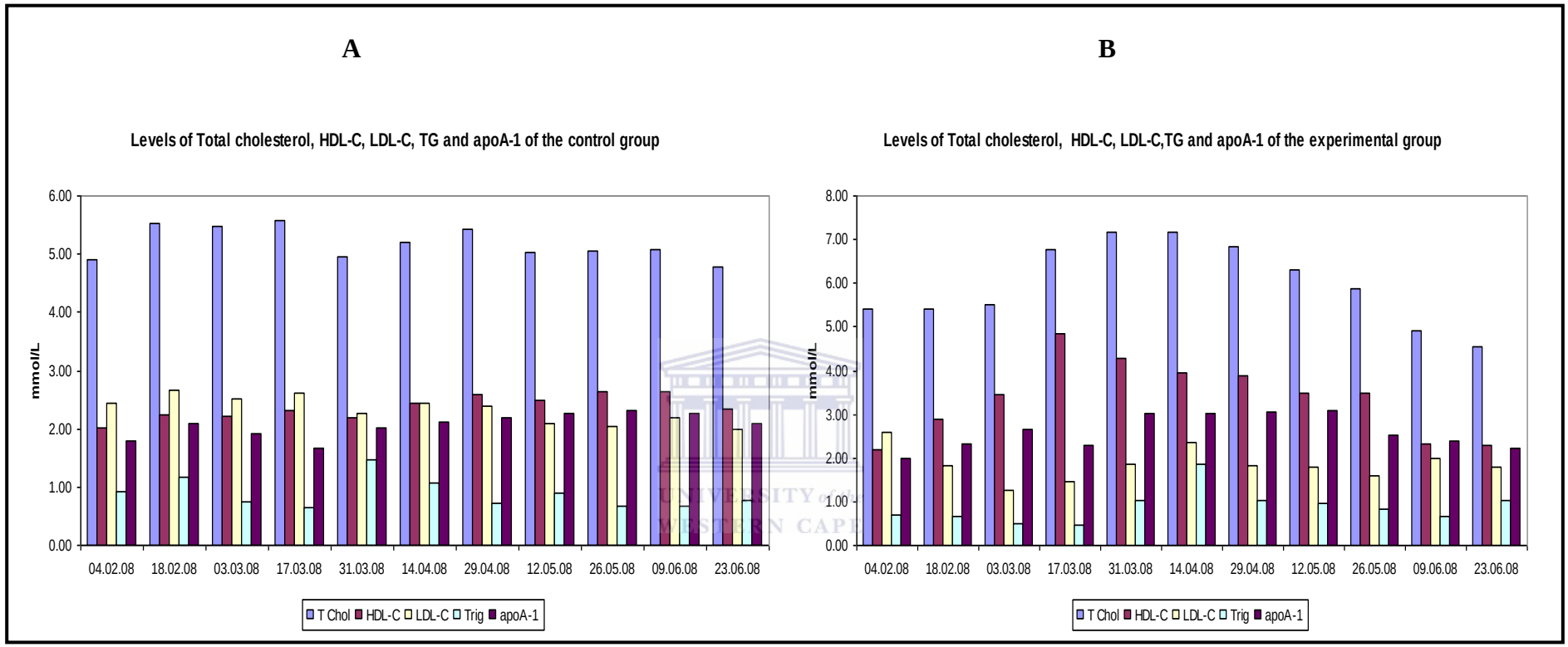

Figure 2.7: Levels of TC, HDL-C, LDL-C, TG and apoA-1 throughout the 4 months study period. A: Control group, B: Experimental group. The treatment schedule was as follows: The treatment group received niacin at 35 mg/kg/d (week 2 to week 3), 70 mg/kg/d (week 4 to week 5), 100 mg/kg/d (week 6 to end of week 16). The control group received the vehicle during the entire study period.

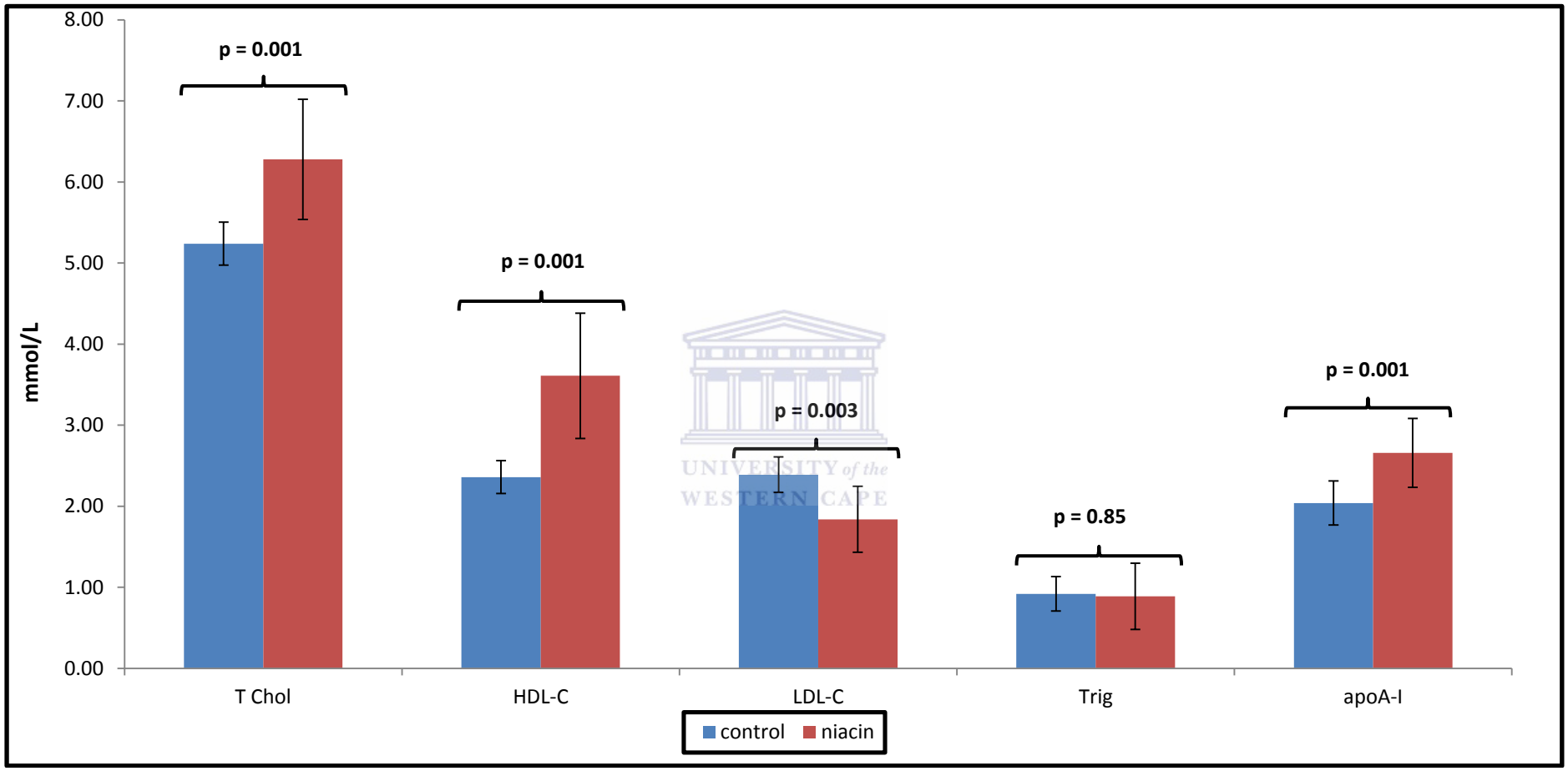

Figure 2.8: Levels of TC, HDL-C, LDL-C, TG and apoA-1 between control and experimental groups during the treatment period. Niacin treatment was associated with reduction in LDL-C, up-regulation of HDL-C synthesis and increase of apoA-1 levels. Data were expressed as mean of all values.

2.3.1.8 Food intake

2.3.1.8.1 Treated food bolus

Group 1 consumed on average 1.5% less than the controls (Figure 2.9). Fluctuations in food consumption were of higher magnitudes in Group 1 when compared to the controls (Figure 2.10).

2.3.1.8.2 Bulk food

The total average consumption of the bulk food bolus was similar for the controls and the treated individuals (Figure 2.11), and fluctuations were minimal (Figure 2.12).

2.3.1.9 Other clinical observations

No sign indicative of unwell-being or distress could be observed throughout the study. No symptoms as defined in our observation logs (Appendix I) could be observed. Motor function and behaviour were normal.

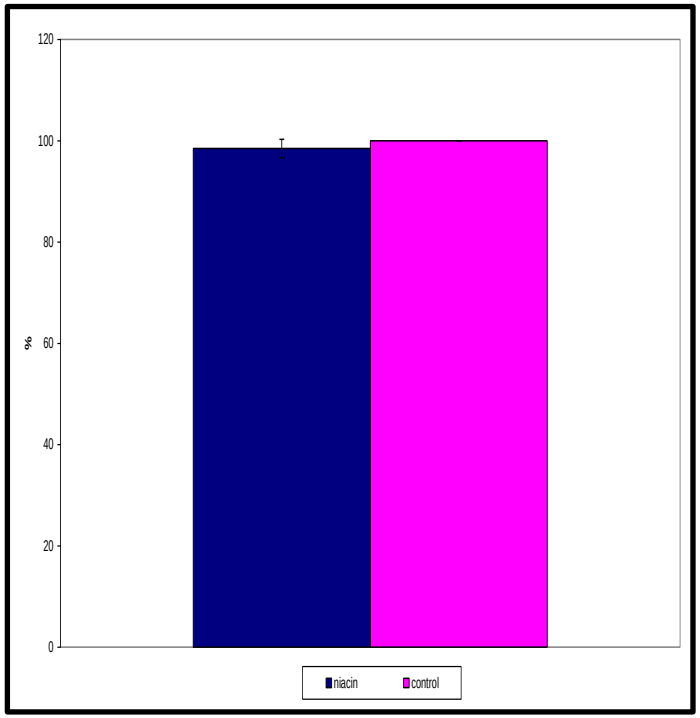

Figure 2.9: Total average treated food consumption.

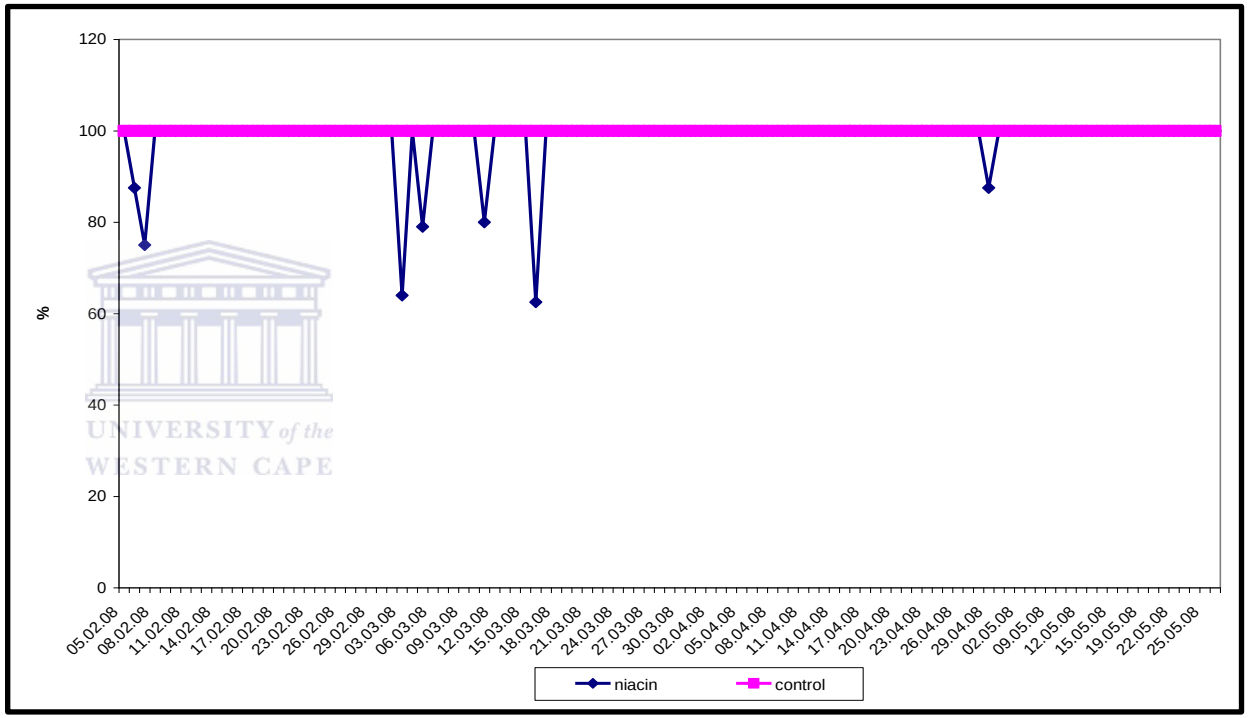

Figure 2.10: Fluctuations in the consumption of treated food.

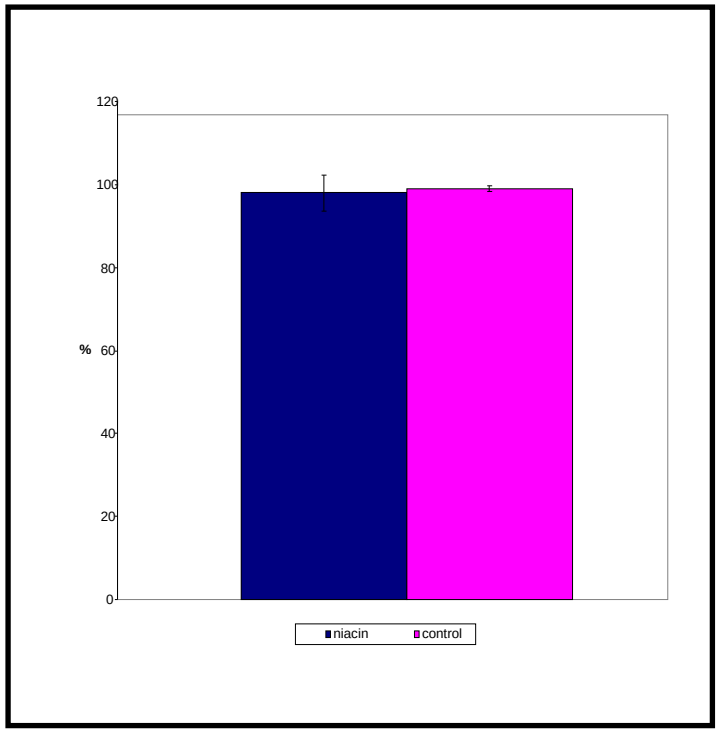

Figure 2.11: Total average consumption of bulk food.

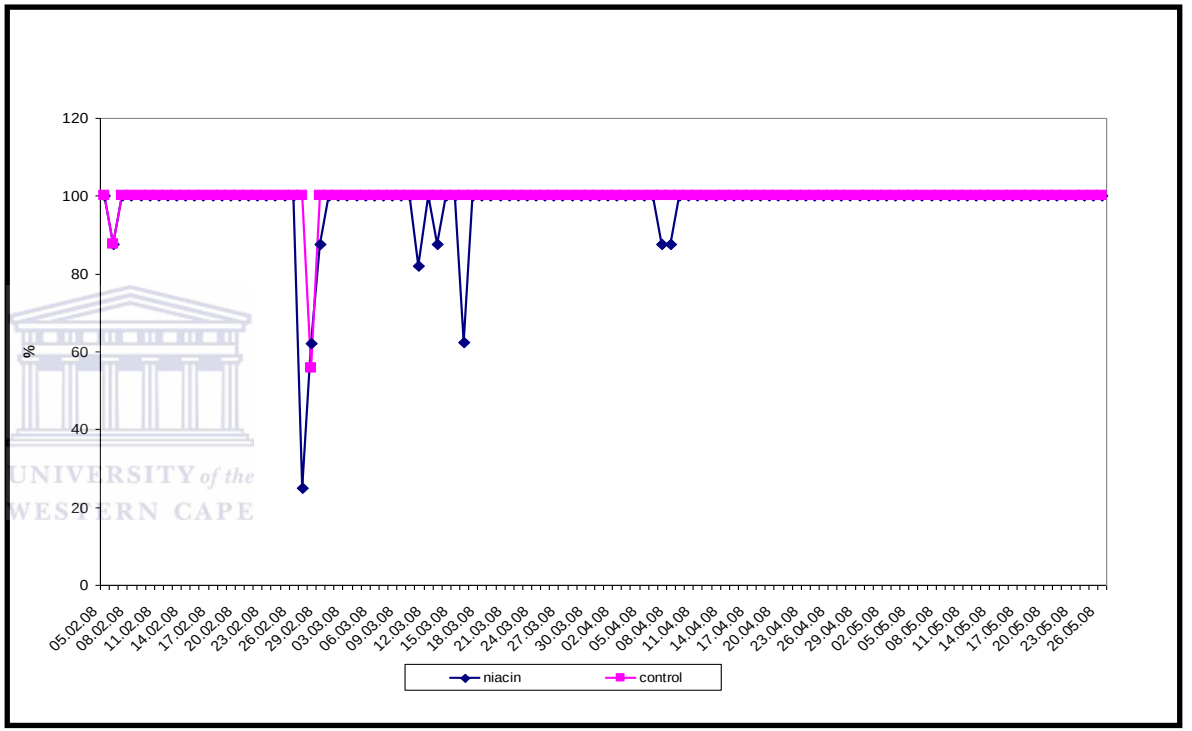

Figure 2.12: Fluctuations in the consumption of bulk food.

2.4 DISCUSSION

In this study, the efficacy of a low dose of niacin was evaluated in the African green monkey over a period of four months. High-density lipoprotein (HDL) cholesterol increased significantly from the beginning of treatment to week 6 and the LDL cholesterol ratio decreased significantly during the same period of treatment. The concentration of HDL-C peaked at week 6, and increased at this stage by about 122% from its own baseline and the control group, although this was not sustained at this level during the remainder of the treatment period. However, differences of between 42 and 55% from baselines and controls were maintained for 12 weeks (between weeks 4 and 16) of the treatment. Considering that a HDL-C increase of 37% would be considered clinically significant in the treatment of dyslipidaemia, the niacin treatment was therefore effected satisfactory changes to further investigate the underlying genetic mechanisms.

Niacin treatment was associated with a reduction in LDL-C, up-regulation of HDL-C synthesis and increase of apoA-I levels when compared to the control group, and statistically significant differences were observed between the two groups illustrated in Figure 2.8. Even though this high level of HDL-C was not sustained throughout the entire intervention period, it remained higher than in the control group until the end of the treatment period. Considerable changes in HDL-C, LDL-C and apoA-I concentrations were observed specifically in week 6 of treatment and this may suggest a change that may be taking place at a cellular level to modify or reduce the effect of niacin when administered at a dose higher than 70 mg/kg in the African green monkey. Further investigation is required at a molecular level to assess and identify

genetic determinants that may be involved in regulating this process, particularly at this point.

Significant changes in triglycerides were not observed since similar patterns were obtained for both treated and control groups.

CONCLUSION

For the purpose of this study, niacin was validated for use as a tool to effect changes in lipid metabolism in preparation for investigating the genetic dynamics of the African green monkey during therapeutic intervention. Results obtained from this study showed that niacin treatment was associated with a reduction in LDL-Cholesterol and a less significant decline in triglycerides. Niacin up-regulated HDL synthesis and also increase apoA-1 levels in the African green monkeys.

Although niacin treatment has been associated with several side effects in humans, the treatment in this study was free of any observable side effects and no sign indicative of unwell-being or distress could be seen throughout Motor function and behaviour were also normal.

EFFECTS OF GENETIC VARIATIONS ON HDL METABOLISM AND REVERSE CHOLESTEROL TRANSPORT PATHWAY IN A NONHUMAN PRIMATE MODEL OF ATHEROSCLEROSIS

3.1 INTRODUCTION

Cardiac and vascular complications are complex multifactorial pathologies, in which both genetic and environmental factors are implicated, thus making them very difficult to prevent. The use of animal models of cardiovascular disease has contributed to increase our knowledge and provided an important insight into the genetic basis of human cardiovascular diseases. This has led to new approaches focused to improve the diagnosis and the treatment of these pathologies.

The availability of genetically modified mouse strains has enabled the elucidation of new pathways involved in the development of cardiovascular disease (Vilahur et al., 2011). However, although their usefulness in uncovering specific gene functions is overwhelming, their utility to extrapolate the findings to human disease or as preclinical models to prove validity pharmacologic agents is less appealing. Furthermore, it has been questioned whether these models are reliable, since compensating mechanisms and redundancies may affect their atherothrombotic phenotype (Moghadasian et al., 2001). In contrast, the development and implementation of animal models of atherosclerosis and thrombosis, has provided valuable tools for the discovery of a number of compounds that, in fact, are now successfully being used for the treatment and prevention of the atherothrombotic diseases (Vilahur et al., 2011).

Rats, rabbits, dogs, pigs, and monkeys are well-established animal models of atherosclerosis. Because of similarities between human and nonhuman primates (Moghadasian et al., 2001), nonhuman primate animal models are believed to be better suited to investigate human cardiovascular pathology. In 1965 Malinov and Maruffo published studies performed using the monkey as animal model to evaluate aortic atherosclerosis (Vilahur et al., 2011) and familial LDL receptor deficiency with atherosclerosis has been reported in rhesus monkey (Kusum et al., 1993).

In addition to shared physiological properties, nonhuman primates and humans possess similar genes of lipid metabolism with similar linkage relationships (Hixson et al.,1990). Nonhuman primate models with mutations affecting various aspects of lipid metabolism have been valuable in the study of the genetic and biochemical basis of similar disorders in human (Vilahur et al., 2011). However, the objective of most animal studies is not to duplicate precisely genetic variations that occur in humans, but rather to understand better the biochemical mechanisms and types of polymorphisms that underlie heritable variations of lipoproteins. Thus, animal models provide a means for defining in detail the molecular mechanisms involved in lipid transport (Reue et al., 1990).

Most research in the field of lipid metabolism is motivated by a desire to understand normal lipid transport in the body and the consequences of abnormalities related to several human diseases, most commonly atherosclerosis (Reue et al., 1990). There are more than 54 genes that code for proteins that directly control lipid metabolism, 10 apolipoproteins that control lipoprotein synthesis, four processing proteins (LPL, HL, LCAT and CETP) and there are at least three receptors (LDL, chylomicron and scavenger

receptors) (Klos et al., 2006). Since 1982, the genes for all of these proteins have been isolated, sequenced and mapped in the human genome (Reue et al., 1990). The exact role of those genes and their proteins in lipid metabolism and lipid transport can only be inferred by physiological studies that perturb the human or animal and affect the level or function of each. To determine their role more precisely, primary genetic alterations must exist that affect the amount or quality of these proteins (Klos et al., 2006).

The application of molecular genetics to inherited cardiovascular disorders has been very successful, especially in the field of single gene disorders. Low levels of HDL-C are a major independent risk factor for atherosclerotic cardiovascular disease and events; and the high prevalence of heart disease is related primarily to CAD. Genetics is known to account for more than 50% of the risk of CAD (Roberts et al., 2007). Genetic screening and early prevention in individuals identified as being at increased risk could dramatically reduce the prevalence of atherosclerotic cardiovascular disease and events, thus necessitating the identification of genes predisposing to these diseases.

Although existing drugs have modest effects on HDL-C levels, this area remains a major unmet medical need in cardiovascular medicine (Duffy et al., 2006). HDL metabolism is exceedingly complex and the protective ability of HDL may relate to the flux of cholesterol through the RCT pathway and to other aspects of HDL functionality.

As with most areas of human biology, studies of human cardiovascular diseases have been enriched and complemented by investigations of animal models. There are, of cause, differences between species that must be considered such as quantitative differences, different biochemical pathways, and different developmental pathways. However, certain

pathological processes, such as atherosclerosis, may occur at similar absolute rates in two species instead of being related to the life span (Reue et al., 1990). Among the nonhuman primates, the African green monkey has been validated to be an excellent animal model for the study of atherosclerosis; and it is pathophysiologically and genetically similar to humans (Moghadasian et al., 2001). This similarity makes the African green monkey particularly useful as an animal model for therapeutic intervention of cardiovascular diseases.

In spite of the comprehensive nature of the studies on cholesterol metabolism in this species, few data are available on the African green monkey's genetics. To date, there is little sequence information on the GenBank database for the African green monkey. Given the importance of this animal model in lipid metabolism research and the fundamental significance of sequence data, it is critical that this information gap be addressed.

The recent focus on HDL-based therapies, presented an opportunity to study the protective action of HDL, its role in the reverse cholesterol transport (RCT) pathway and the expression profile of genes regulating HDL metabolism in the African green monkey. Results obtained from this study will be used as a reference for human studies. Cross-species amplification and genotyping were used to identify SNPs in closely related nonhuman primate species, the African green monkey and humans. Cross-species amplification uses known polymorphisms identified in one species to find potential polymorphic sites in closely related species known as coincident SNPs (cSNPs) (Hodgkinson et al., 2009; Laurent, 2009 and Malhi et al., 2011). A SNP is a polymorphism that occurs within a species and only needs to be present in 1% of a population (Houghton et al., 2006) while a cSNP is defined as a polymorphism that

occurs at the same locus in multiple species (Laurent, 2009). Coincident SNPs are either due to identity by descent (IBD) or identity by state (IBS) (Malhi et al., 2011). For cSNPs that result from IBD, balancing selection near genes may maintain polymorphism in different species. Likewise, cSNPs due to independent mutations would result in IBS and might be expected to occur near CpG dinucleotides and CpG islands at the 5' end of genes (Lloyd et al., 2005 and Hodgkinson et al., 2009).

In this study, SNP genotyping assays developed for humans were used to identify cSNPs in African green monkeys. First, a literature search was conducted to look for previously identified genes and polymorphisms involved in lipid metabolism. These genes were selected for further analyses based upon literature evidence. Those which have been previously implicated in lipid metabolism, and which are the key players in lipid metabolism were selected for further analysis, and were screened for cSNPs previously associated with lipid level changes according to the literature. After careful selection of the genes and prioritized cSNPs, 25 African green monkeys were genotyped and gene expression protocols were standardised. Genotyping protocols including PCR and sequencing were developed and mRNA expression profiles were determined using Real-time PCR in both case and control subjects.

The possible involvement of these prioritized candidate genes and their polymorphisms in cardiovascular disease were assessed by relating them to biochemical factors such as HDL-C and LDL-C, determined in the first study (chapter 2). Drug response (niacin) was correlated to gene expression or single-nucleotide polymorphisms, which is discussed in detail in chapter 4.

3.4 MATERIALS AND METHODS

3.2.1 Identification of candidate genes, sequence variants/polymorphisms and genotyping

Genes were located within the previously published CAD-linked loci by bioinformatic searches of public domain databases, containing annotated genes (NCBI GENBANK), as well as by applying gene prediction programs (ENSEMBL). Previously reported polymorphisms in the prioritized candidate genes were retrieved electronically (NCBI.NIH.NLM.GOV/LOCUSLINK). Potentially new sequence variants were sought by comparing multiple database sequence deposits, and/or by polymerase chain reaction (PCR) and sequencing of specific regions with particular functional significance (thereby increasing the chance of identifying functionally significant polymorphisms). Genotyping protocols were developed, thereafter, the possible involvement of the prioritized candidate susceptibility genes were assessed by analyzing the allelic and genotypic distribution of their polymorphisms in the treated and control groups.

Selection of candidate genes was based on their role in the reverse cholesterol transport process. RCT involves numerous lipid transfer proteins, enzymes, apolipoproteins, and membrane-bound receptors (Fielding et al., 1995). The genes encoding these proteins, as well as genes encoding proteins that regulate their transcription, are candidates for influencing variation in plasma levels of apoA-I, apoB, HDL-C, LDL-C, TC, and TG. Based on a model RCT pathway (von Eckardstein et al., 2001), 10 genes involved in RCT were selected for this study for evaluating the impact of genetic variation on variation in plasma lipid and lipoprotein levels after drug therapy response (Table 3.1).

Table 3.1: Selected candidate genes for RCT

Gene	Symbol	Map position
1. ATP-binding membrane cassette transport protein A	ABCA1	9q31.1
2. Apolipoprotein A-1	apoA-1	11q23.3
3. Apolipoprotein B	apoB	2p24
4. Apolipoprotein C-I	apoC-I	19q13.2
5. Apolipoprotein C-II	apoC-II	19q13.2
6. Apolipoprotein E	apoE	19q13.2
7. Cholesteryl ester transfer protein	CETP	16q13
8. Cholesterol 7 alpha-hydroxylase	CYP7A1	8q11-q12
9. Lecithin-cholesterol acyltransferase	LCAT	16q22.1
10. Scavenger receptor class B member 1	SR-B1	12q24.31

Coincident SNPs were identified from the NCBI dbSNP database in the 10 selected candidate genes (Table 3.1). Twenty two cSNPs were selected for genotyping (Table 3.2). Prioritisation of these cSNPs was based on their function and location within their respective candidate gene and their associated with vascular disease.

Table 3.2: Genotyping for cSNPS in the following genes: CETP. ABCA1, CYP7A1, apoA-1, apoB, apoE, SR-B1, LCAT, apoC-I and apoC-II (Lloyd et al., 2005; Malhi et al., 2011)

GENES	cSNPs	Position
1. CETP	1. CETPu2/I405V (rs5882) 2. Ala373Pro/CETPu1(rs5880)	Exon 14 (A/G) Exon 12 (C/G)
2. ABCA1	3. A3044G, Ile883Met (rs4149313) 4. G3911C, E1172D (Glu/Asp) (rs33918808) 5. G2706A, V771M (rs2066718) 6. G2868A, V825I (rs4149312) 7. R219K (Lys) (rs2230806)	Exon 18 (T/C) H7 Exon 24 (C/G) R_1 Exon 16 (G/A) 5th trm Exon 17 (G/A) 6th trm Exon 7 (C/T) Ex N-term loop
3. CYP7A1	8. A-278C (rs3808607) 9. Asn233Ser (rs8192874)	Promoter (T/G) Exon 3 (C/T)
4. apoE	10. Cys112Arg (rs429358) – E4 = (334T/C - 472C/T) 11. Arg158Cys (rs7412) – E2 = (334T/C - 472C/T)	Exon 3 (T/C) Exon 3 (C/T)
5. apoA-1	12. *Msp*1;C+83T (rs5069) 13. G-75A (rs670)	5'UTR (G/A) Promoter (G/A)
6. apoB	14. T71I (rs1367117 or rs17246849) 15. 4311S (rs1042034 or rs17240958)	Exon 4 (C/T) Exon 29 (T/C)
7. LCAT	16. Ser232Thr (rs4986970) 17. LCATu3 (rs5923)	Exon 5 (T/A) Exon 6 (C/T)
8. apoC-I	18. *Hpa*I	Promoter (T/A)
9. apoC-II	19. Leu96Arg (rs5167) 20. -62 A>C (*rs2288911*)	Promoter (T/G) Promoter (A/C)
10. SR-B1	21. A350A (rs5888) 22. G2S (rs4238001)	Exon 8 (C/T) Exon 1 (G/A)

Genotypes were obtained from 25 monkeys (Table 3.3) and genotyping was performed using polymerase chain reaction amplification of genomic DNA and sequencing. Due to the small gene pool of the MRC Primate colony, same inbreeding occurred resulting in genetic similarity. Genotype variations were therefore not expected, and results obtained for each cSNP were of the same genotype (Table 3.4).

Since genotype variations between case and control subjects were not obtained, four wild caught monkeys from a different animal unit in Potchefstroom University were included in the study. However, similar results were obtained with no genotypic variations. Therefore, eight monkeys (4 controls/group 1 and 4 case subjects/group 2)

were selected out of the original 25 MRC genotyped monkeys for the intervention study discussed in chapter 2 (Table 2.2).

Table 3.3: Selected individuals for genotyping

Monkey ID	Weight (Kg)	Monkey ID	Weight (Kg)
77	3.92	243	4.04
81	4.04	250	3.16
97	4.59	283	3.09
101	4.34	298	3.84
108	3.78	310	3.80
136	4.24	322	3.24
140	4.04	339	4.02
168	3.10	795	3.82
181	3.96	1088	6.22
186	4.12	1080	4.66
215	3.61	1075	5.12
234	3.92	1109	6.52
240	3.52		

Table 3.4: Genotyping results obtained from 25 African green monkeys

SNPs	Genotypes	Allele frequency
1. CETP:I405V	G/G	Minor
2. CETP:Ala373Pro	G/G	Major
3. ABCA1:Ile883Met	G/G	Minor
4. ABCA1:E1172D	G/G	Minor
5. ABCA1:V771M	G/G	Major
6. ABCA1:V825I	T/T	Minor
7.ABCA1: R219K	C/C	Major
8. CYP7A1:A-278C	T/T	Major
9. CYP7A1:Asn233Ser	G/G	Minor
10. apoE:Cys112Arg – E4 = (334T/C - 472C/T)	C/C	Minor
11. apoE:Arg158Cys– E2 = (334T/C - 472C/T)	T/T	Minor
12. apoA-1:*Msp*1;C+83T	T/T	Minor
13. apoA-1:G-75A	A/A	Minor
14.apoB:T71I	T/T	Minor
15. apoB:4311S	C/C	Major
16.LCAT:Ser232Thr	T/T	Minor
17.LCAT:LCATu3	C/C	Minor
18.apoC-I: *Hpa*I	T/T	Minor
19. apoC-II:Leu96Arg	G/G	Minor
20. apoC-II:-62 A>C	C/C	Major
21. SR-B1:A350A	T/T	Minor
22. SR-B1:G2S	A/A	Minor

3.4.1.1 Standard PCRs for genotyping

In order to amplify target DNA, 0.2 ml thin walled tubes were used in a GeneAmp PCR system 2700 (Applied Biosystem) or Eppendorf Mastercycler gradient thermocycler equipped with a heated lid. Unless otherwise stated, the standard 25 µl-PCR reaction contained the following reagents: 2x PCR Mater Mix (Promega), DNA template (50 ng), 0.5 µl of the upstream and downstream primers were made up to 25 µl with Nuclease-Free water (Table 3.5).

Thermocycling conditions: 95 ºC for 5 min followed by 30 cycles of 95 ºC for 30 sec, X ºC for 30 sec and 72 ºC for 1 min; then an extension period of 5 min at 72 ºC (Table 3.6). X denotes the relevant annealing temperature which was chosen 5 ºC below the assumed primer melting temperatures calculated using the following formula (Tm= [no. of GC] x 4 + [no. of AT] x 2 ºC. The oligonucleotides used in this study are listed in Appendix II, Table A9(1). References with regard to which experiments the primers were implemented is included in the text. A DNA Sequencer (Applied Biosystems ABI3730xl DNA analyser) was used to obtained genotyping results.

Table 3.5: PCR reaction used for genotyping

Reagent (Promega)	**Volume (µl)**	**Final Concentration**
PCR Master Mix, 2X	12.5	1X
Forward primer, 10µM	1.25	0.5 µM
Reverse primer, 10µM	1.25	0.5 µM
DNA template	1	50 ng
Nuclease-Free Water to	25	N.A

Table 3.6: PCR program used for genotyping

Step	Temp (°C)	Time	Cycles
Denaturation	95	5 min	1
Denaturation Annealing Extension	95 Primer dependent 72	30 sec 30 sec 1 min	30
Extension	72	5 min	

3.4.2 Gene Expression analysis

Quantitative real-time PCR (qRT-PCR) is one of the most sensitive and commonly used techniques to study gene expression. In this study SYBR® Green gene expression assays from Applied Biosystems were used. SYBR Green bonded to double-stranded DNA, and upon excitation emitted light. Thus, as a PCR product accumulated, fluorescence increased.

SYBR Green is the most economical choice for real-time PCR product detection. Since the dye bonded to double-stranded DNA, there was no need to design a probe for any particular target to be analyzed. However, detection by SYBR Green required extensive optimization. Since the dye could not distinguish between specific and non-specific product accumulated during PCR, follow up assays were needed to validate results.

3.4.2.1 Isolation and quantification of mRNA cDNA synthesis

The PAXgene Blood RNA Kit (PreAnalytiX, Qiagen) was used to isolate and purify intracellular RNA from whole blood collected in the PAXgene Blood Tube (BRT) from eight monkey subjects (4 cases and 4 controls) during the 10 intervention study

time points (Chapter 2). BRT contained a reagent that protected RNA molecules from degradation by RNases and minimised *ex vivo* changes in gene expression. BRT were intended for the collection of whole blood and stabilisation of cellular RNA for up to 5 days at 2-8 ºC or up to 24 months at -20 ºC or -70 ºC. The procedure was simple and purification began with a centrifugation step to pellet nucleic acids in the BRT. The pellet was washed and resuspended, followed by RNA purification as per manual instructions.

The resuspended pellet was incubated in optimised buffer together with proteinase K to bring about protein digestion. An additional centrifugation through the PAXgene Shreder spin column was carried out to homogenise the cell lysate and remove residual cell debris, and the supernatant of the flow-through fraction was transferred to a fresh microcentrifuge tube. Ethanol was added to adjust binding conditions, and the lysate was applied to a PAXgene RNA spin column. During a brief centrifugation, RNA was selectively bonded to the PAXgene silica membrane as contaminants pass through. Remaining contaminants were removed in several efficient wash steps. Between the first and second wash steps, the membrane was treated with DNase I (TURBO DNA-free Kit, AEC Amersham)) to remove trace amounts of bound DNA. After the wash steps, RNA was eluted in elution buffer and heat-denatured. RNA yields from 2.5 ml whole blood were ≥ 3 μg for ≥95% of samples processed. Since yields were donor-dependent, individual yields varied. RNA purities were checked using OD_{260}/OD_{280} ratio and by confirming integrity using Agilent 2100 bioanalyzer (Agilent technologies, Germany).

3.2.2.1.1 RNA quantification and purity

Nucleic acids (RNA and DNA) absorb at 260 nm (A_{260}), while proteins and other contaminants absorb at 280 nm (A_{280}). Thus, the ratio of 260 nm to 280 nm is used to assess the purity of a sample. A ratio of two is generally accepted as pure for RNA. The ratio of A_{260} to A_{230} is used as a secondary measure of purity, and indicates contaminants that absorb at or near 230 nm.

RNA concentration and purity was determined by measuring the absorbance at $A_{260,}$ A_{280} and A_{230} in a Nanodrop 1000 spectrophotometer. The spectrophotometer was initialised by pipetting 2 µl distilled water onto the pedestal of the spectrophotometer, and blanked with 2 µl RNase free water. Thereafter, 2 µl sample was pipetted onto the pedestal and the absorbance determined. Each sample was read in triplicate.

3.2.2.1.2 Analysis of RNA integrity

In eukaryotes, total RNA is comprised of approximately 80% rRNA, whereas only 1 to 3 % is comprised of mRNA. Traditionally, it is assumed that rRNA quality reflects mRNA quality. Thus, mRNA quality is assessed by visualising the 18S or 28S rRNA peaks; a 28S:18S rRNA ratio of 2 indicative of intact RNA.

RNA integrity was determined with the Agilent 2100 bioanalyser (Agilent technologies, Germany) in conjunction with the RNA 6000 Nano-kit as recommended by the manufacturer. The Agilent 2100 bioanalyzer is an improved analytical tool for total RNA analysis, and offers a number of advantages over agarose gel electrophoresis, the traditional method of assessing RNA quality. The bioanalyser 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. The RIN is a numerical assessment of the integrity of the RNA sample, 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 one to 10, with 10 representing high quality RNA**.** An electrophoregram representative of an intact RNA sample is illustrated in Figure 3.1.

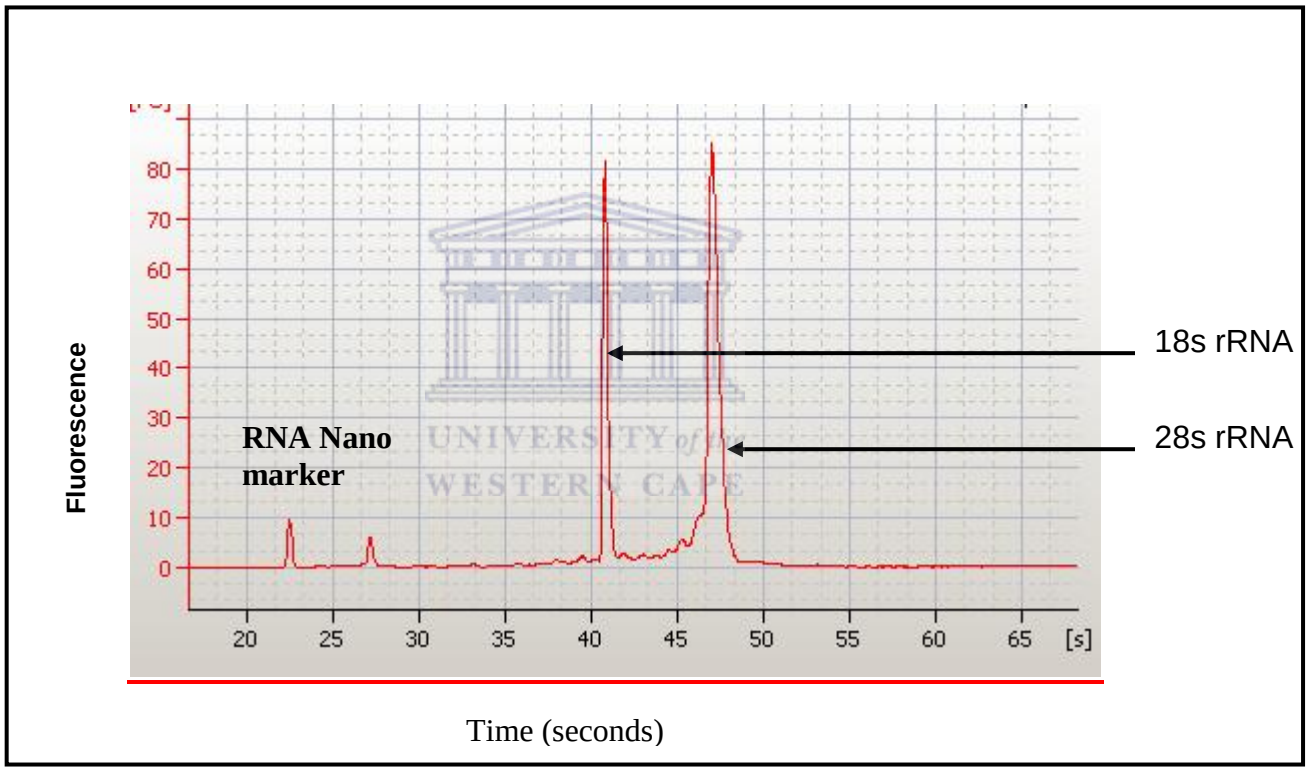

Figure 3.1: **An electrophoretogram of an intact RNA sample.** Distinct 18S and 28S rRNA bands are noted. The marker peak is a control included in the RNA 6000 Nano-kit to correctly align electrophoretograms. Smaller peaks present after the marker may represent 5S and 5.8D subunits, tRNAs and small RNA fragments about 100 bp.

To perform the assay, the RNA 6000 Nano-kit and filtered dye were removed from 4°C and allowed to equilibrate to room temperature for at least 30 minutes. The gel-dye mix was prepared by adding 1 µl of dye to 65 µl of filtered gel, mixed by

vortexing for 10 seconds and then centrifuged at 12 000 g for ten minutes. A RNA 600 Nano chip was placed in the priming station and the well marked dark G was filled with 9 µl of gel-dye mix and primed by depressing the syringe in the chip priming station for 30 seconds. Thereafter, the two wells marked light G were filled with 9 µl of gel-dye mix. Five microlitres of RNA 6000 Nano marker was added to the 12 sample and one ladder well. The RNA ladder and samples (50 to 500 ng/µl) were denatured by placing them at 70°C for two minutes. After brief centrifugation, 1 µl of RNA ladder or RNA sample was pipetted into their respective wells. The chip was vortexed at 2400 g for one minute and then placed in the chamber of the Agilent Bioanalyser which had been decontaminated with RNaseZap and RNase-free water. For decontamination, an electrode cleaner chip containing 350 µl of RNaseZap solution was placed in the bioanalyser for one minute. Thereafter, another electrode cleaner chip, containing 350 µl of RNase-free water was placed in the bioanalyser for 10 seconds, removed and the electrodes allowed to air dry for a further 10 seconds. After selecting the (RNA Eukaryote total RNA Nano series II) programme on the 2100 Agilent Expert software the assay was run.

3.2.2.1.3 DNase treatment

RNA samples were DNase treated to remove contaminating genomic DNA from RNA preparations. For this the TURBO DNase kit was utilized as recommended by the manufacturers. Briefly, 5 µl of 10x DNase buffer and 1.5 µl of DNase was added to 20 µg of RNA and RNase-free water in a total reaction volume of 50 µl. Samples were mixed and incubated at 37°C for 30 minutes, after which another 1.5 µl of DNase was added and incubated at 37°C for a further 30 minutes. The reaction was stopped by adding 10 µl DNase inactivation reagent and mixed by placing tubes on an

orbital shaker for two minutes. Thereafter, the tubes were centrifuged at 10 000 g for 1.5 minutes and the supernatant transferred to a new tube. RNA concentrations were determined using a Nanodrop 1000 spectrophotometer.

3.4.2.2 Reverse Transcription: cDNA synthesis

Total RNA was reverse transcribed into cDNA using the High-Capacity cDNA kit according to the manufacturer's instructions. 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. The RT minus reaction mix tube (negative control) contained the same reaction mix as the RT plus tube, but with the reverse transcription enzyme replaced by water (Table 3.7). After adding the RT plus and RT minus mix components, the prepared reaction mixes were mixed by pipettting and the tubes centrifuged briefly.

Table 3.7: Reaction components for the reverse transcription reaction

Component	Volume (µl)	
	Plus RT	**Minus RT**
1 µg DNase-treated RNA in RNase-free water	10	10
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 volume	20	20

Ten microlitres of plus or minus RT reaction mixes were added to 0.2 ml tubes containing RNA samples. The tube contents were mixed, briefly centrifuged and placed in a 2720 thermal cycler. Reactions were incubated at 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for five seconds to inactivate the reverse transcriptase enzyme. Samples were stored at -20°C until gene expression analysis. The RT minus tube (negative control) was used to calculate the amount of genomic DNA contamination.

3.2.2.1.3 Quantitative Real-time PCR to assess genomic DNA contamination

To assess the extent of genomic DNA contamination in RNA samples, cDNA generated from plus and minus reverse transcription reactions were amplified with exon spanning primers that would amplify both mRNA and genomic DNA. A reaction mix consisting of 12.5 µl SYBR Green mix, 1 µl of 10 µM ActB Forward Primer (400 nM), 1 µl of 10 µM ActB Reverse Primer (400 nM) and H_2O to a final volume of 24 µl was prepared. The reaction mix was scaled up according to the number of test samples. The reaction mix is indicated in Table 3.8.

Twenty four microlitres of reaction mix was aliquoted into the PCR plate, followed by 1 µl (50 ng) of undiluted cDNA (plus or minus RT reactions). The plate was sealed with adhesive film, mixed on a plate shaker for ten minutes and then briefly centrifuged at 3 000 g. The PCR reactions were conducted on the ABI 7500 Sequence Detection System Instrument (Applied Biosystems) using the Absolute Quantification (AQ) Software (SDS V1.4). Universal cycling conditions; 50ºC for two minutes and 95ºC for ten minutes, followed by 40 cycles of 95ºC for 15 seconds and 60ºC for one minute were used. A dissociation curve was added for secondary product detection.

Data was acquired during the extension step (60°C for one minute). After the run, default settings for the threshold cycle (Ct) and baseline were used and Ct values were exported to Microsoft Excel for analysis.

Table 3.8: Reaction mix for quantification of genomic DNA contamination

Component	Volume (µl)	Final Concentration
2x master mix	12.5	1X
ActB Forward primer (10 µM)	1	400 nM
ActB Reverse Primer (10 µM)	1	400 nM
Water	9.5	-
cDNA	1	50 ng
Final volume	25	-

3.4.2.3 Primers design

Twelve QuantiTect Primer Assays (Qiagen) (Table 3.9) were used instead of designed primers since they enable fast and affordable RNA analysis, and eliminate tedious primer design and assay optimization steps. These assays are derived from gene sequences contained in the NCBI Reference Sequence database (www.ncbi.nlm.nih.gov/RefSeq). They are bioinformatically validated, and they detect RNA only, provided that no pseudogenes with high cDNA similarity exist or that the transcript is not derived from a single-exon gene.

Each 10 X QuantiTect Primer Assay contains a mix of forward and reverse primers for a specific target and supplied lyophilized. To reconstitute a tube of 10x QuantiTect Primer Assay a 1.1 ml TE, pH 8.0 was added and mixed by vortexing 4–6 times before being frozen at -20ºC.

Table 3.9: QuantiTech Primer Assay (Qiagen) used in the study

QuantiTech Primer Assay	Assay ID
Cholesteryl ester transfer protein, plasma (CETP)	QT00015344
ATP-binding cassette, sub-family A (ABC1), member 1 (ABCA1)	QT00064869
Cytochrome P450, family 7, subfamily A, polypeptide 1 (CYP7A1)	QT00001085
Apolipoprotein A-I (APOA-1)	QT00015841
Apolipoprotein B (APOB)	QT00020139
Apolipoprotein C-II (APOC-II)	QT00013020
Apolipoprotein E (APOE)	QT00087297
Scavenger receptor class B, member 1 (SCARB1) other names: SR-BI	QT00033488
Lecithin-cholesterol acyltransferase (LCAT)	QT00049336
Apolipoprotein C-I (APOC-1)	QT00018501
Phosphoglycerate kinase 2 (PGK2)	QT00219023
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	QT01192646

3.4.2.4 Generating a Standard Curve

A standard curve was prepared from Total Rat Liver RNA (Ambion) to test the monkey gene expression assays. The reaction mix was scaled up according to the number of samples to be analysed. Nine microlitres of the reaction mix was aliquoted into a well of the PCR plate, followed by 1 µl of a 10-fold dilution series of the standard curve or test samples cDNA. A no-template control (NTC) using water instead of cDNA was used as a negative control in all PCR reactions. All samples were analysed in duplicate. PCR plates were covered with adhesive film and briefly centrifuged. Thereafter, plates were placed in a shaker for 10 minutes and briefly centrifuged at 3 000 g. The PCR reactions were conducted on the ABI 7500 Sequence Detection System Instrument (Applied Biosystems) using universal cycling conditions as described before. Data generated on the ABI 7500 Instrument was analysed with the ABI Standard Quantification (AQ) software (SDS V1.4) using a Ct of 0.1 and a baseline of between 3 and 15 cycles. SYBR® Green gene expression assays, QuantiTech Primer Assay (Qiagen) of Phosphoglycerate kinase 2 (PGK2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 3.9) were used as

endogenous controls to normalize mRNA levels in test samples. Data was normalised to the average of the two endogenous controls.

To generate the standard (or calibration) curve, the threshold cycle for the standard curve reactions was plotted against the fold dilution of the template cDNA on a semi-logarithmic (base 10) plot. The fit to a straight line including the slope and the correlation factor (R^2) was determined. The efficiency and slope obtained for each standard curve was between -3.3 and -3.8.

3.4.2.5 Real-time Quantitative RT-PCR

To determine the yield of the cDNA from the reverse transcription of mRNA, quantitative PCR was used to test various input amounts of RNA for the cDNA yield of different gene targets.

PCR was performed using Power SYBR Green PCR Master Mix kit (Applied Biosystems), 7500 Real-Time PCR System SDS software (Applied Biosystems), optical 96-well plates and optical adhesive covers (Applied Biosystems). The cycling parameters for all genes were the following: hot-start 95 ºC, 15 minutes, 45 cycles of (denaturation 94 ºC, 15 seconds; annealing 56 ºC, 30 seconds; elongation 72 ºC, 30 seconds);final elongation 72 ºC, 5 minutes; melting curve 65-95 ºC.

A 10 µl reaction mixture was prepared in 96-well reaction plates according to Table 3.10. cDNA template(≤5 ng/reaction) was added to the wells containing the reaction mixture. The real-time cycler was then programmed as described above and results

were analysed with the 7500 Real-Time PCR System SDS software (Applied Biosystems).

Table 3.10: Reaction setup for RT-PCR

PCR reaction	**1X (μL)**
2X SYBR Green Mix	5
10X Primer assay	1
cDNA	0.8
Water	3.2
Final volume	**10**

3.4.3 Statistical analysis

Results are presented as means ± SD or as means ± SEM. The association of HDL-C with gene expression was assessed with the use of Pearson's correlation coefficients. Student t-tests (unpaired) were used to assess the effect of pharmacologic intervention (niacin therapy) on gene expression. These changes were compared between the experimental and the control group. All reported p-values are two-tailed, with a p-value < 0.05 indicating statistical significance. Analyses were performed with the use of Minitab software, version 16.2.

3.3 RESULTS

3.3.1 Sequence variants/polymorphisms identification and genotyping

In this chapter an integrated combination of molecular biological and bioinformatic strategies was used to identify reciprocal candidate genes for lipid metabolism in African green monkeys. Literature search was conducted to look for previously reported genes and polymorphisms which are key players in lipid metabolism in humans. Ten genes (CETP. ABCA1, CYP7A1, apoA-1, apoB, apoE, SR-B1, LCAT, apoC-I and apoC-II) were chosen for this study based on their role in the reverse

cholesterol transport process and twenty two cSNPs (reported to play a vital role in RCT) were selected from these genes and genotyped in African green monkeys (Table 3.2). Selection of cSNPs was also prioritized based on their known associated with vascular disease and their function and location within the gene of interest.

Since animal selection for this study was based on results obtained from genotyping, AxyPrep whole blood genomic DNA kit (Axygene Biosc.) was used to isolate DNA from twenty five monkeys. Oligonucleotide primer sets were synthesized and PCR conditions were optimized for all cSNPs (Table 3.6 and Appendix II, Table A9). DNA was amplified and PCR products were sequenced. Genotype variations were not observed due to small gene pool of the MRC Primate colony. Similar results were also obtained from four other wild-caught monkeys acquired from Potchefstroom University. Eight monkeys from the MRC colony were then selected and entered in this study for further analysis. Four monkeys were treated with niacin at an escalating dosage (35, 70 and 100 mg/kg) and the other four were chosen as controls. Their mean lipid-lowering response following drug therapy was analysed, compared to those with the same genotype in a placebo (control) group in chapter 2.

Out of the twenty two cSNPs genotyped in Table 3.2, six cSNPs were identified (Table 3.11). All six cSNPs identified (I405V, I883M, Asn233Ser, cL96R, -62A>C and A350A) have a significant influential role to play in the regulation of reverse cholesterol transport and lipid-lowering drug therapy in both humans and nonhuman primates (Quinet et al., 1991; Lloyd et al., 2005; Isaacs et al., 2007; Nakamoto et al., 2006 and Malhi et al., 2011).

Table 3.11: cSNPs identified in the African green monkeys

Gene	cSNP	Accession number	Chr	Exon	Nucleotide change	Amino acid change	Polarity
CETP	I405V	rs5882	16	14	A/G	I/V	Nonpolar-nonpolar
ABCA1	Ile883Met	rs4149313	9	18	A/G	I/M	Nonpolar-nonpolar
CYP7A1	Asn233Ser	rs8192874	8	3	A/G	N/S	Polar-polar
apoC-II	Leu96Arg -62A>C	rs5167 rs2288911	19	3 Promoter	T/G	L/R	Nonpolar-polar
SR-B1	A350A	rs5888	12	8	C/T	A/A	Nonpolar

3.3.2 Gene expression analysis

3.3.2.1 RNA concentrations and purity

One of the main objectives of this study was to assess the influence of genetic variation on drug response (niacin) by correlating gene expression or single-nucleotide polymorphisms with a drug's efficacy and also to determine the possible involvement of these genes in cholesterol metabolism pathway or reverse cholesterol transport pathway, particularly those identified with cSNPs in our animal model, the African green monkeys.

Gene expression protocols were standardised and mRNA expression profiles were determined using Real-time PCR in both case and control subjects. mRNA was extracted using PAXgene Blood RNA Kit (PreAnalytiX, Qiagen) for all the time points of intervention study described in chapter 2. To remove contaminating genomic DNA, 20 µg of RNA was DNase treated with the TurboDNase kit as described in section 3.2.2.1.3. To assess whether genomic DNA was efficiently removed by DNase treatment, reverse transcription reactions containing (plus RT) or without (minus RT) the reverse transcription enzyme were subjected to quantitative real-time PCR. Taq polymerase, the enzyme responsible for the polymerase chain reaction (PCR), will only amplify double stranded DNA, thus RNA must be reverse

transcribed before being used as a template in PCR. Amplification in minus RT reactions represents genomic DNA contamination. A Ct difference of more than 10 cycles between the plus and minus RT reactions indicate negligible genomic DNA contamination. cDNA prepared from rat liver RNA (Ambion) was included as a positive control, while water was used as a negative control.

RNA integrity was confirmed using Agilent 2100 bioanalyzer (Figure 3.2). Amount and purity of RNA (Table 3.12) were evaluated by Nanodrop 1000 spectrophotometer (Thermo Scientific). cDNA was synthesised with High-Capacity cDNA Reverse Trancsription Kit (Applied Biosystems) in accordance with the manufacturer's recommendations. A standard curve was created and real-time PCR was performed in duplicate for all 10 prioritised genes. As illustrated in Table 3.13, DNase treatment significantly reduced genomic DNA contamination.

Table 3.12: Yield of DNase I treated RNA samples (ng/ul)

Monkey ID	04.02.08	18.02.08	03.03.08	17.03.08	31.03.08	14.04.08	29.04.08	12.05.08	26.05.08	09.06.08
795	4584.8	2369.6	1092.8	3420.0	3084.0	2932.8	4080.0	2100.0	3076.0	5682.4
322	3488.8	3488.8	4797.6	2684.8	3134.4	10871.	7004.0	9062.4	6411.2	14789
339	2466.4	5070.4	4315.2	3580.8	3809.6	7660.8	5742.4	7264.8	4269.6	9602.4
77	7472.8	100.00	5728.8	5701.6	5150.4	9766.4	5681.6	9198.4	6628.0	5392.8
108	2959.2	5962.4	2753.6	4968.8	4800.8	4647.2	1756.0	1656.0	4786.4	884.80
243	2880.0	3421.6	5064.0	8033.6	6002.4	1548.0	6260.8	5671.2	6308.0	4130.4
215	13573	8520.0	8347.2	5515.2	8554.4	3676.0	5508.0	5206.4	2224.0	5919.2
97	5144.8	3956.0	3629.6	5383.2	4352.8	3771.2	1906.4	6796.8	5907.2	2786.4

Table 3.13: Quantitative Real Time PCR analysis of DNase treated RNA

Sample	Ct# (minus RT)	Ct$ (plus RT)	Ct diff†	Ct# (minus RT)	Ct$ (plus RT)	Ct diff†
Time point: 1 (04.02.08)				**Time point: 6 (14.04.08)**		
795	35.16	16.51	21.65	37.21	20.86	16.35
322	35.46	16.82	18.64	37.21	16.35	20.86
339	35.19	17.55	17.64	37.26	15.95	21.31
77	34.53	17	17.53	36.44	16.12	20.31
108	36.5	18.03	18.47	36.15	16.65	19.5
243	35.78	16.51	19.27	36.96	16.18	20.78
215	35.24	17.71	17.53	34.88	18.43	18.43
97	35.7	18.14	17.56	36.06	16.78	19.28
Time point: 2 (02.18.08)				**Time point: 7 (29.04.08)**		
795	35.86	18.54	17.32	37.68	18.25	19.43
322	36.63	18.59	18.03	37.68	18.25	19.42
339	37.69	17.95	19.74	39.82	16.51	23.31
77	36.88	19.35	17.52	38.42	17.16	21.26
108	36.48	17.33	19.15	39.1	21.85	17.25
243	36.4	18.58	17.81	34.83	18.17	16.65
215	36.35	17.6	18.75	36.26	15.92	18.09
97	37.7	17.92	1977	36.48	18.09	20.56
Time point: 3 (03.03.08)				**Time point: 8 (12.05.08)**		
795	35.18	16.45	18.73	39.06	18.42	20.63
322	35.18	18.73	16.45	39.96	19.6	20.36
339	37.39	16.93	20.46	36.15	17.25	18.89
77	37.07	17.21	19.86	33.91	17.58	16.32
108	36.02	19.08	16.94	36.25	18.95	17..29
243	35.41	17.67	17.74	34.32	17.76	16.55
215	35.78	19.26	19.26	33.99	17.53	16.46
97	35.68	17.71	17.96	35.24	18.39	16.84
Time point: 4 (17.03.08)				**Time point: 9 (26.05.08)**		
795	35.01	17.99	17.02	35.51	17.05	18.45
322	35.01	17.02	17.99	37.43	17.22	20.21
339	36.15	16.65	19.5	37.12	17.47	19.64
77	34.37	16.95	17.41	37.29	17.74	19.55
108	36.18	19.59	16.59	36.88	19.35	17.52
243	35.23	16.97	18.26	38.28	17.16	21.11
215	34.2	16.35	16.35	37.71	17.9	19.81
97	37.06	16.8	20.26	>40	17.24	>23
Time point: 5 (31.03.08)				**Time point: 10 (09.06.08)**		
795	35.9	16.99	18.91	39.96	19.6	20.36
322	37.38	16.95	20.43	39	20.45	18.55
339	34.17	16.41	17.76	>40	19.79	>23
77	38.59	16.73	21.86	36.88	19.35	17.52
108	35.57	17.04	18.53	36.25	18.95	17.29
243	37.29	17.74	17.33	19.15	21.03	>23
215	35.26	17.13	18.12	36.35	17.6	19.31
97	35.3	17.51	17.78	35.17	18.19	16.98

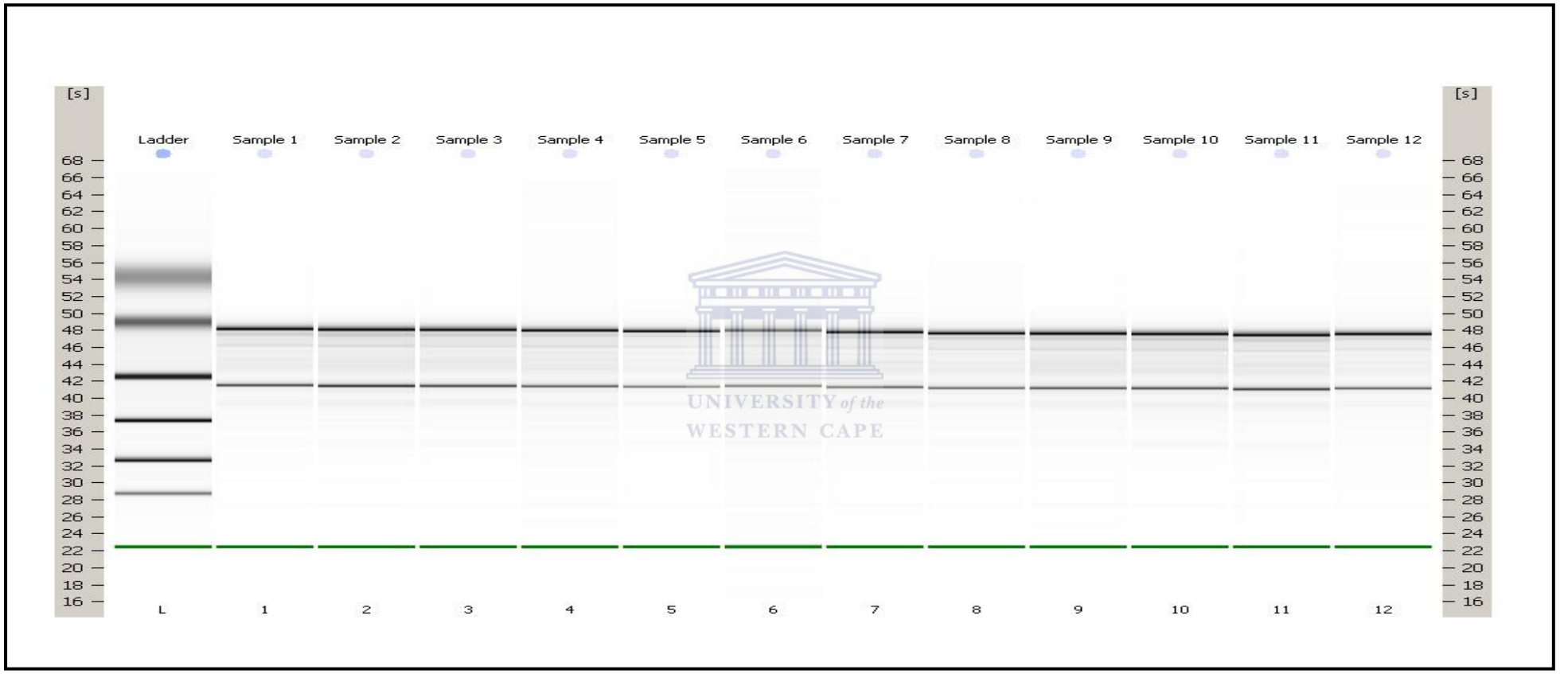

Figure 3.2: mRNA agilent analysis. Results were the same for all 10 time points.

3.5.2.2 PCR efficiency

The slope of a standard curve is used to evaluate the performance of real time PCR reactions and to determine PCR efficiency. Optimal PCR efficiency is indicated by a slope of -3.3. Slopes between -3.1 and -3.6 are acceptable. The slope and R^2 (correlation co-efficient) values obtained for the SYBR® Green gene expression assays used in this study was calculated using a rat cDNA standard curve (Table 3.14). The slopes for all the genes investigated were within the acceptable range.

Table 3.14: Slope and R^2 values of the genes investigated in the study

Gene	Slope	R^2
Cholesteryl ester transfer protein, plasma (CETP)	-3.1	0.99
ATP-binding cassette, sub-family A (ABC1), member 1 (ABCA1)	-3.2	0.99
Cytochrome P450, family 7, subfamily A, polypeptide 1 (CYP7A1)	-3.4	0.99
Apolipoprotein A-I (APOA-1)	-3.3	0.99
Apolipoprotein B (APOB)	-3.1	0.97
Apolipoprotein C-II (APOC-II)	-3.1	0.99
Apolipoprotein E (APOE)	-3.3	0.99
Scavenger receptor class B, member 1 (SCARB1) other names: SR-BI	-3.3	0.99
Lecithin-cholesterol acyltransferase (LCAT)	-3.3	0.98
Apolipoprotein C-I (APOC-1)	-3.2	0.99
Phosphoglycerate kinase 2 (PGK2)	-3.1	0.97
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	-3.2	0.97

3.3.2.3 The effect of niacin treatment on gene expression

The effects of niacin treatment on the expression of the ten prioritized reverse cholesterol transport genes (CETP. ABCA1, CYP7A1, apoA-1, apoB, apoE, SR-B1, LCAT, apoC-I and apoC-II) were determined using quantitative real time PCR (qRT-PCR). The expression of the reference genes PGK2 and GAPDH was used to normalize mRNA levels.

The treatment of niacin affected the expression of genes differently at each time point and this is illustrated in Figures 3.3 to 3.12. Gene expression differences that were statistically significant were only observed in four genes (CETP with $p = 0.04$ at time point 14.04.08, CYP7A1 with $p = 0.04$ at baseline and time point 17.03.08, SR-B1 with $p = 0.03$ at washout (09.06.08) and apoC1 with $p = 0.04$ at time point 14.04.08). Up-regulation of the level of mRNA in the treated group compared to the controls was only seen in SR-B1 from the time point when niacin treatment was maintained at 100 mg/kg, however statistically significant differences were not obtained throughout the treatment phase (Figure 3.6). mRNA expression level of CETP, ABCA1, LCAT, apoC-I, CYP7A1 and apoE in the treated group compared to the controls were decreased with increased dosage of niacin leading to a downregulation (Figures 3.3, 3.4, 3.5, 3.7and 3.12). ApoB mRNA was only expressed at a very low level in both experimental and control groups after two months of intervention treatment with niacin (Figure 3.8). ApoA-1 and apoC-II were poorly expressed in both experimental and control groups throughout the study (Figure 3.9 and 3.10).

During the intervention phase of this study (described in chapter 2), levels of total cholesterol, HDL-C, LDL-C, triglycerides and apoA-1 were determined at different time

points from baseline to the end of the washout period. The effect of niacin treatment was also determined at genetic level by the expression profile of the selected candidate genes at these time points as mentioned above. A considerable change in the concentrations of HDL-C, LDL-C and apoA-1 was observed at week 6 (time point 17.03.08). A change in gene expression of CYP7A1 mRNA was also observed at this time point with a statistical significant difference of p-value 0.04 (Figure 3.13).

Since the levels of HDL-C are significant determinant of cholesterol efflux capacity a correlation analysis was conducted to determine the relationship between the levels of HDL-C and mRNA expression of the 10 selected RCT candidate genes. HDL-C concentrations showed a strong inverse correlation with CETP, SR-B1, apoA-1, apoB, CYP7A1, apoC-I and apo C-II concentrations ($r = -0.14, -0.27, -0.01, -0.95, -0.30, -0.69, -0.33$; $p < 0.001$) and a positive correlation with ABCA1, LCAT and apoE concentrations ($r = 0.48, 0.16, 0.56$; $p < 0.001$) (Appendix II, Table A10).

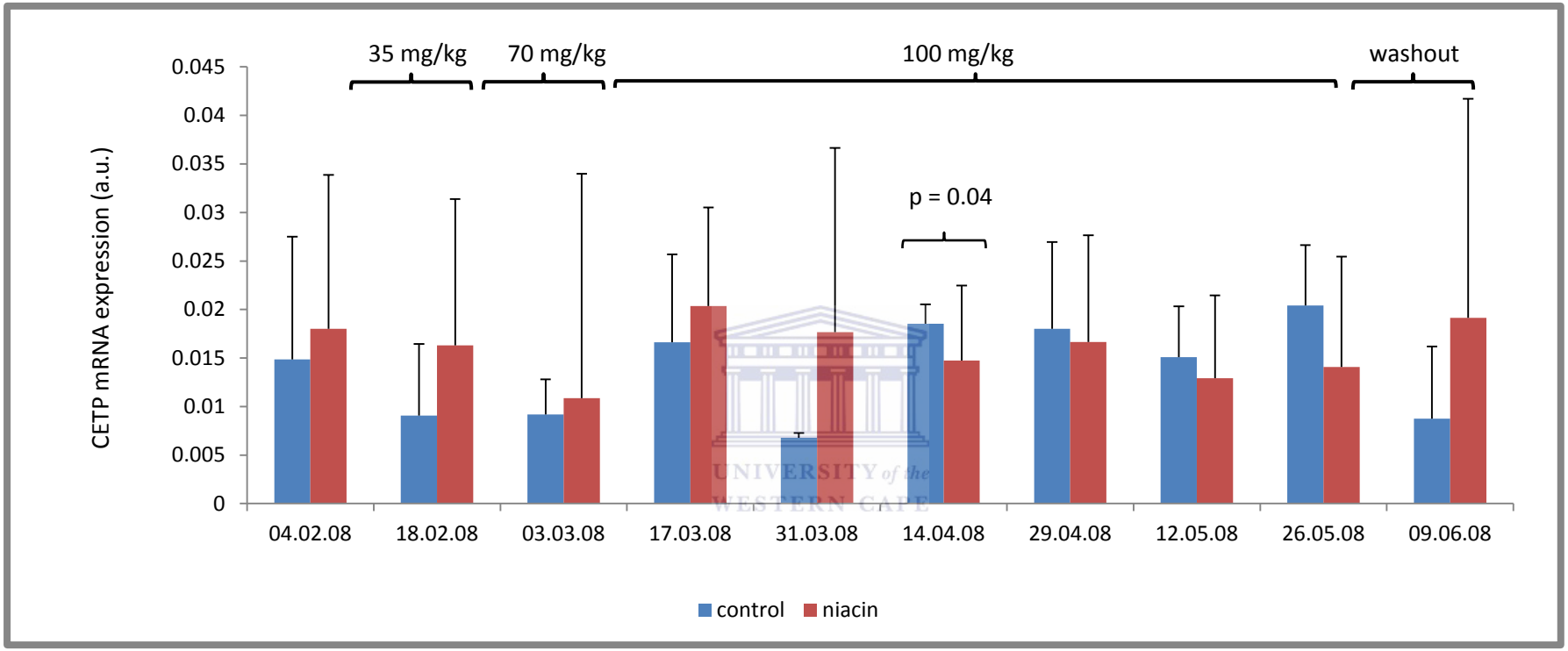

Figure 3.3: The effect of niacin treatment on mRNA expression of CETP in the African green monkey. The experimental group received niacin at an escalating dose (35 to 100 mg/kg) while the control group received a maintenance diet throughout the study period of four months. Messenger RNA levels were determined by quantitative real time PCR for all 10 sampling points of the study period from baseline (04.02.08) to washout (09.06.08). CETP expression decreased when niacin treatment was maintained at a higher dosage and increased again after the washout period. Data were expressed as mean ± SD and mRNA expression. A p-value of 0.04 was calculated at time point 14.04.08 when the treatment of niacin was maintained at 100 mg/kg. P-values for other time points were greater than 0.05, therefore no difference indicated between the groups.

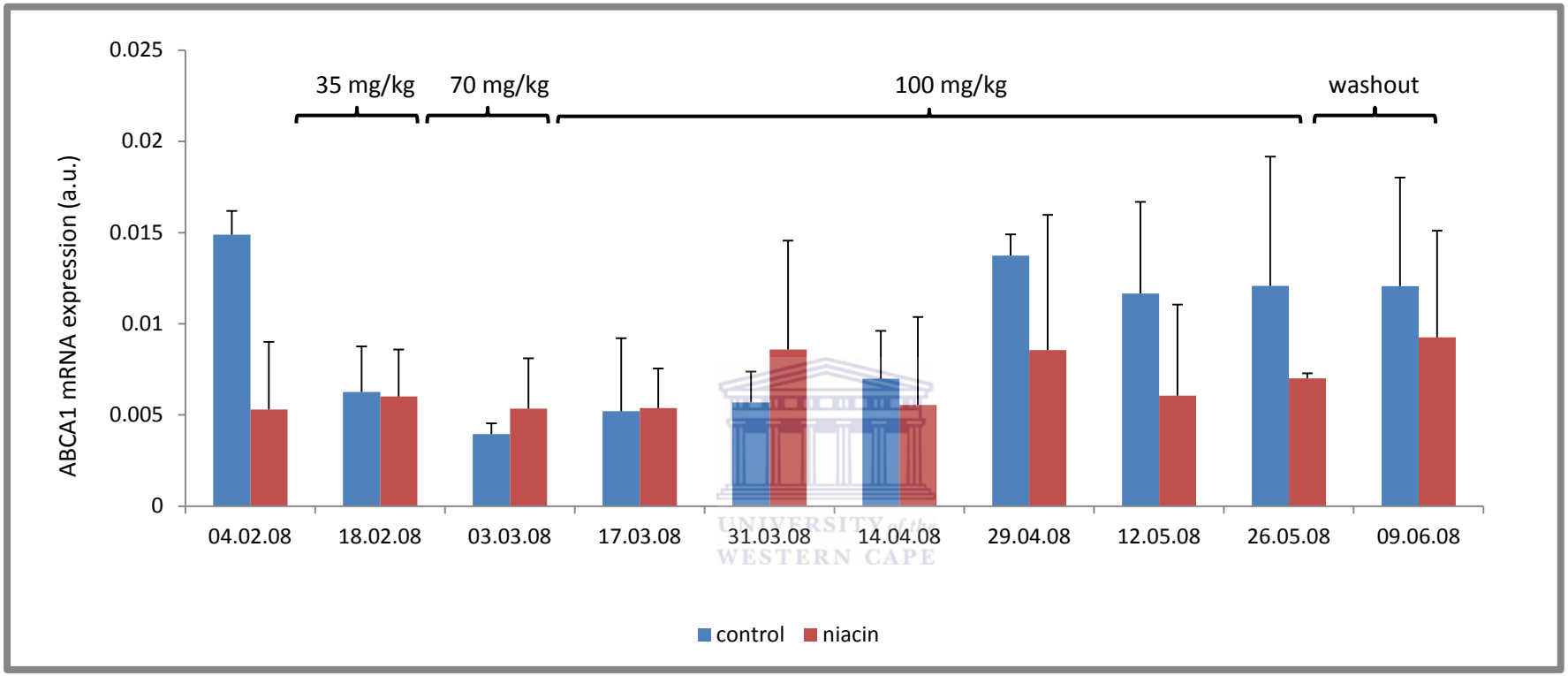

Figure 3.4: The effect of niacin treatment on mRNA expression of ABCA1 in the African green monkey. The experimental group received niacin at an escalating dose (35 to 100 mg/kg) while the control group received a maintenance diet throughout the study period of four months. Messenger RNA levels were determined by quantitative real time PCR for all 10 sampling points of the study period from baseline (04.02.08) to washout (09.06.08). ABCA1 expression of the experimental group decreased with increased dosage of niacin treatment as compared to the control group. Data were expressed as mean ± SD and mRNA expression in a.u. (arbitrary units). P-values for all time points were greater than 0.05, therefore no difference between the groups.

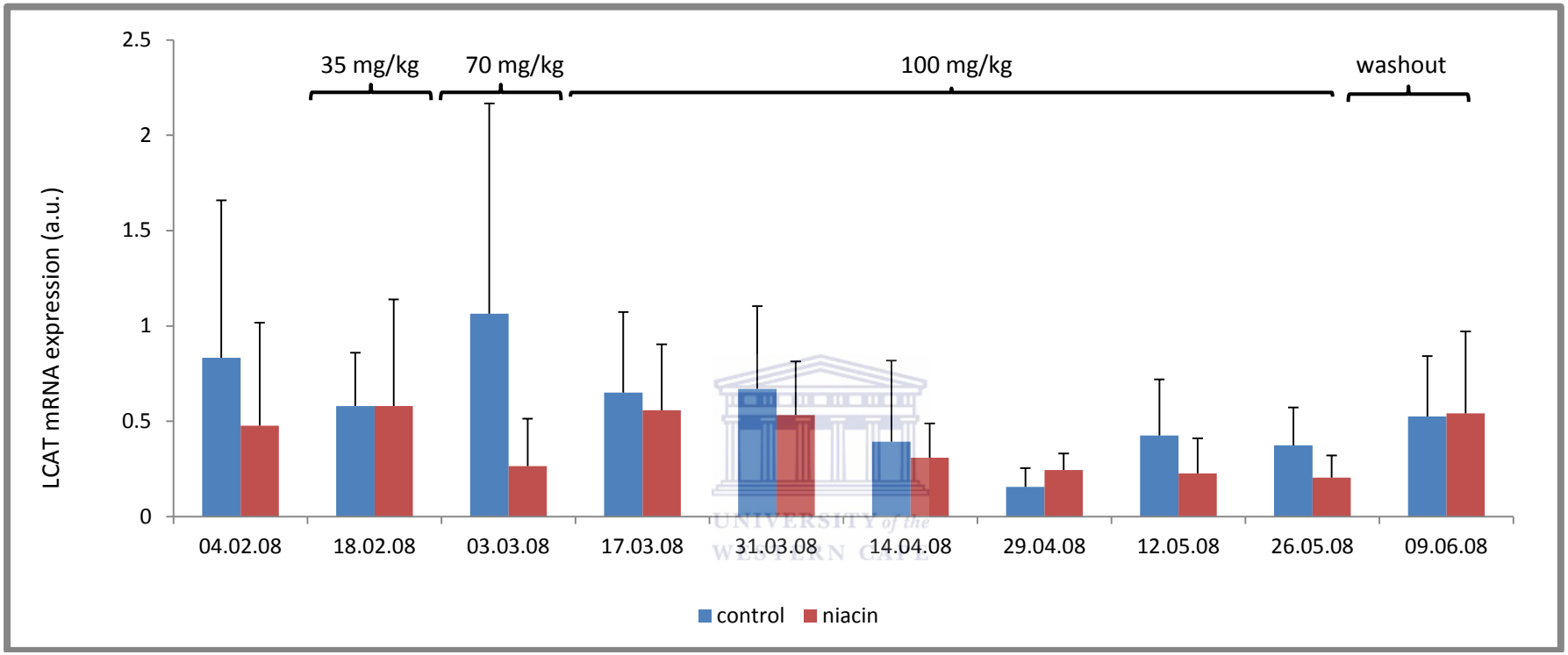

Figure 3.5: The effect of niacin treatment on mRNA expression LCAT in the African green monkey. The experimental group received niacin at an escalating dose (35 to 100 mg/kg) while the control group received a maintenance diet throughout the study period of four months. Messenger RNA levels were determined by quantitative real time PCR for all 10 sampling points of the study period from baseline (04.02.08) to washout (09.06.08). LCAT expression decreased from the time niacin was administered at 70 mg/kg (03.03.08) until the end of treatment (26.05.08). Data were expressed as mean ± SD and mRNA expression in a.u. (arbitrary units). P-values for all time points were greater than 0.05, therefore no difference indicated between the groups.

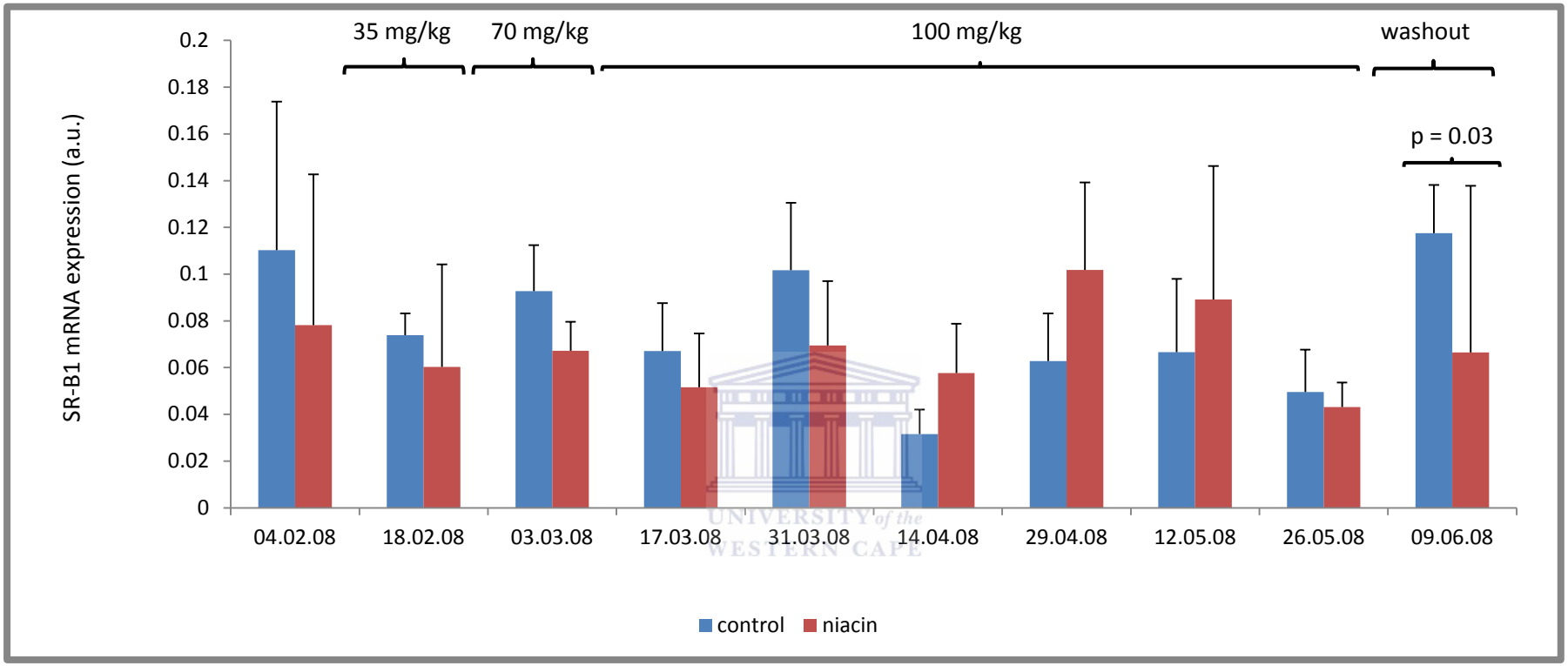

Figure 3.6: The effect of niacin treatment on mRNA expression of SR-B1 in the African green monkey. The experimental group received niacin at an escalating dose (35 to 100 mg/kg) while the control group received a maintenance diet throughout the study period of four months. Messenger RNA levels were determined by quantitative real time PCR for all 10 sampling points of the study period from baseline (04.02.08) to washout (09.06.08). SR-B1 expression of the experimental group increased as compared to the controls from the time niacin was maintained at 100 mg/kg (14.04.08). Data were expressed as mean ± SD and mRNA expression in a.u. (arbitrary units). A p-value of 0.03 was calculated at washout. P-values for other time points were greater than 0.05, therefore no difference indicated between the groups.

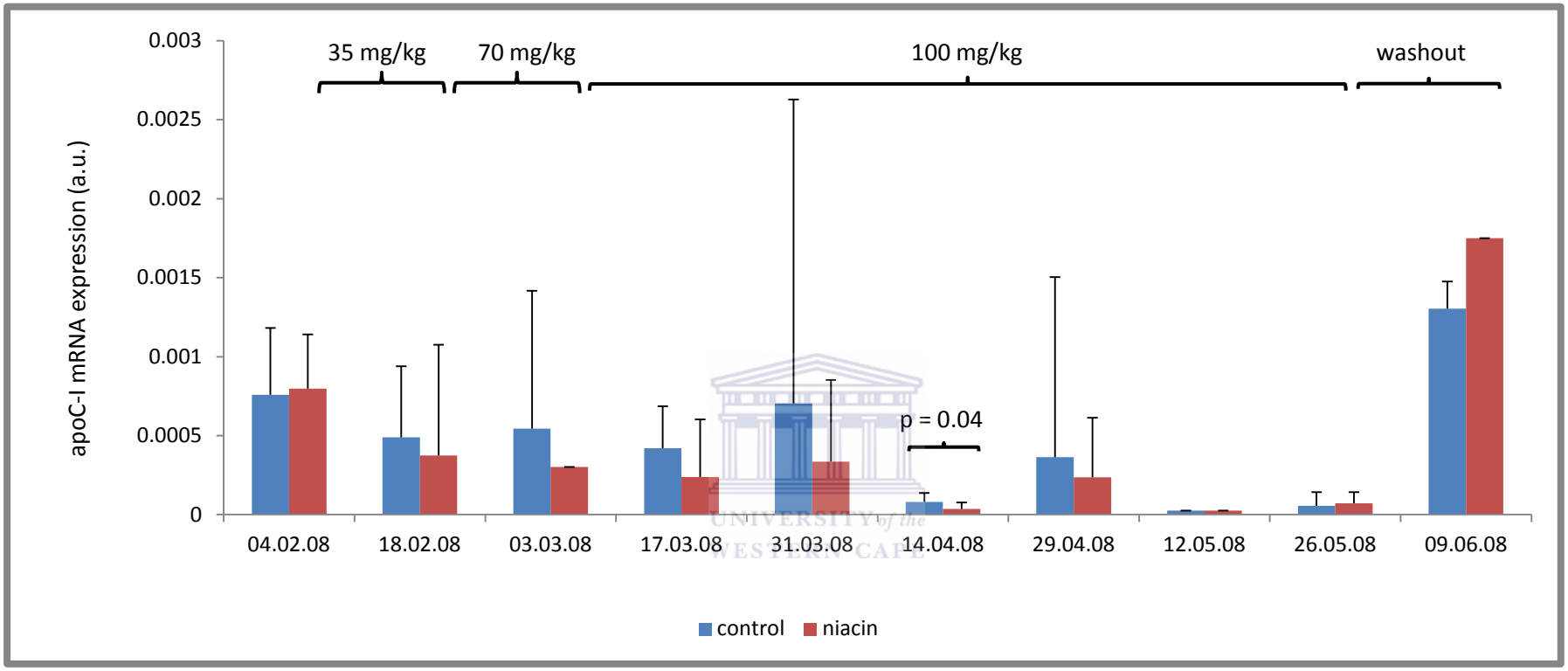

Figure 3.7: The effect of niacin treatment on mRNA expression apoC-1 in the African green monkey. The experimental group received niacin at an escalating dose (35 to 100 mg/kg) while the control group received a maintenance diet throughout the study period of four months. Messenger RNA levels were determined by quantitative real time PCR for all 10 sampling points of study period from baseline (04.02.08) to washout (09.06.08). ApoC-1 expression of both experimental and control groups decreased after the 5th time point (31.03.08). Reduced expression of apoC-1 was observed in the experimental group as compared to the controls, and a p-value of 0.04 was calculated at time point 14.04.08 when the treatment of niacin was maintained at 100 mg/kg. P-values for other time points were greater than 0.05, therefore no difference indicated between the groups. Data were expressed as mean ± SD and mRNA expression in a.u. (arbitrary units).

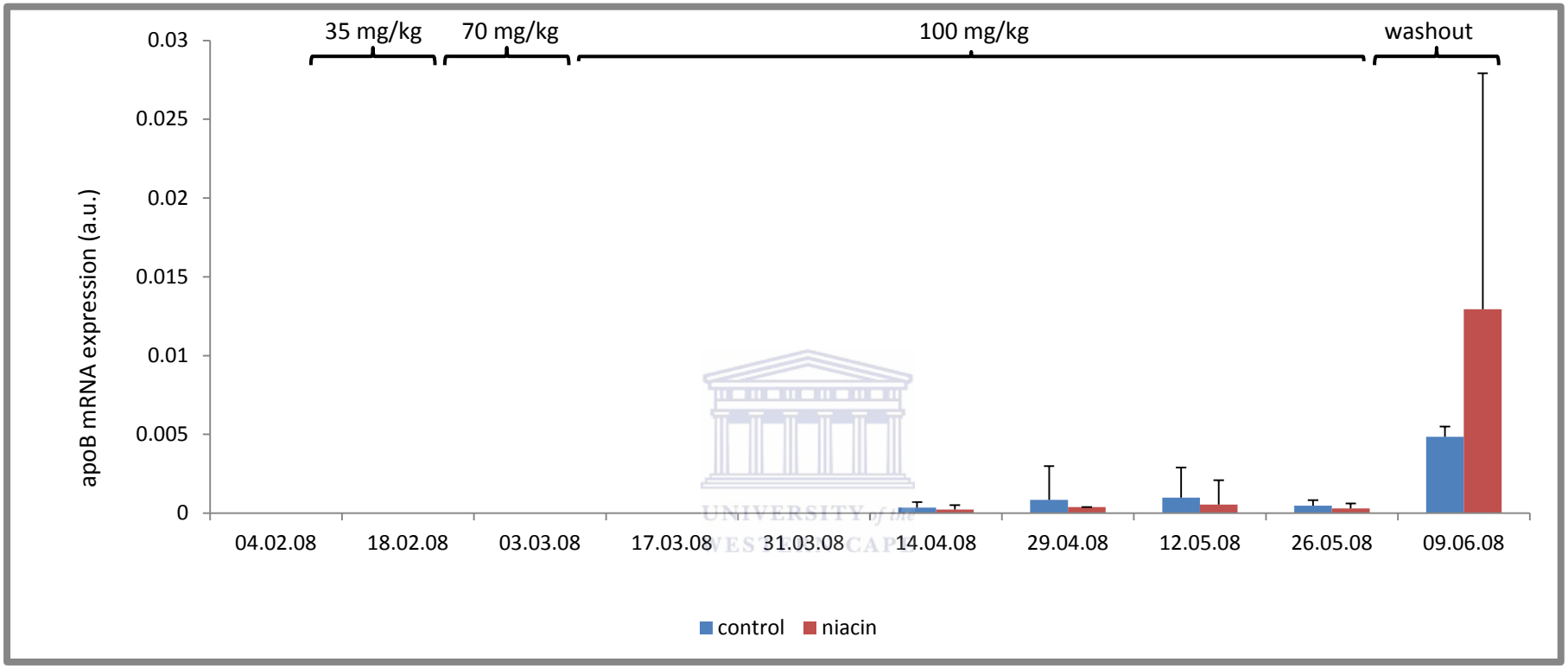

Figure 3.8: The effect of niacin treatment on mRNA expression apoB in the African green monkey. The experimental group received niacin at an escalating dose (35 to 100 mg/kg) while the control group received a maintenance diet throughout the study period of four months. Messenger RNA levels were determined by quantitative real time PCR for all 10 sampling points of the study period from baseline (04.02.08) to washout (09.06.08). The expression of apoB for both experimental and control groups was very low and only observed from time point 14.04.08. Data were expressed as mean ± SD and mRNA expression in a.u. (arbitrary units). P-values for all time points were greater than 0.05, therefore no difference indicated between the groups.

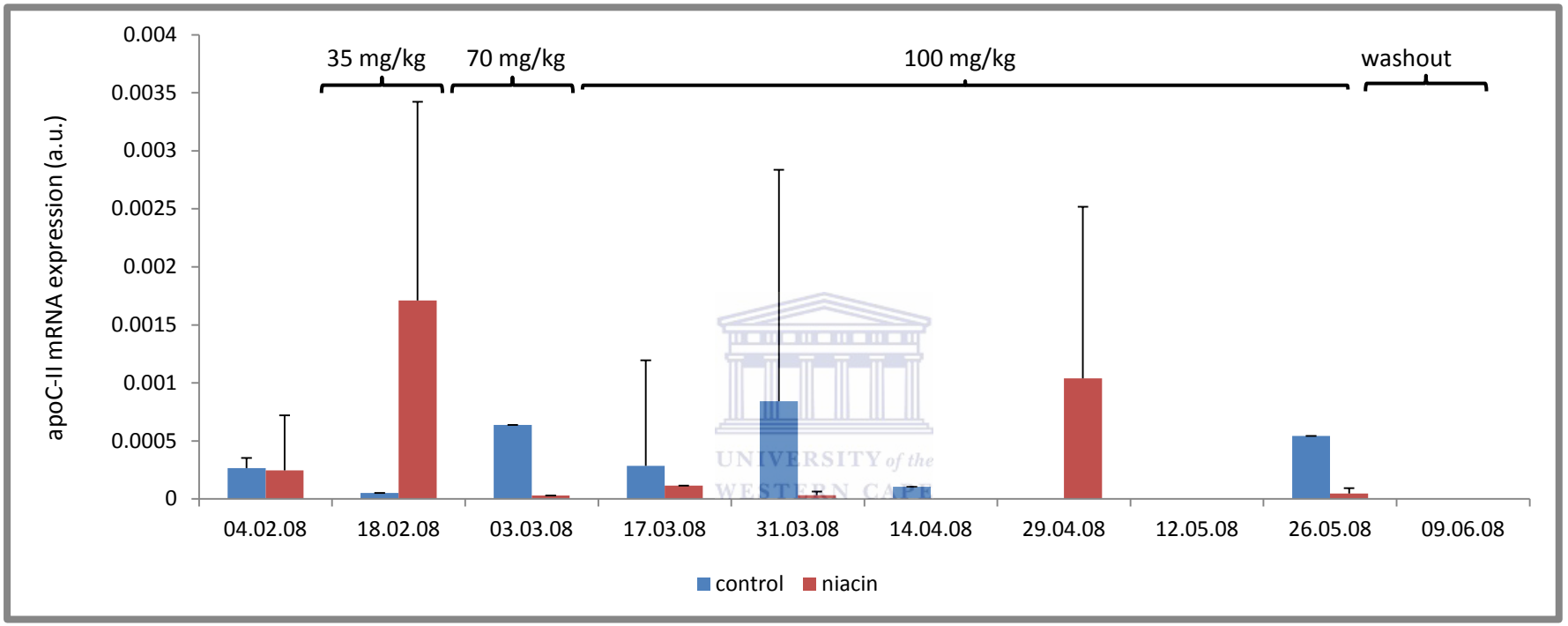

Figure 3.9: The effect of niacin treatment on mRNA expression of apoC-II in the African green monkey. The experimental group received niacin at an escalating dose (35 to 100 mg/kg) while the control group received a maintenance diet throughout the study period of four months. Messenger RNA levels were determined by quantitative real time PCR for all 10 sampling points of the study period from baseline (04.02.08) to washout (09.06.08). The apoC-II expression of both experimental and control groups was weak and only observed in some of the time points. Data were expressed as mean ± SD and mRNA expression in a.u. (arbitrary units). P-values for all time points were greater than 0.05, therefore no difference indicated between the groups.

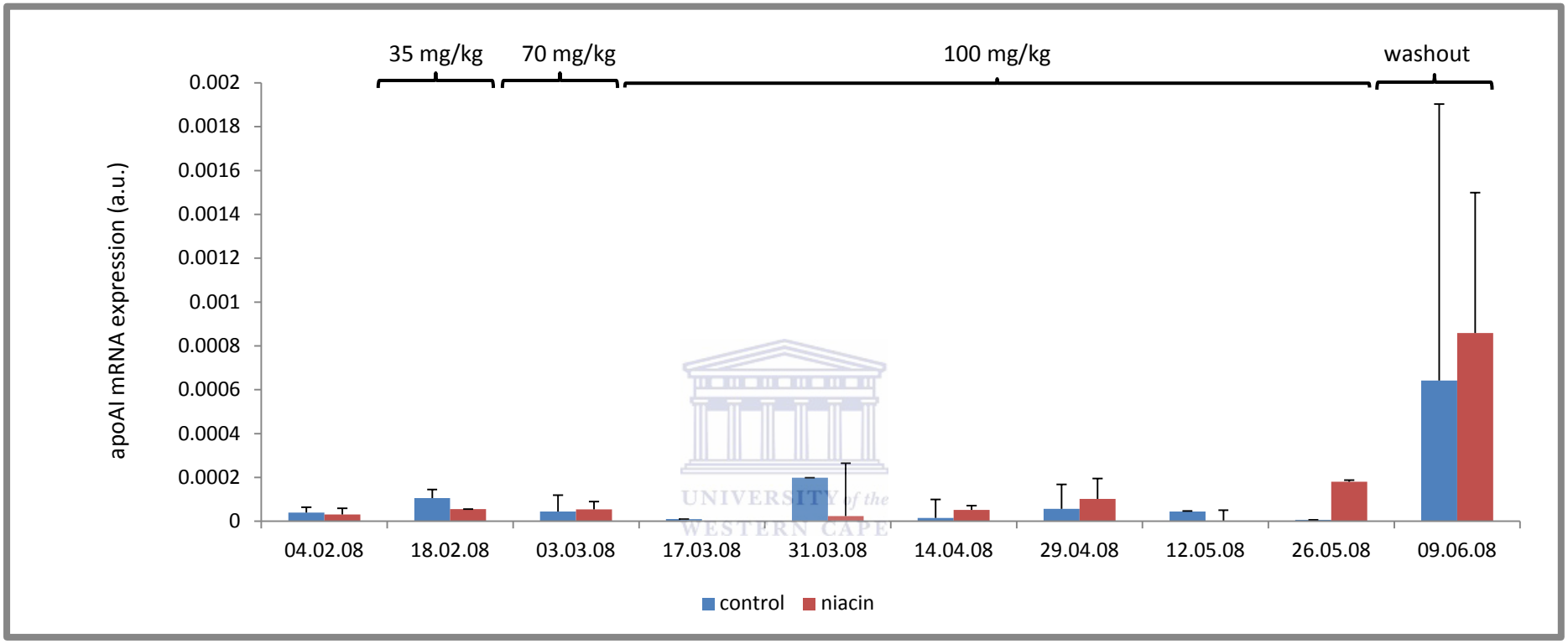

Figure 3.10: The effect of niacin treatment on mRNA expression of apoA-1 in the African green monkey. The experimental group received niacin at an escalating dose (35 to 100 mg/kg) while the control group received a maintenance diet throughout the study period of four months. Messenger RNA levels were determined by quantitative real time PCR for all 10 sampling points of the study period from baseline (04.02.08) to washout (09.06.08). ApoA-1 was weakly expressed in both experimental and control groups throughout the study period. Data were expressed as mean ± SD and mRNA expression in a.u. (arbitrary units). P-values for all time points were greater than 0.05, therefore no difference indicated between the groups.

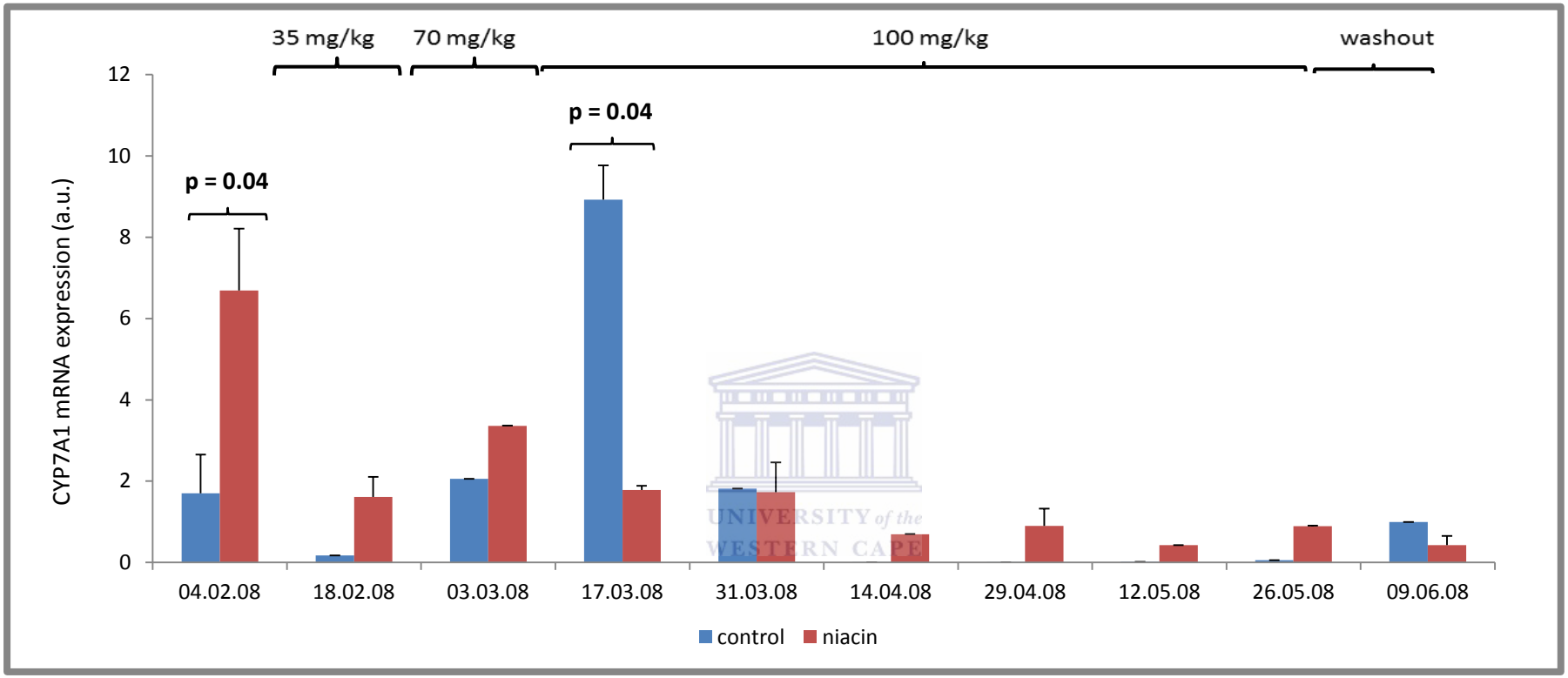

Figure 3.11: The effect of niacin treatment on mRNA expression of CYP7A1 in the African green monkey. The experimental group received niacin at an escalating dose (35 to 100 mg/kg) while the control group received a maintenance diet throughout the study period of four months. Messenger RNA levels were determined by quantitative real time PCR for all 10 sampling points of the study period from baseline (04.02.08) to washout (09.06.08). A decline in CYP7A1 expression was observed in the experimental group from baseline to washout period. P-values of 0.04 were calculated at baseline (04.02.08) and at time point 17.03.08. P-values for the other time points were greater than 0.05, therefore no difference were indicated between the groups. Data were expressed as mean ± SD and mRNA expression in a.u. (arbitrary units).

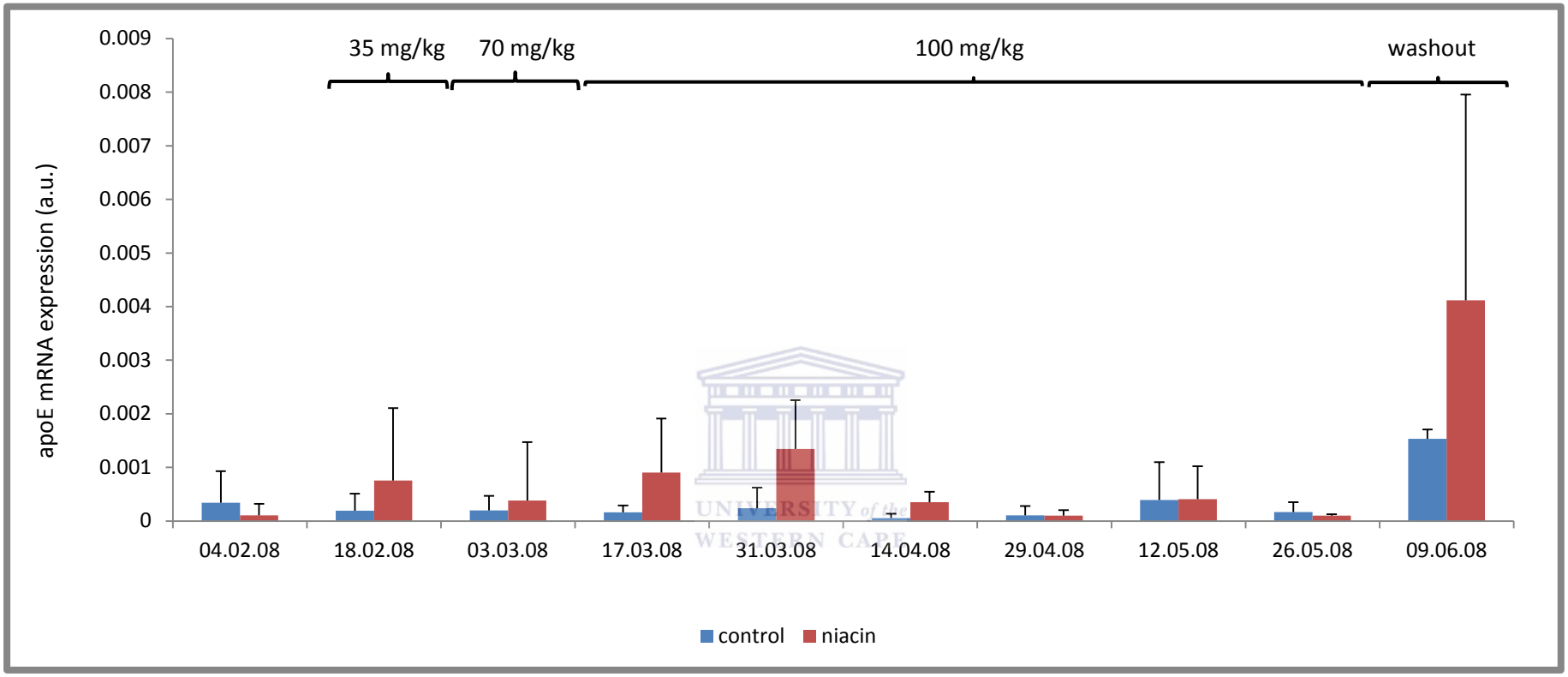

Figure 3.12: The effect of niacin treatment on mRNA expression of apoE in the African green monkey. The experimental group received niacin at an escalating dose (35 to 100 mg/kg) while the control group received a maintenance diet throughout the study period of four months. Messenger RNA levels were determined by quantitative real time PCR for all 10 sampling points of the study period from baseline (04.02.08) to washout (09.06.08). ApoE expression of experimental group decreased after the 5th time point (31.03.08) when niacin treatment was maintained at 100 mg/kg. P-values for all time points were greater than 0.05, therefore no difference indicated between the groups. Data were expressed as mean ± SD and mRNA expression in a.u. (arbitrary units).

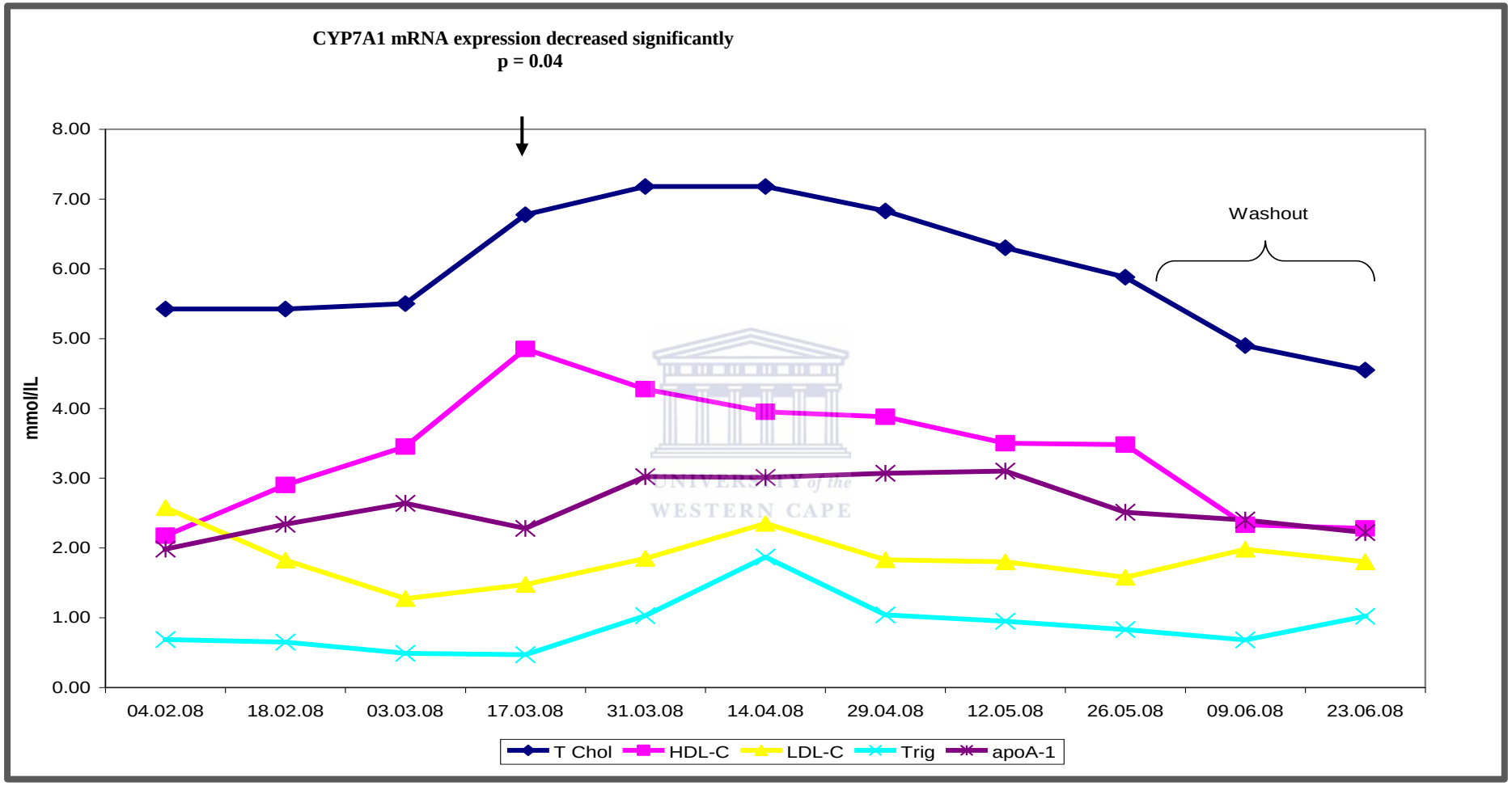

Figure 3.13: The effect of niacin treatment on Total Cholesterol, HDL-C, LDL-C, Triglycerides, and apoA-1 in the African green monkey. Overall, niacin treatment decreased LDL-C by 39%, increased HDL-C by 37% and apoA-I by 26%. Considerable changes in the concentrations of HDL-C, LDL-C, apoA-1 and a significant decline of CYP7A1 mRNA expression were observed at time point 17.03.08.

3.4 DISCUSSION

This study has shown for the first time that the African green monkey share six cSNPs (I405V, I883M, Asn233Ser, cL96R, -62A>C and A350A) with humans (Table 3.11). These cSNPs are known to have a significant influential role in the regulation of reverse cholesterol transport and lipid-lowering drug therapy.

To genetically and phenotypically characterise the African green monkey, 22 cSNPs found at the same position in both humans and nonhuman primates were prioritised and blasted against a dataset of human and monkey SNPs. Six out of the 22 genotyped cSNPs were identified. Since genotype variations between case and control subjects were not obtained in this study, four wild caught monkeys from Potchefstroom University were included. However, no genotypic variations were obtained with these individuals, presumably due to the small gene pool. Therefore, inbreeding is a well-defined genetic consequence with an increase of homozygosity in the whole genome of this species (Charpentier et al., 2007). Based on this information and the fact that MRC wild caught monkeys used as controls originated from the same area than the monkeys from Potchefstroom, associating the effect of niacin with the individual genotypes could not be achieved.

Exploring the genetic basis of HDL-C, a well-studied endophenotype for cardiovascular disease (CVD), has several attractive features as a target for genetic analysis. Since the prevention of CVD is critically dependent on lipid-lowering therapy, in this study six cSNPs in five “candidate genes” associated with HDL-C levels, lipid-lowering drugs response and known to regulate RCT were identified and subsequently genotyped in niacin treated cases and controls of the African green

monkey. The rationale for using the African green monkey in this study was based on the knowledge that this animal model shares a common ancestor with humans, is known to develop atherosclerosis in a fashion similar to humans with respect to morphology and cytology (Fernandez et al., 2008), and is well suited and established as translational model for drug testing (Ebeling et al., 2011). Niacin therapy response was evaluated in healthy monkeys since this model has shown response to HDL-C increases with other reference lipid-lowering compounds such as statin (Yin et al., 2011).

In the last few years there has been growing evidence of the influence of genetic variation/polymorphisms in the determination of plasma lipid concentrations, especially for genes involved in lipid transport and metabolism. More recently, there is a growing interest in the role of these genetic polymorphisms in predicting susceptibility to disease and responsiveness to drug interventions. Plasma lipid abnormalities are risk factors for coronary atherosclerosis and determination of these plasma lipid concentrations may be influenced by genetic variations.

This study presented several challenges. Firstly, spontaneous or induced primate models of low plasma HDL are not available. This is unlike LDL, which can be easily modulated by diet. Secondly, the availability of African green monkeys for research is limited and maintenance is expensive hence the relatively small sample size in the intervention study. Thirdly the reference sequence of the African green monkey is currently unavailable and fourthly, the MRC Primate colony utilized for this study is captive-bred and genetically very similar. Identification of genotypes linked with a particular SNP was therefore not possible due to the small gene pool of

the colony. Additionally, invasive techniques such as liver biopsies would have been required for gene expression analysis of genes involved in lipid metabolism and RCT pathway.

CETP

The relationship of I405V polymorphism in CETP gene expression with plasma CETP level, HDL-C and RCT was investigated in this study. It was shown that carriers of 405V allele have increased HDL-C concentration. Niacin treatment appeared to have decreased CETP mRNA expression as compared to the control mRNA expression profile. However, a statistically significant difference between the experimental and control group was only observed at one time point (14.04.08) with a p-value of 0.04 (Figure 3.3). The lack of statistically significant differences in other time points could be attributed to the fact that this might be a conservative modification of CETP at residue 405 from an isoleucine to a valine and this might not be a direct cause for the alteration in CETP activity and HDL-C values. Therefore, the amino acid change may not have altered the specific activity values of CETP, but its presence may have influenced the effect of lipid-lowering treatment. A negative correlation coefficient of -0.14 with a $p < 0.001$ was obtained between the level of HDL-C and the mRNA expression profile of CETP (Appendix II, Table A10).

It is apparent that a change in the up-regulation of HDL-C by the niacin treatment was accompanied by down-regulation of CETP gene expression especially at time point 14.04.08 when it was statistically significant ($p=0.04$). These findings suggest that CETP I405V polymorphism could modify the effect of niacin on TG reduction and HDL-C elevation at that particular time point. This observation could support the

hypothesis (Brousseau, 2004) that V405 alleles are associated with reduced CETP activity and increased HDL-C levels, however, based on this current study, it cannot be concluded up to what extent physiological variation of CETP could influence RCT pathway, particularly in the African green monkey.

Several studies have reported that carriers of 405V allele have increased HDL-C concentration (Lewis et al., 2005). The I405V polymorphism is located in exon 14 of the CETP gene and is caused by A to G transition in position 20206. It is characterized by alteration in the primary structure of the protein having either an isoleucine or a valine at codon 405. VV genotype of this polymorphism has been associated with lower plasma CETP concentration, higher HDL-C concentration and elevation of the risk for CHD (Isaacs et al., 2007 and Okumura et al., 2002). Since plasma CETP plays a key role in RCT, the presence of I405V polymorphism may therefore alter the susceptibility to atherosclerosis and may have an influential role to play in the effect of niacin therapy to the African green monkeys.

CETP has both pro- and anti-atherogenic effects (Shah, 2007). If CETP is totally absent the lipoprotein profile is atherogenic (Berard et al., 2003 and Tall et al., 1999) though some studies found that low CETP activity correlated with an anti-atherogenic state (Barter, 2001). VV genotype of I405V polymorphism is reported with low CETP, high cholesterol and elevation of the risk for CHD (Isaacs et al., 2007).

ABCA1

A cSNP analysis of the ABCA1 gene provided evidence here that common genetic variations within ABCA1 are associated with altered plasma lipid levels and risk of CAD in the vervet monkey. Five cSNPs (I883M, E1172D, V771M, R219K and V825I) known to influence plasma lipid levels and CAD were genotyped and only one cSNP (Ile883Met) was identified. Clee et al., (2001) have shown that I883M is a common variant that is possibly associated with an increased risk of coronary artery disease (CAD) in the homozygous state. I883M is located in the middle part of the protein corresponding to the seventh hydrophobic segment (H7) (Frikke-Schmidt et al., 2004). Because this variant is associated with little functional effect, it might demarcate the border of the region in which structural alterations significantly impair ATP-binding cassette function. Kyriakou et al., 2007 have reported that I883M has an effect on the activity of ABCA1 in facilitating cholesterol efflux. They observed a trend towards an increased rate of apoAI-mediated cholesterol efflux in cells expressing the 883M variant compared with cells expressing the wild-type (883I).)

Results obtained in mRNA expression of ABCA1 in the African green monkey may have shed some light on the involvement of I883M in RCT regulation, and may play any significant role in influencing ABCA1 activity to niacin therapy in this species. During the experimental intervention the levels of ABCA1 mRNA expression of the experimental group appeared to have been down-regulated as compared to the control group (Figure 3.4) along with increased level of HDL-C and apoA-1 (Figures 2.2 and 2.5). However, statistically significant differences between the two groups were not observed. A negative correlation coefficient was obtained between the level of HDL-C and the mRNA expression profile ($r = -0.17$, $p < 0.001$). Since I883M is known to

increase the risk of CAD, its presence which did not show any relevant statistical significance, did not appear to have influenced the effect of ABCA activity in facilitating cholesterol efflux with niacin up-regulating HDL-C synthesis in the African green monkey.

SR-B1

A polymorphism in exon 8 of the SR-B1 gene (c.1119C.T) was identified in the African green monkey for the first time. This polymorphism is also known as A350A and SR-B1 is best known for its role in facilitating the uptake of cholesteryl esters from high-density lipoproteins in the liver. This process drives the movement of cholesterol from peripheral tissues towards the liver for excretion. This movement of cholesterol is known as reverse cholesterol transport and is a protective mechanism against the development of atherosclerosis. The identification of a polymorphism in the SR-B1 of the African green monkey has possibly shed some light in evaluating the influence of SR-B1 in niacin therapy within this species. SR-B1 A350A is known to have gender-specific and age-related effects on cholesterol transport (Morabia et al., 2004). The common T variant of exon 8 (A350A) has been reported to be significantly associated with having an atherogenic versus non-atherogenic lipid profile, with significantly opposite gender-specific clinical effects, atheroprotective HDL-C-wise in men and atherogenic LDL-C-wise in women (Morabia et al., 2004). Although this polymorphism is a synonymous mutation, it is likely to be a marker for another functional mutation.

In light of prior reports of the effect of this polymorphism on fasted lipid concentration (Morabia et al., 2003), It can be speculated that that this polymorphism

is associated with an anti-athrogenic profile in the African green monkey. Considering that in this study the association between SR-B1 and niacin therapy response was investigated through gene expression profiles, results obtained have indeed reflected the possibility of this influence that SR-B1 A350A can have on RCT and HDL-C synthesis. Since this polymorphism is known to have gender-specific effects on cholesterol transport, female monkeys were used for this study, and biochemistry analysis demonstrated elevated levels of HDL-C and apoA-1 with reduced levels of LDL-C and triglycerides. SR-B1 mRNA expression levels of the experimental group appeared to have been up-regulated when compared with the control group, especially at time points when niacin treatment was maintained at a higher dose of 100 mg/kg. However, statistically significant differences were not observed for all time points except at the washout period ($p = 0.03$) (Figure 3.6).

SR-B1 is known to play a crucial role in RCT pathway by mediating selective uptake of HDL cholesteryl esters by the liver. According to Morabia et al., (2003), the presence of SR-B1 A350A will only favour the atherogenic effect on females, which is contradictory to our findings. A negative correlation coefficient of -0.27 ($p < 0.001$) was obtained between the level of HDL-C and the mRNA expression profile of SR-B1 (Appendix II, Table A10). When a negative correlation coefficient is obtained, a decline in one variable is accompanied by an increase of another variable; therefore, in this case the presence of SR-B1 A350A did not seem to have any effect on niacin therapy response since HDL-C synthesis was up-regulated and is atheroprotective towards the female African green monkey (Figure 3.6).

CYP7A1

The CYP7A1 gene was screened for polymorphisms in the African green monkey. An average of two cSNPs (Asn233Ser and A-278C) was genotyped and a C/T SNP in exon 3, causing an amino acid change at Asn233Ser was identified. The association of this polymorphism to plasma lipid levels and risk to atherosclerosis has possibly contributed to the understanding of the mechanisms driving RCT and niacin response in the African green monkey model. In gene expression analysis, the level of CYP7A1 mRNA expression appeared to have been down-regulated during niacin treatment intervention (Figure 3.11). It was interesting to note statistically significant differences between the experimental and the control group ($p = 0.04$) at week 6 (time point 17.03.04) where a considerable change in the concentrations of HDL-C, LDL-C and apoA-1were observed. (Figure 3.11 and Figure 3.13). It is obvious that niacin treatment had an effect on the expression level of CYP7A1 mRNA, especially at this time point when compared to the baseline which was also statistically significant with a p-value of 0.04. CYP7A1 mRNA of the experimental group was highly expressed at baseline as compared to the control group, however, the expression pattern was reversed and down-regulated at week 6 (time point 17.03.08) when niacin was maintained at 100 mg/kg. The expression remained low from this time point to the end of treatment. A correlation coefficient of -0.30 was obtained between HDL-C levels and mRNA expression profile of CYP7A1 (Appendix 11, Table A10). Since Asn233Ser polymorphism has been associated with variations in plasma LDL-cholesterol concentrations and is known to be involved in drug metabolism, it may play an integral role in metabolising niacin and influencing the up-regulation of HDL-C synthesis.

CYP7A1 catalyses the first reaction in the cholesterol catabolic pathway in liver. This pathway converts cholesterol to bile acids, which is the primary mechanism for the removal of cholesterol from the body. The CYP7A1 catalytic reaction is the rate-limiting step and the major site for regulating homeostasis of cholesterol and bile acids. The gene encoding CYP7A1 was mapped to chromosome 8q11 and it spans about 10 kb and contains 6 exons (Nakamotoet al., 2006). Genetic variations in the CYP7A1 gene associated to disorders of cholesterol and bile acid metabolism have been studied extensively (Nakamoto et al., 2006). Asn233Ser of the CYP7A1 gene has been associated with variations in plasma LDL-cholesterol concentrations and is known to be involved in drug metabolism (Nakamoto et al., 2006).

apoC-II

Out of the six polymorphisms identified in this study, CETP I405V, ABCA1 I883M, SR-B1 A350A and CYP7A1 Asn233Ser have been discussed. The remaining two polymorphisms identified are located in the apoC-II gene which has been mapped on chromosome 19 in a gene cluster containing the apoE/C-I/C-I'/C-IV/C-II gene (Chen et al., 2007). It was interesting to note that the difference between mRNA expression of the control and experimental group was not statistically significant and the expression profile was minimal throughout the intervention phase of the study (Figure 3.9). It is obvious that both polymorphisms did not have any impact on niacin therapy. According to literature, the end result of apoC-II is increased levels of LDL-C due to its influence on LPL (Vorgia et al., 1998; Chen et al., 2007), however in this study, the LDL-C levels remained low, and therefore, the low expression of apoC-II had no influence on this process.

The exact function of apoC-IV is unknown, although it appears to play a role in lipid metabolism. ApoC-II is synthesized primarily by the liver and is a 79 amino acid residue protein that plays a pivotal role in lipid metabolism as an activator for the lipoprotein lipase (LPL), and leads to increased levels of LDL-C (Chen et al., 2007). ApoC-II Leu96Arg has been associated with triglyceride concentrations in women.

LCAT, apoC-I, apoA-1, apoE and apoB

LCAT, apoC-I, apoA-1, apoE and apoB were investigated through the intervention phase of this study. These genes are known to play a key role in the RCT pathway and have been implicated in lipid metabolism (Tall, 1990). SNPs/polymorphisms which are known to influence the function and regulation of these genes, and which have also been reported to have an influential role in drug response, were selected and genotyped (Appendix II, Table A9), however, none of these SNPs were identified in the African green monkey.

Gene expression analysis was conducted to evaluate if any of these genes had a direct impact on niacin response and RCT pathway. LCAT was expressed throughout the intervention phase of the study; however, no statistically significant differences were obtained even though the expression profile appeared to have decreased from the time niacin was maintained at a higher dose of 100 mg/kg (Figure 3.5). Although LCAT accentuates reverse cholesterol transport (RCT) from plasma to liver and induces cholesterol degradation, LCAT's role in RCT and protection against atherosclerosis is the subject of on-going research (Brown et al., 2010). In the absence of LCAT, RCT can still continue (Brown et al., 2010). In this study, LCAT did not play a significant role in either niacin therapy or on the RCT pathway.

To attempt the evaluation of independent effects of apoC-I on RCT and niacin response, *HpaI* SNP was genotyped; however, it was not identified in our animal model. Gene expression analysis revealed less expression of apoC-I in the experimental group as compared to the controls, and a statistical significant difference with a p-value 0.04 was only observed at the 6th time point (14.04.08) when treatment of niacin was maintained at 100 mg/kg (Figure 3.7). apoC-I is a constituent of triglyceride-rich lipoprotein, and high density lipoproteins whose importance in plasma lipoprotein metabolism is increasingly evident (Xu et al., 1999). Its function is to displace apoE from triglycerides-rich emulsions and lipoproteins (Gao et al., 2002). The fact that apoC-I mRNA expression appeared to have been down-regulated in experimental groups as compared to the control group, especially with statistically significant difference at time point 14.04.08, highlighted the fact that apoC-I might have carried out its function by displacing apoE which was also evident by its poor gene expression profile in Figure 3.12.

ApoE is known to be an exchangeable protein that plays an important role in lipid metabolism, especially in the removal of atherogenic remnants of triglyceride-rich lipoproteins (Hixson et al., 1990). The presence of apoE allele e4 is known to be associated with the pathogenesis of peripheral and coronary artery disease and the e2 allele is known to be protective from atherosclerosis. Both polymorphisms were genotyped, but not identified in the African green monkey. Due to its poor expression profile and the fact that it was not statistically significant, it is therefore clear that apoE had no effect on niacin response in the African green monkey (Figure 3.12).

Both apoA-1 and apoB are known to play an essential role in the RCT pathway. SNPs from apoA-1 (Msp1 and G75A) and apoB (T71I and 4311S) known to be involved in lipid metabolism and drug response were genotyped but were not identified in the African green monkey in this study. Gene expression analysis of both genes was very poor and statistically insignificant with apoB only expressed half way through the intervention study when niacin treatment was maintained at its highest dosage (Figure 3.8 and Figure 3.10). Since apoB is a protein component of LDL particles and is known to be cleared from plasma mainly by binding to LDL receptors and subsequent internalization and degradation in the liver, it's expression at the beginning of the intervention study would not have been expected, and our findings are in agreement with that, albeit not statistically significant. Due to niacin therapy, LDL-C level was reduced as indicated in chapter 2; apoB expression was expected to be less and was only observed towards washout period at the end of the study. Considering the fact that only a small sample size of healthy monkeys instead of diseased monkeys were used in this study, data generated would have been more significant if an access to a larger sample size was possible with baseline values depicting a diseased state.

3.5 CONCLUSION

This study suggests that a cascade of genes and the potential effect of polymorphisms located in these genes may be responsible for the regulation and function of RCT pathway and drug treatment response in the African green monkey

. Considering that only six out of 22 cSNPs involved in human and rhesus RCT were identified in the African green monkey, it is possible that the outcome of this study may be due to yet to be identified genetic variation or those that are not present in multi-species but species specific to the African green monkey. Results may therefore

be associated with the experiment rather than possible genetic heterogeneity between treatment and control groups.

Niacin treatment showed an effect on the expression level of CYP7A1 mRNA when a considerable change in the concentrations of HDL-C, LDL-C and apoA-1 were observed. This change may have been influenced by the presence of CYP7A1:Asn233Ser polymorphism which may have been involved in metabolising niacin and influencing the up-regulation of HDL-C synthesis in this animal model.

GENERAL DISCUSSION AND CONCLUSIONS

4.3 DISCUSSION

Lipid metabolism is a dynamic process that involves multiple pathways with both intracellular, and extracellular regulation and modulation. While the cross-talk and interactions among these pathways are essential to maintain lipid homeostasis, dysregulation of each pathway can contribute substantially to the development of cardiovascular diseases such as dyslipidemia and atherosclerosis.

Low levels of HDL-C are a major independent risk factor for atherosclerotic cardiovascular disease and events. Epidemiological and interventional studies have clearly established the critical role of lipoprotein metabolism in atherosclerotic disease, in which risk increases with plasma levels of LDL-cholesterol and is inversely proportional to HDL-cholesterol (Duffy et al., 2006). The success of LDL-cholesterol-lowering therapy has stimulated increased interest in HDL-cholesterol-directed approaches, and several HDL-cholesterol-elevating drugs are currently available or under development (Carlson, 2005). Some of the most widely used HDL-cholesterol-elevating drugs include statins (~5–10% increase), fibrates (peroxisome proliferator-activated receptor α (PPARα) agonists; ~5–20% increase), and nicotinic acid or niacin (~15–35% increase) (Ohashi et al., 2005). Although existing drugs have modest effects on HDL-C levels, this area remains a major unmet medical need in cardiovascular medicine.

HDL metabolism is exceedingly complex, and the protective ability of HDL may relate to the flux of cholesterol through the RCT pathway, and to other aspects of HDL functionality. The actual mechanism by which HDL protects against atherosclerosis is likely multifactorial and not yet fully elucidated; however, there are several proposed major atheroprotective mechanisms of HDL. The most popular has been that HDL promotes and facilitates the process of reverse cholesterol transport (RCT), whereby excess macrophage cholesterol is effluxed to HDL and ultimately returned to the liver for excretion in the bile and feces (Duffy, 2006). However, several additional properties of HDL have been described over the last decade, including inhibition of LDL oxidation, inhibition of endothelial inflammation, promotion of endothelial nitric oxide production, promotion of prostacyclin bioavailability, and inhibition of platelet aggregation and coagulation (Khera et al., 2011). The *in vivo* relevance of these mechanisms remains uncertain, but in concept many of these functions of HDL could contribute to reduction in atherosclerosis, increased vascular protection, and reductions in clinical events.

It has been shown in both humans and nonhuman primates that low levels of HDL-C are an important and prevalent risk factor in the development of cardiovascular disease. Over the last decade, cholesterol-lowering therapy has been considered to be the central approach in the prevention of cardiovascular disease. Currently, niacin is considered to be the most potent available pharmacological agent for increasing HDL cholesterol. The mechanism by which niacin raises HDL-C levels is the topic of continued investigation. In vivo studies in humans suggest that the catabolic rate of apoA-I is slowed by niacin therapy (Carlson, 2005). Studies in hepatocytes in vitro have shown reduced uptake of HDL apoA-I after niacin treatment (Kamanna et al.,

2008). The paradigm has been that in addition to indirect effects on HDL metabolism through reduction in triglycerides, niacin acts on an unknown pathway in the liver to slow HDL and apoA-I catabolism. The recent discovery of a niacin receptor, a G protein–coupled receptor known as HM74A (also known as GPR109A) (Shen et al., 2009), has fueled tremendous new interest in the mechanisms of niacin action and the development of new compounds that act on this receptor. Intriguingly, activated macrophages express the niacin receptor, and niacin treatment of macrophages upregulates ABCA1 expression (Shen et al., 2009).

Given the apparent similarities in lipoprotein cholesterol metabolism between human beings and nonhuman primates, niacin therapy may have the same effects on HDL metabolism of nonhuman primates. Nonhuman primates that have consumed an atherogenic diet for several years develop lesions that are comparable to those found in human beings. Amongst the nonhuman primates, the African green monkey has been recognised as a good model for the study of diet-induced atherosclerosis (Moghadasian et al., 2001). However, notwithstanding their closeness to humans, validity for their use as models for lipid-lowering drug interventions still has to be demonstrated. It is noteworthy that the Primate Unit of the Medical Research Council has already utilised the African green monkey in lipid-lowering research, with lipid modulating compounds such as CETP inhibitors. A specific diet was not needed to induce the disease and the model responded with HDL-C increases. Besides, the diets that induce dyslipidaemia can only modulate LDL not HDL.

The African green monkey is the most commonly used Old World primate in biomedical research, but, in many areas, the least well defined. In order to utilise this

species fully, there is a need to understand and define as much of its biology as possible. However, there is currently very little sequence data available for this species in the GenBank and in order to conduct genetic studies on the African green monkey, the need exists to rely on available human and rhesus macaque sequence. To further characterise the African green monkey as an animal model for atherosclerosis, human ortholog genes known to be linked to lipid metabolism were sequence and genotype in a case:control colony of the African green monkey. Since African green monkeys are closely related to humans both in evolutionary and genetic terms, shared ancestral polymorphisms (cSNPs) were prioritized and assessed if they had any effect on niacin treatment. It is a fact that SNPs, by their very nature, are uncommon and need only to be present in 1% of a population; and it is also known that most SNPs are not shared among species with the prime exceptions among primates being those that occur at CpG sites. Since these sites are frequently methylated, they are much more prone to de novo mutation and hence independent occurrence.

The main objective of this study was therefore to make an original contribution to primatology by defining the molecular genetics of the African green monkey in relation to CHD. Data on the controlled pharmacological intervention linked to genetic determinants of lipid metabolism in the African green monkey are provided here for the first time. Since the African green monkey is closely related to humans both in evolutionary and genetic terms, shared ancestral polymorphisms known as coincident SNPs (cSNPs) were prioritized for this study. Furthermore, the application of bioinformatics with molecular techniques such as real-time PCR were used along with orthologous human reference sequence to identify for the first time in the African green monkey specific lipid metabolism candidate genes and cSNPs corresponding to

ancestral polymorphisms present in both humans and nonhuman primates, and preserved in both lineages.

Results obtained from this study reflect the careful selection of biologically relevant candidate genes, and the effective conditioning of their putative effects upon known risk factors. The case-control design based on normolipidaemic African green monkeys is a critical element of the study and merits several comments.

A literature search was conducted to look for previously reported genes and polymorphisms involved in lipid metabolism in humans. Ten "candidate genes" (CETP, ABCA1, CYP7A1, apoA-1, apoB, apoE, SR-B1, LCAT, apoCI and apoCII) were chosen based on their role in the reverse cholesterol transport process. Twenty two Single nucleotide polymorphisms (SNPs) were identified and subsequently genotyped in niacin treated cases and controls of the African green monkey.

As a first step towards elucidating the effect of niacin on HDL-C, the possible involvement of these prioritised candidate genes and their polymorphisms were assessed biochemically by analysing known risk factors of coronary artery disease such as HDL-C and LDL-C, and also by analysing gene expression. Eight healthy monkeys were selected and entered in this study. Four monkeys were treated with niacin at an escalating dosage (35, 70 and 100 mg/kg) and the other 4 were chosen as controls. Levels of HDL-C, LDL-C, TC, TG and apoA-1 were then measured at baseline and after every two weeks for four months up to a washout period. Niacin treatment was associated with a considerable reduction in LDL-Cholesterol and a less

significant decline in Triglycerides. Niacin was associated with up-regulation of HDL synthesis, and associated increased apoA-1 levels.

Out of the twenty two cSNPs genotyped, six were identified in the African green monkeys (I405V, I883M, Asn233Ser, cL96R, -62A>C and A350A). All six cSNPs are known to have a significant influential role in the regulation of reverse cholesterol transport and lipid-lowering drug therapy.

Niacin consumption was associated with increased plasma HDL-C and apoA-1 as well as decreased plasma LDL-C and triglycerides (Figure 2.8). Gene expression analysis was correlated with biochemistry results by the association between a combination of cSNPs identified in the prioritized candidate genes, and plasma lipid levels in the African green monkey. Niacin appeared to have minimal effect if any on gene expression of SRB1, CETP, ABCA1, LCAT, apoC-I, and apoC-II within the context of specific cSNPs identified. However, an interesting observation was made of CYP7A1 mRNA expression, especially at time point 17.03.08 (week 6) when a considerable change in the concentrations of HDL-C, LDL-C and apoA-1 were observed. It is apparent that niacin treatment had an effect on the expression level of CYP7A1 mRNA, especially at this time point when compared to the baseline. This change may have been influenced by the presence of CYP7A1:Asn233Ser polymorphism which may have been involved in metabolising niacin and influencing the up-regulation of HDL-C synthesis in this animal model.

This study provides for the first time data on a controlled pharmacological intervention linked to genetic determinants of lipid metabolism in the African green monkey model.

However, there are also several limitations:

1. The genome of the African green monkey has not yet been sequenced; therefore there is no available reference sequence. This limitation has made primer design of "candidate" genes used in this study more challenging. However, available reference sequences from human and rhesus monkeys were adopted and used as the template for primer design and for bioinformatics analysis. The rationale for this was the fact that nonhuman primates are closely related to humans, both in evolutionary and genetic terms. The human reference was used for the bulk of the genome colinearity and gene content analysis. The information was then projected to the rhesus to obtain a nucleotide level read out that is even closer to the African green monkey genomic sequence since they belong to the same family group, the Old World monkey.
2. The African green monkeys of the MRC Primate Unit are genetically very similar. Since genotype variations were not expected, results obtained from genotyping SNPs were the same for both control and experimental groups. Identification of genotypes linked with a particular SNP was therefore not possible. To address this, four wild caught monkeys from a different animal centre (Potchefstroom) were included in the study. However, results obtained from these wild caught monkeys were similar to those obtained from the MRC

captive-bred monkey colony. Based on this information, associating the effect of niacin with the individual genotypes could not be achieved.

3. Niacin therapy response was evaluated only on healthy monkeys since dietary interventions only modulate LDL. There is no known primate model of spontaneously low HLD. To induce advanced lesions atherosclerotic lesions in this animal model would have taken 3 to 4 years of feeding and atherogenic diet, and invasive techniques such as liver biopsies would have been required for gene expression analysis. Since liver is the driving engine for cholesterol efflux and RCT pathway, a much more higher and positive gene expression would have been achieved if liver tissue was used instead of blood cells which were convenient for this study.
4. Niacin is known to cause side effects when taken in high doses, due to ethical considerations and abiding to the National Guidelines for the Care and Use of Animals for Scientific Purposes escalation dose higher than 100 mg/kg was not recommended for this study.
5. Relative scarcity in terms of availability for research, and high cost of the African green monkey are two factors that make a larger sample size difficult, therefore only eight monkeys were available for this study, hence poor gene expression profile were obtained and statistical significant values were difficult to achieve.

However, several advantages were also identified:

1. There was no need to induce disease or use a disease model. Ethically, this is considered as a refinement and promotes the concept of the ‘three Rs’

(replacement, reduction and refinement) according to the National Guidelines for the Care and Use of Animals for Scientific Purposes.

2. Due to the close evolutionary relationship between humans and the African green monkey, results obtained from this study can be extrapolated to humans, and this could make a contribution to the understanding of cardiovascular diseases.
3. The study applies a number of original approaches to identifying candidate susceptibility genes for lipid metabolism. Furthermore, it is the first time that many genetic aspects have been defined in the African green monkey; therefore, this study does not only focus on human diseases, but animal diseases as well.
4. The results have contributed to defining the molecular genetics of the African green monkey especially in the light that its genome sequence has not yet been mapped.

Pharmacological therapy development through genetic studies has had a significant impact in clinical medicine. Pharmacogenetics and genetic testing have provided the experimental basis that has shown that the variability in a drug response is a function of an individual's genetic makeup. Genetic polymorphisms can influence the response to medication through a number of mechanisms (Schmitz et al., 2003). It is noteworthy that this study provided further impetus for the development of niacin (nicotinic acid) in pharmacogenetic processes. However, it must be acknowledged that a large scale SNP discovery initiative should be undertaken for the African green monkey in order to have enough information to validate this species as a good model in pharmacogenomic studies.

Low HDL cholesterol levels are the most common lipid abnormality in patients with CAD, and are the primary lipid disorder in half of this population. Raising HDL levels has been difficult, and statins only moderately affect HDL, raising levels by approximately 5% to 10% (Schmitz et al., 2003). Niacin has a long-standing history as an effective lipid-altering therapeutic agent with well-established clinical benefits (Kamanna et al., 2008). In addition to its beneficial effects on standard lipoprotein levels, niacin has shown further benefits in patients with coronary artery disease by significantly increasing HDL and LDL particle size (Tavintharan et al., 2001).

Niacin favourably alters all major lipid subfractions at pharmacologic doses. Alone or in combination, it promotes regression of coronary artery disease, decreases coronary events, stroke, and total mortality (Kamanna et al., 2008). Major recent progress in niacin is in four areas. Firstly, recent data indicate that it increases HDL and lowers triglycerides and LDL by mechanisms different from statins, fibrates, and bile-sequestrants, providing a rationale for combination therapy to achieve synergistic effects for complete lipid goal achievement (Schmitz et al., 2003). Secondly, new data on an extended-release preparation of niacin taken once nightly indicates that it is as effective, and has greater tolerability than immediate-release niacin (Tavintharan et al., 2001). Thirdly, preliminary data with single tablet formulation extended-release niacin and an HMG CoA reductase inhibitor (lovastatin) shows it to be safe and very effective, especially for raising HDL. Finally, emerging evidence indicates that niacin can be used effectively and safely in patients with type 2 diabetes mellitus, who often have low HDL levels (Ganji et al., 2003).

The focus of lipid-altering therapies has been largely on their abilities to lower LDL-C and triglyceride levels and raise HDL-C levels. This study demonstrates that in

addition to these effects, genetic determinants of lipid transport and metabolism may also provide additional significant benefit in nonhuman primates such as the African green monkey. Treatment with 100 mg/kg of niacin produced improvements in HDL-C, and apoA-1 concentrations, and additionally resulted in significant reduction of 39% in LDL-C. Even though most of the time points were not statistically significant, following just 18 weeks of treatment, niacin appeared to have upregulated SR-B1 gene expression and downregulated the expression level of the other nine important genes involved in driving RCT pathway and lipid metabolism. Thus, niacin treatment at such low dose (100 mg/kg) appeared to cause different effects on lipoproteins, beyond the conventionally measured responses observed in serum lipid. It will be important to clarify whether this response is specific to blood cell gene expression or seen more generally in various tissues and in the liver *in vivo*, because it could suggest that regulation of these genes by niacin is tissue-dependent. It would have also been interesting to observe if a larger sample size and a higher dosage of niacin treatment would have had a better effect with significant statistical values, however due to side effects that may be caused by niacin when taken in high doses, escalation higher than 100 mg/kg was not recommended.

There is ample evidence in human studies that niacin acutely or chronically alters gene expression and cellular signalling in various tissues and cells. The underlying mechanism may involve signalling through the niacin-receptor, but there is substantial evidence that *in vivo* effects on gene expression may be mediated indirectly by changes in circulating lipid or hormone levels induced by niacin treatment (Kang et al., 2011). Future studies are warranted to elucidate these mechanisms, which would

contribute not only to the full understanding of niacin effects in human and nonhuman primates, but also to the discovery of novel pathways for gene regulation by lipids.

The data obtained from this study indicated that niacin increased the accumulation of apoA-I, however, niacin did not affect the de novo synthesis of apoA-I and the mRNA expression of apoA-I, suggesting that niacin has no effect on apoA-I synthetic processes. Therefore it was hypothesized that niacin may influence the removal or reuptake of HDL by hepatocytes and this is supported by the findings of Zhang et al., 2008. Niacin may have selectively inhibited the uptake of HDL-apoA-I but not HDL-cholesterol esters. These findings suggest that niacin may increase the capacity of retained apoA-I to augment cholesterol efflux and reverse cholesterol transport pathway. Based on these data it was suggested that niacin inhibits the removal of HDL-apoA-I at the level of yet unidentified putative pathways, but not SR-B1-mediated events, which is selective to HDL-cholesterol esters.

The apoB content of the lipid profile can also differ substantially in response to interventions, because this measurement provides an estimate of atherogenic (non-HDL) particle number (Ganji et al., 2003). ApoB is present at a fixed ratio of one molecule per particle, and does not exchange between particles as the other apolipoproteins do. In addition, therapies that result in lowering apoB levels can translate into lower risk of cardiovascular disease (Ganji et al., 2003). In this study, apoB expression was suppressed and only observed towards the end of the treatment phase. It is speculated that niacin-mediated inhibition of TG synthesis may have decreased the lipidation of apoB and translocation through endoplasmic reticular (ER) membrane which may have led to increased intracellular apoB degradation. Increased

hepatocyte apoB degradation by niacin would then decrease the number of VLDL and their catabolic products, LDL particles, which explains the lower apoB and LDL concentrations observed clinically after niacin treatment in the African green monkey. In 1999 Jin and colleagues also reported similar findings of niacin to have shown to accelerate intracellular apoB degradation by inhibiting TG synthesis in human HepG2 cells (Kang et al., 2011); however, this matter has been extensively discussed in literature and is still being debated.

4.4 CONCLUSION

The purpose of this study was not to add new knowledge to lipidology, genetics or the mechanism of action of niacin, but to produce new insights into medical primatology by defining the African green monkey biology aspects that have not been studied before. The main objective of the study was to investigate the influence of genetic variation in a therapeutic intervention using niacin as a lipid-lowering drug in the African green monkey. Niacin was only used as a tool to effect changes in lipid metabolism, not as an object of the research.

The following objectives were achieved:

1. Ten “candidate genes” linked to lipid metabolism were identified and screened for coincident single-nucleotide polymorphisms (cSNPs) known to be involved in reverse cholesterol transport pathway, six of which were identified for the first time in the African green monkey.
2. A four months pharmacological intervention study using niacin as the drug-lowering agent was conducted. Niacin successfully increased plasma HDL and over a period of 12 weeks during the treatment phase.

3. The expression profile of the selected “candidate” genes.

A combined use of biochemistry and molecular biology offered the basis for systematic dissection of the effects of niacin treatment in a nonhuman primate model closely related to humans. The characterisation of individual polymorphisms implicated in lipid metabolism is challenging, but essential for optimising the regulation and function of the RCT pathway. It is noteworthy that linkage disequilibrium may exist between genes involved in RCT; therefore the influence of one cSNP on the effect of drug therapy may be inter-depended within different genes in the African green monkey as it is in humans.

The results in this study showed that niacin treatment is associated with considerable reduction in LDL-C and up-regulation of HDL synthesis. Niacin treatment influenced the expression of genes involved in RCT pathway and the genetic variation in these genes could further modify the anti-atherosclerotic effect of niacin in the African green monkey. However, the findings of this study based on the biochemical and expression differences would be more attributed to the treatment, and not the presence of response modifying cSNPs (at the loci tested, as well as at other untested loci), especially due to the genetic identical nature of this colony.

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APPENDIX I: BIOCHEMISTRY ANALYSIS

1. Analyses performed at PathCare Pathology Laboratories

1.1 Plasma cholesterol:

CHOL reagent is used to measure cholesterol concentration by a timed-endpoint method. In the reaction, cholesterol esterase (CE) hydrolyzes cholesterol esters to free cholesterol and fatty acids. Free cholesterol is oxidized to cholestene-3-one and hydrogen peroxide by cholesterol oxidase (CO). Peroxidase catalyzes the reaction of hydrogen peroxide with 4-aminoantipyrine (4-AAP) and phenol to produce a coloured quinoneimine product (SYNCHRON LX Systems Manual, 2000).

1.2 HDL-cholesterol:

Two point calibration **c**olorimetric end point. SYNCHRON Systems HDL Cholesterol (HDLD) Reagent, when used in conjunction with SYNCHRON Systems Lipid calibrator, is intended for the quantitative determination of HDL cholesterol in the high density lipoprotein (HDL) fraction of serum or plasma on SYNCHRON systems (SYNCHRON LX Systems Manual, 2000).

1.3 Triglycerides:

Triglycerides GPO reagent is used to measure the triglycerides concentration by a timed endpoint method. ^{T}riglycerides in the sample are hydrolyzed to glycerol and free fatty acids by the action of lipase. A sequence of three coupled enzymatic steps using glycerol kinase (GK), glycerophosphate oxidase (GPO), and horseradish

peroxidase (HPO) causes the oxidative coupling of 3,5-dichloro-2-hydroxybenzenesulfonic acid (DHBS) with 4-aminoantipyrine to form a red quinoneimine dye (SYNCHRON LX Systems Manual, 2000).

1.4 LDL-cholesterol:

The liquid, ready-to-use SYNCHRON® Systems Direct LDL Cholesterol reagent is a homogeneous assay that eliminates off-line pretreatment and centrifugation steps. The method depends on the properties of a unique detergent, which solubilizes only the non-LDL lipoprotein particles and releases cholesterol to react with cholesterol esterase and cholesterol oxidase to produce a non-color forming product. A second detergent solubilizes the remaining LDL particles and a chromogenic coupler allows for colour formation (SYNCHRON LX Systems Manual, 2000).

2. FIGURES AND RAW DATA TABLES

Table A1: Total cholesterol (mmol/L), values during treatment phase highlighted: means (yellow), SD (green) and P-value (pink)

Treatment	No.	04.02.08	18.02.08	03.03.08	17.03.08	31.03.08	14.04.08	29.04.08	12.05.08	26.05.08	09.06.08	23.06.08
Group 1 Niacin	**795**	8.00	6.80	6.70	7.50	7.80	8.30	8.10	6.60	7.00	6.70	4.20
	322	4.50	4.80	5.6	8.10	7.30	7.30	7.00	6.30	5.40	3.80	3.20
	339	4.50	5.20	5.2	5.20	5.70	7.10	5.70	7.40	5.90	4.70	4.80
	77	4.70	4.90	4.5	6.30	7.90	6.00	6.50	4.90	5.20	4.40	6.00
	Mean	**5.43**	**5.43**	**5.50**	**6.78**	**7.18**	**7.18**	**5.66**	**6.30**	**5.88**	**4.90**	**4.55**
	SD	**1.72**	**0.93**	**0.92**	**1.29**	**1.02**	**0.94**	**1.00**	**1.04**	**0.81**	**1.26**	**1.17**
Group 2 Control	**108**	4.70	6.30	6.0	5.4	5.1	5.20	5.60	4.90	4.60	4.80	4.80
	243	6.00	6.90	7.2	7.4	5.9	6.20	6.30	5.50	5.50	5.40	5.50
	215	5.20	5.00	4.7	5.5	4.7	5.40	5.60	5.70	5.70	5.80	5.10
	97	3.70	3.90	4.0	4.0	4.1	4.00	4.20	4.00	4.40	4.30	3.70
	Mean	**4.90**	**5.53**	**5.48**	**5.58**	**4.95**	**5.20**	**5.43**	**5.03**	**5.05**	**5.08**	**4.78**
	SD	**0.96**	**1.34**	**1.42**	**1.40**	**0.75**	**0.91**	**0.88**	**0.76**	**0.65**	**0.66**	**0.77**
p-value		0.62	0.91	0.98	0.25	0.01	0.02	0.08	0.10	0.16	0.82	0.76

Table A2: HDL-cholesterol (mmol/L), values during treatment phase highlighted: means (yellow), SD (green) and P-value (pink)

Treatment	No.	04.02.08	18.02.08	03.03.08	17.03.08	31.03.08	14.04.08	29.04.08	12.05.08	26.05.08	09.06.08	23.06.08
Group 1 Niacin	**795**	3.50	3.10	3.60	5.00	5.40	5.00	5.10	3.80	4.20	3.10	2.00
	322	1.80	2.70	3.3	4.70	4.60	4.10	3.90	3.40	3.30	1.90	1.50
	339	2.00	2.80	2.7	3.40	3.60	4.00	3.30	4.30	3.60	2.50	2.40
	77	1.40	2.10	2.2	3.50	3.50	2.70	3.20	2.50	2.80	1.80	3.20
	Mean	**2.18**	**2.90**	**3.45**	**4.85**	**4.28**	**3.95**	**3.88**	**3.50**	**3.48**	**2.33**	**2.28**
	SD	**0.92**	**0.42**	**0.62**	**0.82**	**0.90**	**0.95**	**0.87**	**0.76**	**0.59**	**0.60**	**0.72**
Group 2 Control	**108**	1.00	1.60	1.60	1.40	1.70	1.60	1.80	1.80	1.90	1.80	1.50
	243	2.80	3.00	3.10	3.50	2.80	3.50	3.70	3.00	3.00	3.00	3.00
	215	2.70	2.40	2.20	2.40	2.20	2.50	2.60	3.00	3.20	3.40	2.90
	97	1.60	2.00	2.00	2.00	2.10	2.20	2.30	2.20	2.40	2.30	2.00
	Mean	**2.03**	**2.25**	**2.23**	**2.33**	**2.20**	**2.45**	**2.60**	**2.50**	**2.63**	**2.63**	**2.35**
	SD	**0.87**	**0.60**	**0.63**	**0.88**	**0.45**	**0.79**	**0.80**	**0.60**	**0.59**	**0.71**	**0.72**
p-value		0.82	0.29	0.15	0.02	0.01	0.05	0.08	0.09	0.09	0.54	0.89

Table A3: LDL-cholesterol (mmol/L), values during treatment phase highlighted: means (yellow), SD (green) and P-value (pink)

Treatment	No.	04.02.08	18.02.08	03.03.08	17.03.08	31.03.08	14.04.08	29.04.08	12.05.08	26.05.08	09.06.08	23.06.08
Group 1 Niacin	**795**	3.1	2.00	1.60	1.40	1.00	1.80	1.50	1.40	1.20	2.10	1.90
	322	2.10	1.40	0.60	1.60	1.10	2.50	2.10	1.80	1.60	1.50	1.30
	339	2.10	1.70	1.50	0.90	1.60	2.10	1.00	2.20	1.50	1.90	1.80
	77	3.00	2.20	1.40	2.00	3.70	3.00	2.70	1.80	2.00	2.40	2.20
	Mean	**2.58**	**1.83**	**1.28**	**1.48**	**1.85**	**2.35**	**1.83**	**1.80**	**1.58**	**1.98**	**1.80**
	SD	**0.55**	**0.35**	**0.46**	**0.46**	**1.26**	**0.52**	**0.74**	**0.33**	**0.33**	**0.38**	**0.37**
Group 2 Control	**108**	3.60	4.20	3.70	3.60	3.10	3.20	3.40	2.70	2.60	3.00	3.00
	243	2.80	3.00	3.20	3.00	2.50	2.50	2.20	2.10	2.00	1.90	2.00
	215	2.00	2.30	1.90	2.50	2.10	2.60	2.50	2.30	2.10	2.30	1.80
	97	1.40	1.20	1.30	1.40	1.40	1.40	1.40	1.30	1.40	1.50	1.20
	Mean	**2.45**	**2.68**	**2.53**	**2.63**	**2.28**	**2.43**	**2.38**	**2.10**	**2.03**	**2.18**	**2.00**
	SD	**0.96**	**1.26**	**1.11**	**0.93**	**0.71**	**0.75**	**0.83**	**0.59**	**0.49**	**0.64**	**0.75**
p-value		0.41	0.14	0.05	0.04	0.29	0.44	0.17	0.21	0.09	0.31	0.31

Table A4: triglycerides (mmol/L), values during treatment phase highlighted: means (yellow), SD (green) and P-value (pink)

Treatment	No.	04.02.08	18.02.08	03.03.08	17.03.08	31.03.08	14.04.08	29.04.08	12.05.08	26.05.08	09.06.08	23.06.08
Group 1 Niacin	**795**	0.8	0.59	0.56	0.41	0.91	1.9	1.33	0.46	0.54	1.09	0.79
	322	0.42	0.71	0.42	0.53	0.69	2.04	0.44	0.97	0.81	0.63	1.94
	339	0.33	0.37	0.19	0.40	0.31	0.87	0.65	0.48	0.42	0.26	0.43
	77	1.22	0.92	1.51	0.92	2.21	2.66	1.75	1.87	1.56	0.74	0.92
	Mean	**0.69**	**0.65**	**0.49**	**0.47**	**1.03**	**1.87**	**1.04**	**0.95**	**0.83**	**0.68**	**1.02**
	SD	**0.40**	**0.23**	**0.58**	**0.24**	**0.82**	**0.74**	**0.61**	**0.66**	**0.51**	**0.34**	**0.65**
Group 2 Control	**108**	0.41	0.78	0.61	0.36	0.87	1.72	0.36	1.24	0.57	0.63	0.44
	243	0.37	0.96	0.90	0.80	1.58	0.60	0.36	0.66	0.82	0.54	0.39
	215	1.21	1.77	0.76	0.64	1.85	1.13	0.81	0.56	0.69	0.62	0.95
	97	1.69	1.19	0.7	0.75	1.56	0.79	1.34	1.08	0.64	0.88	1.24
	Mean	**0.92**	**1.18**	**0.74**	**0.64**	**1.47**	**1.06**	**0.72**	**0.89**	**0.68**	**0.67**	**0.76**
	SD	**0.64**	**0.43**	**0.12**	**0.20**	0.42	0.49	**0.47**	**0.33**	**0.11**	**0.15**	**0.41**
p-value		0.57	0.09	0.82	0.66	0.40	0.13	0.43	0.88	0.60	0.95	0.52

Table A5: apoA-1 (gl/L), values during treatment phase highlighted: means (yellow), SD (green) and P-value (pink)

Treatment	No.	04.02.08	18.02.08	03.03.08	17.03.08	31.03.08	14.04.08	29.04.08	12.05.08	26.05.08	09.06.08	23.06.08
Group 1 Niacin	**795**	2.6	2.69	2.65	2.20	3.24	3.18	3.61	3.32	1.60	3.20	2.93
	322	1.80	2.41	2.63	2.36	3.07	3.14	2.96	3.00	2.68	2.01	1.74
	339	1.78	2.14	2.28	1.93	2.51	3.05	2.87	3.32	3.09	2.35	2.19
	77	1.73	2.12	2.36	2.49	3.27	2.66	2.84	2.77	2.65	2.05	2.00
	Mean	**1.99**	**2.34**	**2.64**	**2.28**	**3.02**	**2.64**	**3.07**	**3.10**	**2.51**	**2.40**	**2.22**
	SD	**0.37**	**0.27**	**0.19**	**0.24**	**0.35**	**0.24**	**0.36**	**0.27**	**0.64**	**0.55**	**0.51**
Group 2 Control	**108**	1.14	1.77	1.63	1.25	1.62	1.61	1.74	1.8	1.91	1.75	1.62
	243	2.02	2.49	2.22	2.22	2.29	2.55	2.53	2.52	2.58	2.5	2.46
	215	2.09	1.95	1.84	1.59	1.92	2.17	2.13	2.42	2.42	2.51	2.14
	97	1.92	2.17	2.01	1.66	2.14	2.14	2.3	2.28	2.36	2.32	2.12
	Mean	**1.79**	**2.10**	**1.93**	**1.68**	**1.99**	**2.12**	**2.18**	**2.26**	**2.32**	**2.27**	**2.09**
	SD	**0.44**	**0.31**	**0.25**	**0.40**	**0.29**	**0.39**	**0.33**	**0.32**	**0.29**	**0.36**	**0.35**
p-value		0.56	0.28	0.01	0.06	0.00	0.01	0.01	0.00	0.62	0.70	0.69

Table A6: Bodyweight (Kg)

Treatment	No.	04.02.08	18.02.08	03.03.08	17.03.08	31.03.08	14.04.08	29.04.08	12.05.08	26.05.08	09.06.08	23.06.08
Group 1 Niacin	**795**	3.56	3.38	3.30	3.20	3.17	3.12	3.18	3.28	3.29	3.38	3.62
	322	3.02	3.04	2.96	2.90	2.94	2.96	2.88	2.92	3.04	3.12	3.25
	339	3.29	3.35	3.28	3.20	3.05	3.12	2.90	3.10	3.33	3.40	3.42
	77	3.30	3.37	3.28	3.17	3.26	3.28	3.46	3.40	3.53	3.50	3.71
	Mean	**3.29**	**3.29**	**3.21**	**3.12**	**3.11**	**3.12**	**3.11**	**3.18**	**3.30**	**3.35**	**3.50**
	SD	**0.22**	**0.16**	**0.16**	**0.15**	**0.14**	**0.13**	**0.27**	**0.21**	**0.20**	**0.16**	**0.21**
Group 2 Control	**108**	3.55	3.75	3.75	3.72	3.78	3.7	3.78	3.81	3.76	3.84	3.76
	243	3.84	3.93	3.93	3.9	3.90	3.84	3.9	3.95	4.0	4.00	4.05
	215	3.68	3.66	3.66	3.58	3.63	3.52	3.58	3.6	3.64	3.58	3.6
	97	3.50	3.55	3.62	3.54	3.74	3.7	3.82	3.4	3.9	4.02	4.06
	Mean	**3.64**	**3.72**	**3.74**	**3.69**	**3.76**	**3.69**	**3.77**	**3.69**	**3.83**	**3.86**	**3.87**
	SD	**0.15**	**0.16**	**0.14**	**0.16**	**0.11**	**0.13**	**0.14**	**0.24**	**0.16**	**0.20**	**0.23**
p-value		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.05

3. LOG SHEET SAMPLE

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	vocalization

Check list: motor function and activity

0 = absent, not observed, not displayed + = weak, poor, minimal

++ = normal, average, moderate +++ = excessive, strong

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

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

APPENDIX II: MOLECULAR BIOLOGY

1. PURIFICATION OF GENOMIC DNA FROM WHOLE BLOOD

AxyPrep Blood Genomic DNA Miniprep Kit was used to extract DNA from whole blood of the African green monkeys. This method is based on the efficient release of genomic DNA from anti-coagulated whole blood by a special cell lysis and heme/protein precipitation buffer (Buffer AP1) coupled with the selective adsorption of the genomic DNA to a special AxyPrep column. The purified genomic DNA is eluted in a low-salt Tris buffer containing 0.5 mM EDTA, which enhances DNA solubility and helps to protect the high molecular weight DNA against subsequent nuclease degradation. Blood genomic DNA is directly isolated from the white blood cell (WBC) component of whole blood, without the need to remove the red blood cells (RBCs) in advance.

The procedure was performed according to the manufacturer's instructions. For maintaining the integrity and reactivity of the genomic DNA, particularly in PCR, the purified genomic DNA was eluted and stored in low-salt Tris buffer containing 0.5-1 mM EDTA.

2. SPECTROPHOTOMETRIC QUANTIFICATION OF NUCLEIC ACIDS (Maniatis, 1989)

DNA quantification was performed at the wavelengths of 260 nm and 280 nm. The reading at 260nm allows calculation of the concentration of nucleic acid in the sample, where an OD of 1 corresponds to approximately 50u g/mL for double-stranded DNA. The ratio between the readings at 260nm and 280 nm (OD_{260}/OD_{280})

provides an estimate of the purity of the nucleic acid, with pure preparations of DNA giving OD_{260}/OD_{280} values of 1.8. Accurate quantification of the amount of nucleic acid is only possible if there is no protein or phenol contamination of the sample.

3. SEQUENCING REACTIONS

Sequencing was performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). Approximately 200 ng DNA was used per reaction, and half shots were used (Table A7)

Table A7: Sequencing reactions, as described in the Perkin-Elmer ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit manual (1998).

Reagent	Quantity
Terminator Ready Reaction mix (dye terminators, dUTP*, dCTP, dATP, dITP**, AmpliTaq DNA polymerase, r*Tth* pyrophoshatase, magnesium chloride, buffer)	8 μL
DNA template	100 - 200 ng
-21 M13 Primer (forward)	1.6 pmol
$dddH_2O$	x
Total volume	10 μL

* dITP is used in place of dGTP to minimise band compressions

** dUTP is used in place of dTTP as it results in a better T patterns because dUTP improves incorporation of T terminators.

The reagents were vortexed then spun briefly before the PCR sequencing reaction was started (Table A8).

Table A8: Sequencing reaction using a Hybaid PCR Sprint Thermal Cycler

	Temperature	**Time**
Denaturing	96°C	10 seconds
Annealing	50°C	5 seconds
Extension	60°C	4 minutes
Repeat this sequence for 25 cycles, then store at 4°C before purification		

4. ELECTROPHORESIS

4.1 AGAROSE GEL ELECTROPHORESIS

Agarose gels were made by dissolving the appropriate amount of agarose in 1X TBE buffer (12.1 g Tris, 0.37 g EDTA and 5.14 g Boric acid made up to 1L and adjusted to pH 8.4 with 1 M HCl) for 0.8 - 1.5 % gels, depending on the fragment size loaded onto the gel. Genomic DNA was run on 0.8% gels, whereas 1.5% gels were used with fragment sizes of 190 bp and 550 bp. The agarose gels were electrophoresed in TBE buffer at a voltage range between 80 - 120 V for approximately 1 hour. Samples were loaded into the wells with 10% tracking dye. 0.5 μg/mL ethidium bromide added to the gels to allow visualisation of DNA when it was placed on a UV transilluminator, which caused any DNA bound to ethidium bromide to fluoresce.

Tracking Dye III (Maniatis *et al*, 1993)

0.25% bromophenol blue
0.25% xylene cyanol FF
30% glycerol in ddH_2O
Store at 4°C

Markers

A 100bp DNA Ladder from Promega was used. This Ladder is ready for 5´ end-labeling with radioisotopes using T4 Polynucleotide Kinase, allowing visualization by autoradiography. A Blue/Orange Loading Dye, 6X, was provided.

5. DNA SEQUENCES

Table A9(1): Oligonucleotide sequences of SNPs

Gene	SNP Id	Forward 5'	Reverse 3'	Position	Allele 1/2	Temp (°C)
ABCA1	Ile883Met	GAGAAGAGCCACCC TGGTTCCAACCAGA AGAGGAT	AAGGCAGGAGACA TCGCTT	Exon 18 (H7)	T/C	54
	E1172D	GAGCAGTTCTGATGC TGGCCTGGGCAGCGA CCACGA	TCTGCACCTCTCCTC CTCTG	Exon 24 (R_1)	C/G	51
	V771M	CAAGTGAGTGCTTGG GATTG	TGCTTTTATTCAGGG ACTCCA	Exon 16 (5^{th} trm)	G/A	55
	V825I	CCCATGCACTGCAGA GATTC	GCAAATTCAAATTTC TCCAGG	Exon 17 (6^{th} trm)	G/A	46
	R219K	GTATTTTTGCAAGGCT ACCAGTTACATTTGACAA	GATTGGCTTCAGGAT GTCCATGTTGGAA	Exon 7 (Ex N-term loop)	C/T	54
CETP	I405V	CTCACCATGGGCATTT GATTGCAGAGCAGCTC CGACTCC	AATGGGAAGCTCTGT CAGCCTCGGCCACCC AG	Exon 14	A/G	56
	Ala373Pro	CACAGCAAATTTGGTT TCTCTCC	CCCAGTCTATCCAAG ACTAC	Exon 12	C/G	54
CYP7A1	Asn233Ser	TCAGTTCTGAGATGCT TTCCC	AGTCTTTCCAGCCCTG GTAG	Exon 3	C/T	54
	A-278C	AGTCCACAGGTATCAG AAGTG	CCCCAGGTCCGAATG TTAAG	Promoter	T/G	53
apoE	Cys112Arg (E4)	TAAGCTTGGCACGGCT GTCCAAGGACCCGGCT GGGCGCGGACAT	AGAGAATTCGCCCCG GCCTGGTACACCGCTT CGCGGATGGCGCTGA	Exon 3	T/C	52
	Arg158Cys (E2)	CCCGGCTGGGCGCGGA CAT	CGCTTCGCGGATGGC GCTGA	Exon 3	C/T	52
apoA-1	*Msp1*	ACTCTTAAGTT CCACATTGCCAGGAC	CTCTGTGCCCTTCTC CTCAC	5'UTR	G/A	56
	G-75A	AGGGACAGAGCTGATCC TTGAACTCTTAAG	TTAGGGGACACCTAG CCCTCAGGAAGAGCA	Promoter	G/A	51
apoB	T71I	AATGCTCTGCTACCCTG AAT	AACACACAAGTTCAT ACCTC	Exon 4	C/T	55
	4311S	CTGGCTTGCTAACCTCTCT G	GAGAAGCTTCCTGAA GCTCG	Exon 29	T/C	52
LCAT	Ser232Thr	CTCATTGGCCACAGCCTC	GGTGAGACCAAGCTG ATCCT	Exon 5	T/A	53
	LCATu3	TATGAGGATGGTGATGAC ACGGT	TTTGCTACCGTAAGCCC TG	Exon 6	C/T	50
apoC-I	*HpaI*	ATCGATCACGACCCTCTC	GCTACATTCTGAGTG GGGGA	Promoter	T/A	51
apoC-II	Leu96Arg	CTATGACGACCACCTGAGGG	TGTCCCTGTCTGGATCC TTC	Promoter	T/G	53
	-62 A>C	GAGAAGGTTCCCTGTGACGT GACCTT	CAAAGATCGATAAAGCA	Promoter	A/C	52
SR-B1	A350A	TCCTTGTCTGATGTCCCCT C	TAAGGAACTTTGGTG GCTCG	Exon 8	C/T	51
	G2S	CCTCACCCACCTTAAGGA CC	CCTGTGTCGTCTCTGT CGC	Exon 1	G/A	49

Table A9(2): The pattern of coincident SNPs (Hodgkinson et al., 2009)

	SNP	Old World Monkey					
		C/T	G/A	C/A	G/T	C/G	A/T
Human	C/T	3 840	11	181	98	197	73
	G/A	14	3 708	95	171	189	101
	C/A	226	107	291	3	48	27
	G/T	114	254	0	304	48	16
	C/G	190	194	46	51	217	3
	A/T	81	89	33	19	0	532

6. STATISTICAL ANALYSIS

6.1 Standard deviation

Statistical computations were performed using Computer software. The data pertaining to anthropometric measurements were subjected to the mean value analysis.

$$\overline{X} \quad \frac{\Sigma X}{n}$$

Where X = Arithmetic Mean

ΣX = Arithmetic Mean

n = Total number of variables

To find out the degree of dispersion of recorded data around the mean, standard

$$SD = \sqrt{\frac{\sum X-\overline{X})^2}{n}}$$

Where SD = Standard deviation

$X-\overline{X}$ = Deviation of value from the mean

n = Total number of observations in the sample.

6.2 Student 't' test

Two –tailed Student 't' test was performed to find the difference in the mean values of control and experimental groups of the African green monkeys.

$$t = \frac{|\overline{X}_1 - \overline{X}_2|}{S\sqrt{1/n_1 + 1/n_2}}$$

$$S = \sqrt{\frac{S_{i2}n1 + S_2n2}{n_1+n_2-2}}$$

Where $\overline{X}_1$ = Mean of first sample with standard deviation S_1.

$\overline{X}_2$ = Mean of Second Sample with standard deviation S_2

S = Combined standard deviation

The level of significance and noted from 't' table against the degree of freedom (df).

$$df = n_1+n_2-2$$

6.3 The Pearson correlation coefficient

The Pearson correlation measures the correlation or strength of linear dependence between two variables X and Y.

It returns values between +1 and −1 inclusive.

- 1 implies that Y increases as X increases.
- 0 implies that there is no linear correlation between the variables.
- −1 implies that Y decreases as X increases.

A correlation coefficient 'r' is an index that measures the strength of a relationship between the two variables in a given set of data. It was used to find the correlation between HDL-C and expression profiles of selected lipid metabolism genes. The values of the correlation coefficient lie between +1 and -1 for complete positive and negative correlation. Complete correlation describes a situation where all readings lie on a straight line having either positive or negative slope. Correlation coefficient near 'zero' represents situation where there is no particular tendency for two sets of data to cover linearly.

Correlation coefficient 'r' is given by the following formula:

$$r = \frac{[(\Sigma XY)] - [\{(\Sigma X)\ (\Sigma Y)\}/n]}{[\{(\Sigma X^2) - (\Sigma X)^2\}/N]\ [\{(\Sigma Y^2) - (\Sigma Y)^2\}/N]}$$

where r = Correlation coefficient.

ΣXY = Sum of cross-products of variables 'X' and 'Y' for 'n' subjects.

ΣX = Sum of individual values of 'X' of 'n' subjects.

ΣY = Sum of individual values of 'Y' and 'n' subjects

X and Y = Mean values of variables 'X' and 'Y'

N = Number of pairs of observations

A significant correlation was tested by applying 't' test.

$$t = r\sqrt{(n-2) / (1-r)}$$

The value was checked at degree of freedom (df = n-2).

Table A10: Correlation coefficient between HDL-C and gene expression profiles during niacin treatment intervention study

Time point	HDL-C	CETP	ABCA1	LCAT	SR-B1	APOA-1	APOE	APOC1	APOB	CYP7A1	APOC11
04.02.08	2.18	1.21	0.36	0.45	0.71	0.80	0.31	1.05	0.00	4.57	0.93
18.02.08	2.90	1.80	0.96	1.00	0.82	0.52	3.93	0.76	0.00	1.33	3.28
03.03.08	3.45	1.18	2.35	0.25	0.73	1.22	1.95	0.55	0.00	1.64	0.05
17.03.08	4.85	1.22	1.03	0.86	0.77	0.00	5.65	0.57	0.00	0.54	0.40
31.03.08	4.28	2.60	1.51	0.80	0.68	0.12	5.62	0.48	0.00	0.95	0.04
14.04.08	3.95	0.79	0.79	0.79	1.83	3.34	6.51	0.45	0.64	3.36	0.00
29.04.08	3.88	0.92	0.62	1.56	1.62	1.78	0.94	0.65	0.45	2.88	0.00
12.05.08	3.50	0.86	0.52	0.53	1.34	0.04	1.04	1.00	0.56	2.29	0.00
26.05.08	3.48	0.69	0.58	0.55	0.87	2.58	0.60	1.29	0.63	1.55	0.26
09.06.08	2.33	2.19	2.77	1.03	2.67	1.34	2.69	1.34	2.67	0.43	0.00
p-value		**p < 0.001**	**p < 0.001**	**p < 0.001**	**p < 0.001**	**p < 0.001**	**0.49**	**p < 0.001**	**p < 0.001**	**0.03**	**1.45**
ation coefficient (r)		**-0.14**	**-0.17**	**0.16**	**-0.27**	**-0.01**	**0.56**	**-0.69**	**-0.95**	**-0.30**	**-0.33**

* r = (a value between -1 and +1),

7. GENE EXPRESSION DATA

Table: A11: CETP mRNA expression (a.u)

CETP	Baseline:	04.02.08	Week 2:	18.02.08	Week 4:	03.03.08	Week 6:	17.03.08	Week 8:	31.03.08	Week 10:	14.04.08	Week 12:	29.04.08	Week 14:	12.05.08	Week 16:	26.05.08	Week 18:	09.06.08
	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin
	0.01	0.03	0.01	0.03	0.00	0.00	0.03	0.00	0.01	0.02	0.01	0.01	0.05	0.03	0.01	0.00	0.00	0.01	0.00	0.03
	0.01	0.03	0.00	0.03	0.01	0.01	0.00	0.02	0.01	0.00	0.00	0.02	0.00	0.00	0.02	0.00	0.01	0.02	0.00	0.00
	0.03	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.00	0.01	0.00	0.03	0.01	0.02	0.02	0.00	0.01	0.00
	0.01	0.01	0.02	0.00	0.01	0.01	0.01	0.02	0.01	0.05	0.00	0.02	0.00	0.01	0.01	0.01	0.01	0.03	0.00	0.04
mean	0.01	0.02	0.01	0.02	0.01	0.01	0.02	0.02	0.01	0.02	0.02	0.01	0.02	0.02	0.02	0.01	0.02	0.01	0.01	0.02
sd	0.01	0.02	0.01	0.02	0.00	0.02	0.01	0.01	0.00	0.02	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
p-value	**0.77**		**0.43**		**0.76**		**0.78**		**0.37**		**0.04**		**0.84**		**0.33**		**0.41**		**0.29**	

Table: A12: ABCA1 mRNA expression (a.u)

ABCA1	Baseline:	04.02.08	Week 2:	18.02.08	Week 4:	03.03.08	Week 6:	17.03.08	Week 8:	31.03.08	Week 10:	14.04.08	Week 12:	29.04.08	Week 14:	12.05.08	Week 16:	26.05.08	Week 18:	09.06.08
	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin
	0.01	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.01	0.02	0.00	0.01	0.01	0.01	0.02	0.01	0.00	0.01	0.00	0.01
	0.03	0.01	0.01	0.01	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.01	0.02	0.01
	0.01	0.00	0.01	0.01	0.00	0.00	0.01	0.00	0.01	0.01	0.01	0.00	0.01	0.02	0.01	0.01	0.02	0.01	0.01	0.00
	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.03	0.00	0.02	0.00	0.01	0.01	0.01	0.02
mean	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
sd	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.01	0.00	0.01	0.00	0.01	0.01
p-value	**0.21**		**0.92**		**0.36**		**0.94**		**0.39**		**0.62**		**0.48**		**0.16**		**0.20**		**0.97**	

Table: A13: LCAT mRNA expression (a.u)

LCAT	Baseline:	04.02.08	Week 2:	18.02.08	Week 4:	03.03.08	Week 6:	17.03.08	Week 8:	31.03.08	Week 10:	14.04.08	Week 12:	29.04.08	Week 14:	12.05.08	Week 16:	26.05.08	Week 18:	09.06.08
	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin
	0.00	1.25	0.26	0.83	0.65	0.58	0.39	1.00	0.56	0.93	0.24	0.58	0.06	0.33	0.24	0.24	0.28	0.11	0.00	1.18
	1.12	0.38	0.69	1.24	0.25	0.15	0.51	0.35	0.29	0.54	0.10	0.21	0.08	0.23	0.31	0.00	0.43	0.31	0.29	0.30
	0.36	0.00	0.47	0.25	0.66	0.33	0.43	0.22	0.54	0.37	0.20	0.20	0.24	0.29	0.28	0.21	0.16	0.10	0.40	0.40
	1.85	0.28	0.91	0.00	2.69	0.00	1.28	0.66	1.29	0.30	1.02	0.25	0.24	0.13	0.87	0.45	0.62	0.30	0.89	0.28
mean	0.83	0.48	0.58	0.58	1.06	0.26	0.65	0.56	0.67	0.53	0.39	0.31	0.16	0.24	0.42	0.23	0.37	0.20	0.53	0.54
sd	0.83	0.54	0.28	0.56	1.10	0.25	0.42	0.35	0.43	0.28	0.42	0.18	0.10	0.09	0.30	0.19	0.20	0.12	0.32	0.43
p-value	**0.50**		**1.00**		**0.24**		**0.75**		**0.62**		**0.73**		**0.23**		**0.31**		**0.20**		**0.62**	

Table: A14: SRB1 mRNA expression (a.u)

SRB1	Baseline:	04.02.08	Week 2:	18.02.08	Week 4:	03.03.08	Week 6:	17.03.08	Week 8:	31.03.08	Week 10:	14.04.08	Week 12:	29.04.08	Week 14:	12.05.08	Week 16:	26.05.08	Week 18:	09.06.08
	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin
	0.03	0.16	0.08	0.10	0.12	0.08	0.05	0.08	0.10	0.11	0.02	0.03	0.04	0.15	0.03	0.04	0.03	0.06	0.00	0.26
	0.18	0.07	0.06	0.08	0.07	0.05	0.09	0.04	0.07	0.05	0.02	0.06	0.08	0.10	0.08	0.17	0.06	0.05	0.04	0.18
	0.11	0.00	0.07	0.06	0.09	0.06	0.05	0.03	0.10	0.06	0.04	0.08	0.07	0.10	0.06	0.08	0.03	0.04	0.08	0.18
	0.12	0.09	0.08	0.00	0.09	0.08	0.07	0.05	0.14	0.05	0.04	0.06	0.06	0.06	0.10	0.06	0.07	0.03	0.08	0.09
mean	0.11	0.08	0.07	0.06	0.09	0.07	0.07	0.05	0.10	0.07	0.03	0.06	0.06	0.10	0.07	0.09	0.05	0.04	0.12	0.07
sd	0.06	0.06	0.01	0.04	0.02	0.01	0.02	0.02	0.03	0.03	0.01	0.02	0.02	0.04	0.06	0.03	0.02	0.01	0.02	0.07
p-value	**0.51**		**0.58**		**0.08**		**0.35**		**0.16**		**0.08**		**0.13**		**0.52**		**1.00**		**0.03**	

Table: A15: apoCI mRNA expression (a.u)

apoCI	Baseline:	04.02.08	Week 2:	18.02.08	Week 4:	03.03.08	Week 6:	17.03.08	Week 8:	31.03.08	Week 10:	14.04.08	Week 12:	29.04.08	Week 14:	12.05.08	Week 16:	26.05.08	Week 18:	09.06.08
	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin
	3E-04	3E-04	2E-04	7E-04	5E-04	0E+00	7E-04	4E-04	4E-04	5E-04	7E-05	4E-05	2E-04	4E-04	0E+00	0E+00	0E+00	7E-05	0E+00	0E+00
	2E-03	2E-04	1E-03	4E-04	5E-04	3E-04	1E-04	3E-04	3E-04	4E-04	8E-05	2E-05	5E-05	5E-04	0E+00	0E+00	2E-05	0E+00	4E-03	2E-03
	2E-04	2E-03	2E-04	4E-04	3E-04	4E-04	6E-04	3E-04	2E-04	2E-04	1E-04	4E-05	5E-05	8E-05	2E-05	2E-05	1E-04	0E+00	6E-05	2E-03
	4E-04	3E-04	5E-04	0E+00	9E-04	2E-04	3E-04	0E+00	2E-03	2E-04	6E-05	4E-05	1E-03	0E+00	0E+00	0E+00	9E-05	0E+00	2E-04	1E-03
mean	8E-04	8E-04	5E-04	4E-04	5E-04	3E-04	4E-04	2E-04	7E-04	3E-04	8E-05	4E-05	4E-04	2E-04	2E-05	2E-05	6E-05	7E-05	1E-03	2E-03
sd	4E-04	3E-04	5E-04	7E-04	9E-04	0E+00	3E-04	4E-04	2E-03	5E-04	6E-05	4E-05	1E-03	4E-04	0E+00	0E+00	9E-05	7E-05	2E-04	0E+00
p-value	**0.96**		**0.68**		**0.08**		**0.32**		**0.44**		0.04		**0.68**		**1.00**		**0.29**		**0.76**	

Table: A16: apoB mRNA expression (a.u)

apoB	Baseline:	04.02.08	Week 2:	18.02.08	Week 4:	03.03.08	Week 6:	17.03.08	Week 8:	31.03.08	Week 10:	14.04.08	Week 12:	29.04.08	Week 14:	12.05.08	Week 16:	26.05.08	Week 18:	09.06.08
	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin
											9E-05	3E-04	7E-04	0E+00	7E-04	2E-03	2E-04	3E-04	1E-02	1E-02
											7E-04	1E-04	3E-04	6E-04	8E-04	0E+00	3E-04	2E-04	5E-04	1E-02
											2E-04	2E-04	2E-04	5E-04	4E-04	4E-04	1E-03	4E-04	6E-04	2E-02
											4E-04	3E-04	2E-03	4E-04	2E-03	3E-04	4E-04	2E-04	0E+00	7E-03
mean											3E-04	2E-04	8E-04	4E-04	1E-03	5E-04	5E-04	3E-04	5E-03	1E-02
sd											4E-04	3E-04	2E-03	0E+00	2E-03	2E-03	4E-04	3E-04	6E-04	1E-02
p-value											**0.42**		**0.38**		**0.39**		**0.43**		**0.06**	

Table: A17: apoCII mRNA expression (a.u)

apoCII	Baseline:	04.02.08	Week 2:	18.02.08	Week 4:	03.03.08	Week 6:	17.03.08	Week 8:	31.03.08	Week 10:	14.04.08	Week 12:	29.04.08	Week 14:	12.05.08	Week 16:	26.05.08	Week 18:	09.06.08
	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin
	2E-05	5E-04	7E-05	2E-03	1E-03	0E+00	2E-05	0E+00	0E+00	3E-05	2E-04	0E+00	0E+00	1E-03	0E+00	0E+00	0E+00	5E-05	0E+00	0E+00
	9E-04	2E-05	4E-05	0E+00	3E-05	2E-05	1E-04	0E+00	5E-04	0E+00	1E-04	0E+00	0E+00	0E+00	0E+00	0E+00	1E-04	0E+00	0E+00	0E+00
	5E-05	1E-03	0E+00	0E+00	7E-04	0E+00	0E+00	1E-04	4E-05	0E+00	2E-05	0E+00	0E+00	0E+00	0E+00	0E+00	3E-04	0E+00	0E+00	0E+00
	9E-05	0E+00	0E+00	0E+00	0E+00	4E-05	9E-04	1E-04	2E-03	6E-04	0E+00	0E+00	0E+00	6E-04	0E+00	0E+00	0E+00	0E+00	0E+00	0E+00
mean	3E-04	2E-04	5E-05	2E-03	6E-04	3E-05	3E-04	1E-04	8E-04	3E-05	1E-04	0E+00	0E+00	1E-03	0E+00	0E+00	0E+00	5E-05	0E+00	0E+00
sd	9E-05	5E-04	0E+00	2E-03	0E+00	0E+00	9E-04	0E+00	3E-05	2E-03	0E+00	0E+00	0E+00	1E-03	0E+00	0E+00	0E+00	5E-05	0E+00	0E+00
p-value	**0.63**		**0.42**		**0.20**		**0.43**		**0.41**		**0.15**		**0.23**				**0.28**			

Table: A18: apoAI mRNA expression (a.u)

apoAI	Baseline:	04.02.08	Week 2:	18.02.08	Week 4:	03.03.08	Week 6:	17.03.08	Week 8:	31.03.08	Week 10:	14.04.08	Week 12:	29.04.08	Week 14:	12.05.08	Week 16:	26.05.08	Week 18:	09.06.08
	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin
	1E-05	8E-06	0E+00	8E-05	3E-05	2E-04	0E+00	0E+00	0E+00	0E+00	0E+00	8E-07	7E-06	0E+00	0E+00	0E+00	0E+00	0E+00	1E-03	0E+00
	6E-05	6E-05	1E-04	3E-05	0E+00	3E-05	1E-05	0E+00	0E+00	0E+00	2E-06	6E-06	9E-07	2E-05	3E-05	4E-06	1E-05	5E-04	0E+00	2E-03
	0E+00	0E+00	0E+00	0E+00	2E-05	1E-05	0E+00	0E+00	4E-04	2E-05	3E-05	1E-04	2E-04	0E+00	1E-04	0E+00	0E+00	2E-06	2E-04	3E-04
	4E-05	3E-05	0E+00	0E+00	8E-05	1E-05	2E-05	0E+00	0E+00	3E-05	2E-04	0E+00	0E+00	0E+00	4E-06	2E-05	0E+00	2E-06	0E+00	0E+00
mean	4E-05	3E-05	1E-04	6E-05	4E-05	5E-05	1E-05	2E-04	0E+00	2E-05	2E-05	5E-05	6E-05	1E-04	0E+00	2E-06	7E-06	2E-04	6E-04	9E-04
sd	3E-05	3E-05	0E+00	4E-05	4E-05	7E-05	0E+00	0E+00	2E-04	2E-07	2E-05	8E-05	9E-05	1E-04	5E-05	3E-06	7E-06	3E-04	6E-04	1E-03
p-value	**0.77**		**0.97**		**0.64**		**0.23**		**0.45**		**0.81**		**0.43**		**0.31**		**0.40**		**0.63**	

Table: A19: CYP7A1 mRNA expression (a.u)

CYP7A1	Baseline:	04.02.08	Week 2:	18.02.08	Week 4:	03.03.08	Week 6:	17.03.08	Week 8:	31.03.08	Week 10:	14.04.08	Week 12:	29.04.08	Week 14:	12.05.08	Week 16:	26.05.08	Week 18:	09.06.08
	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin
	0.00	10.34	0.04	0.50	4.34	0.00	14.74	0.11	5.42	0.74	0.00	0.01	0.00	0.00	0.00	0.42	0.00	0.15	1.30	0.23
	4.85	7.35	0.38	3.90	0.30	0.19	10.02	6.58	0.00	0.29	0.00	0.05	0.00	0.29	0.04	0.00	0.11	2.53	1.70	0.62
	0.00	3.00	0.27	0.43	3.58	0.30	6.09	0.44	0.04	0.12	0.00	0.13	0.00	0.00	0.00	0.03	0.00	0.00	0.15	0.00
	1.95	6.08	0.00	0.00	0.00	12.95	4.84	0.00	0.00	5.76	0.00	2.60	0.00	1.51	0.01	0.00	0.00	0.00	0.00	0.00
mean	1.70	6.69	0.17	1.61	2.05	3.36	4.42	2.38	1.82	1.73	0.00	0.70	0.00	0.90	0.02	0.42	0.06	0.90	1.00	0.43
sd	0.95	1.52	0.00	0.50	0.00	0.00	0.84	0.11	0.00	0.74	0.00	0.01	0.00	0.42	0.00	0.00	0.00	0.01	0.00	0.23
p-value	0.04		**0.34**		**0.72**		0.04		**0.86**		**0.35**		**0.30**		**0.40**		**0.38**		**0.27**	

Table: A20: apoE mRNA expression (a.u)

apoE	Baseline:	04.02.08	Week 2:	18.02.08	Week 4:	03.03.08	Week 6:	17.03.08	Week 8:	31.03.08	Week 10:	14.04.08	Week 12:	29.04.08	Week 14:	12.05.08	Week 16:	26.05.08	Week 18:	09.06.08
	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin	control	niacin
	6E-05	1E-04	1E-04	1E-03	2E-04	1E-03	9E-04	1E-03	2E-04	3E-04	1E-04	2E-04	1E-04	5E-05	5E-04	6E-04	7E-05	3E-05	0E+00	4E-03
	1E-03	2E-04	2E-04	1E-03	2E-04	1E-04	3E-03	0E+00	7E-04	7E-05	2E-04	2E-04	8E-05	4E-05	3E-04	0E+00	9E-05	2E-04	4E-03	0E+00
	9E-05	0E+00	9E-05	2E-04	1E-04	9E-05	1E-04	9E-04	1E-04	1E-04	9E-05	2E-04	5E-05	4E-05	1E-04	5E-04	3E-04	1E-04	3E-04	9E-03
	1E-04	1E-04	3E-04	0E+00	3E-04	2E-04	1E-03	2E-03	4E-04	1E-04	5E-05	4E-04	2E-04	8E-05	7E-04	1E-04	2E-04	1E-04	2E-04	3E-03
mean	3E-04	1E-04	2E-04	8E-04	2E-04	4E-04	2E-04	9E-04	2E-04	1E-03	5E-05	3E-04	1E-04	1E-04	4E-04	4E-04	2E-04	1E-04	2E-03	4E-03
sd	6E-04	2E-04	3E-04	1E-03	3E-04	1E-03	1E-04	1E-03	4E-04	9E-04	8E-05	2E-04	2E-04	1E-04	7E-04	6E-04	2E-04	3E-05	2E-04	4E-03
p-value	**0.43**		**0.24**		**0.49**		**0.58**		**0.24**		**0.06**		**0.14**		**0.67**		**0.35**		**0.23**	

8. STANDARD CURVES

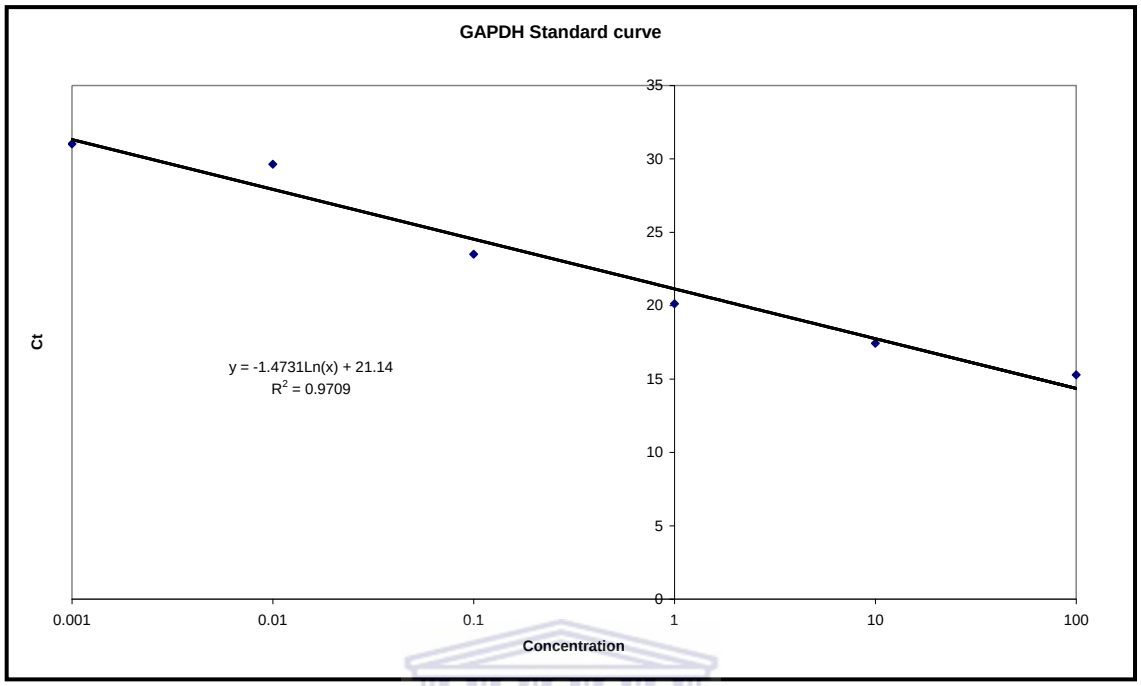

Figure A1: GAPDH Standard curve

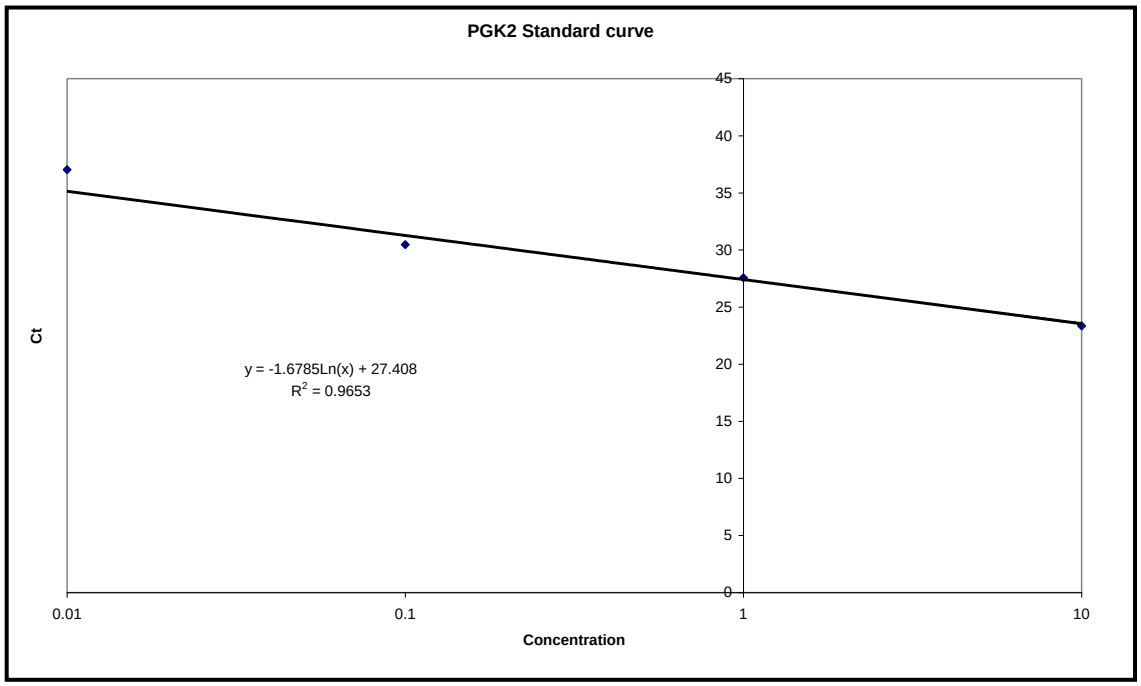

Figure A2: PGK2 Standard curve

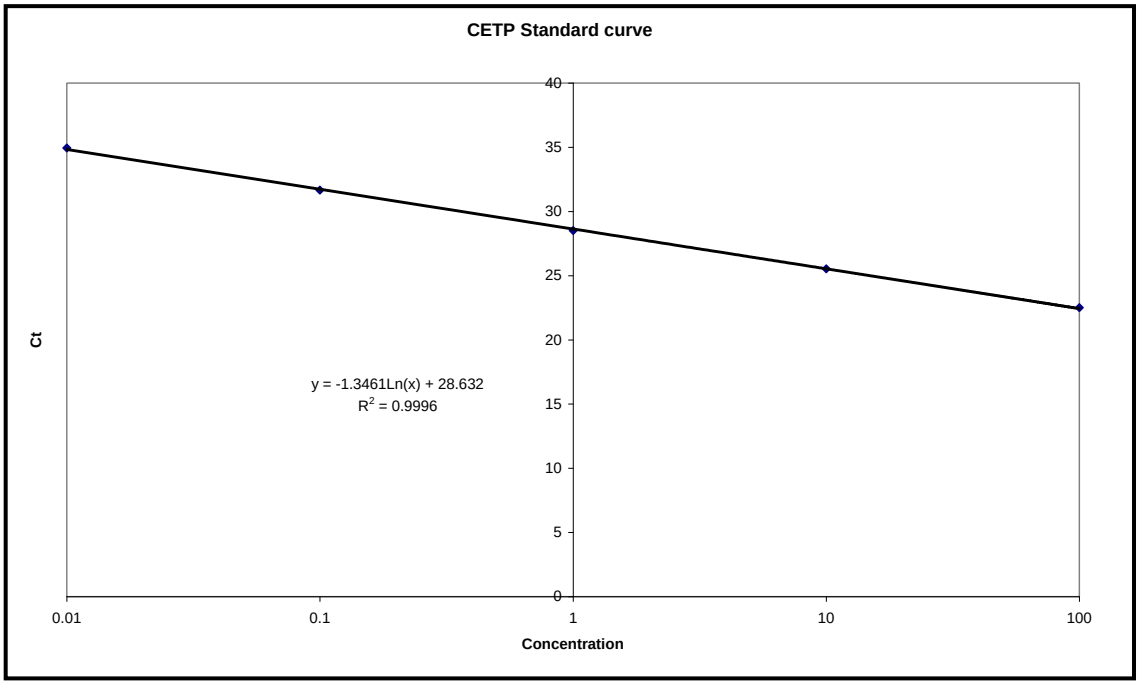

Figure A3: CETP Standard curve

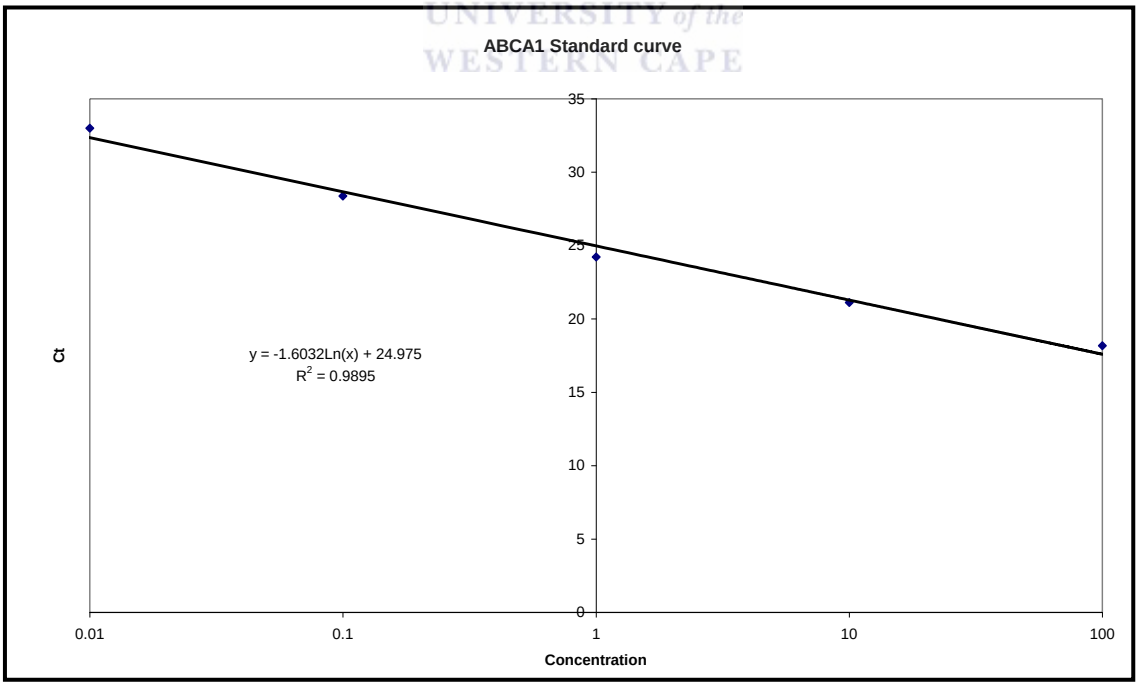

Figure A4: ABCA1 Standard curve

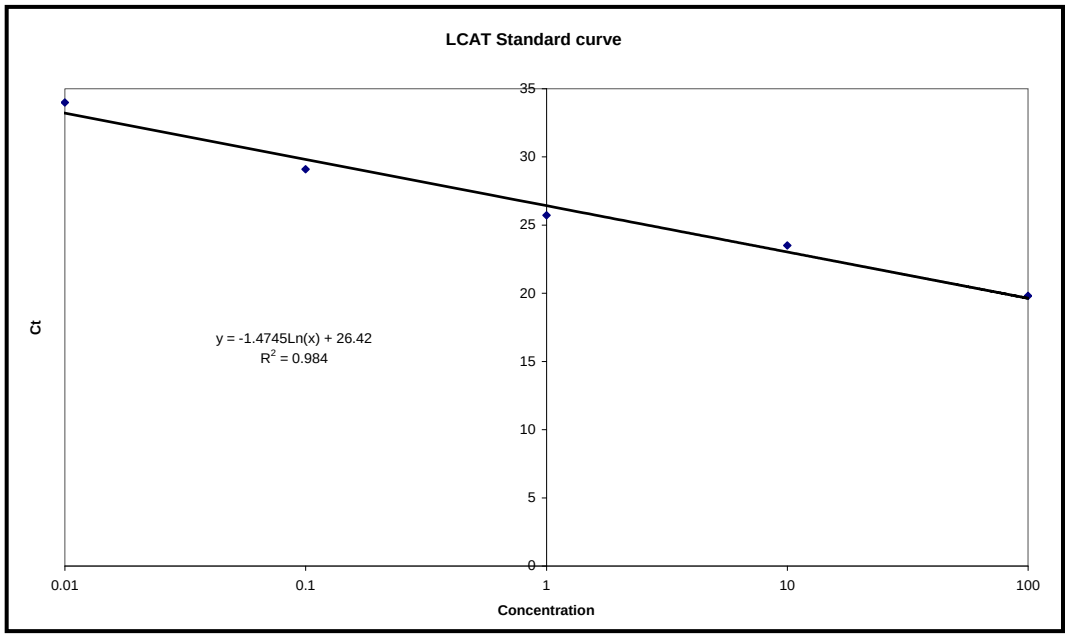

Figure A5: LCAT Standard curve

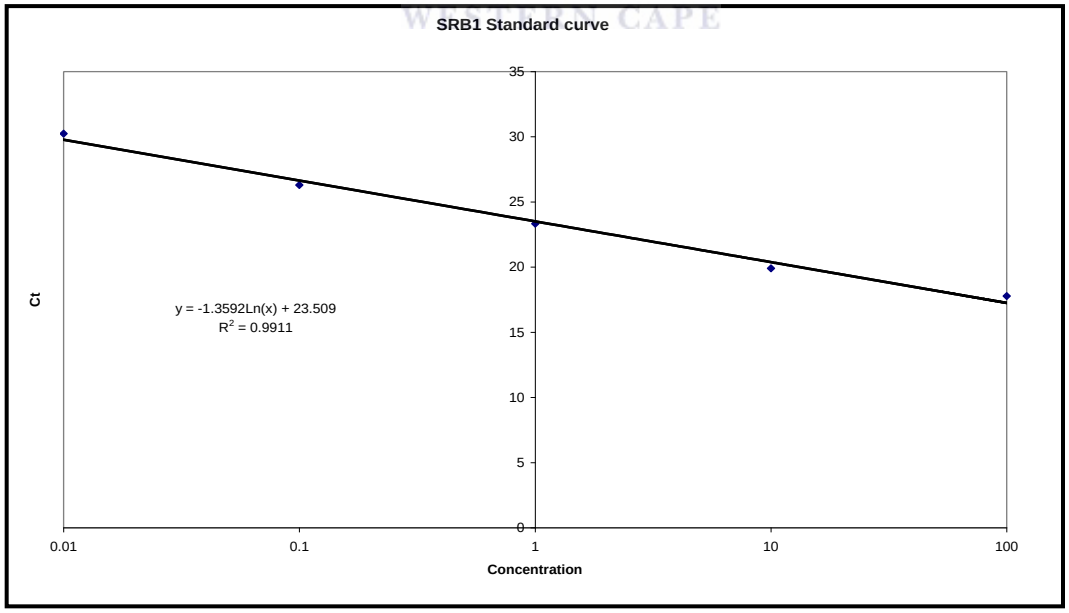

Figure A6: SRB1 Standard curve

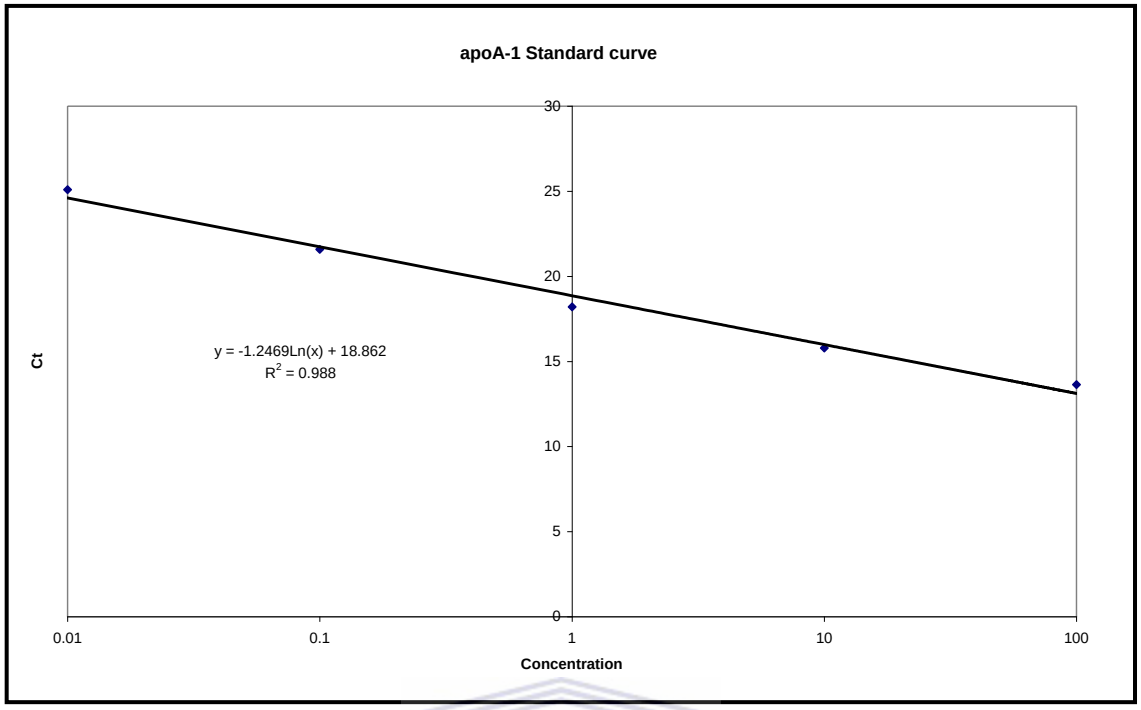

Figure A7: apoA-1 Standard curve

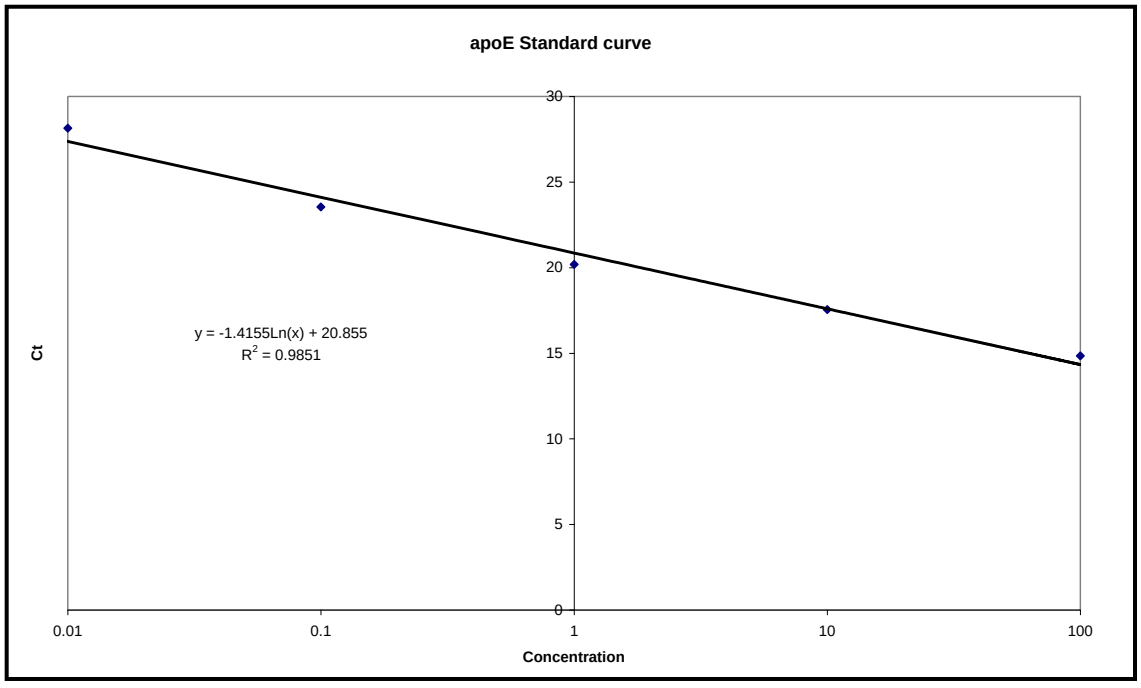

Figure A8: apoE Standard curve

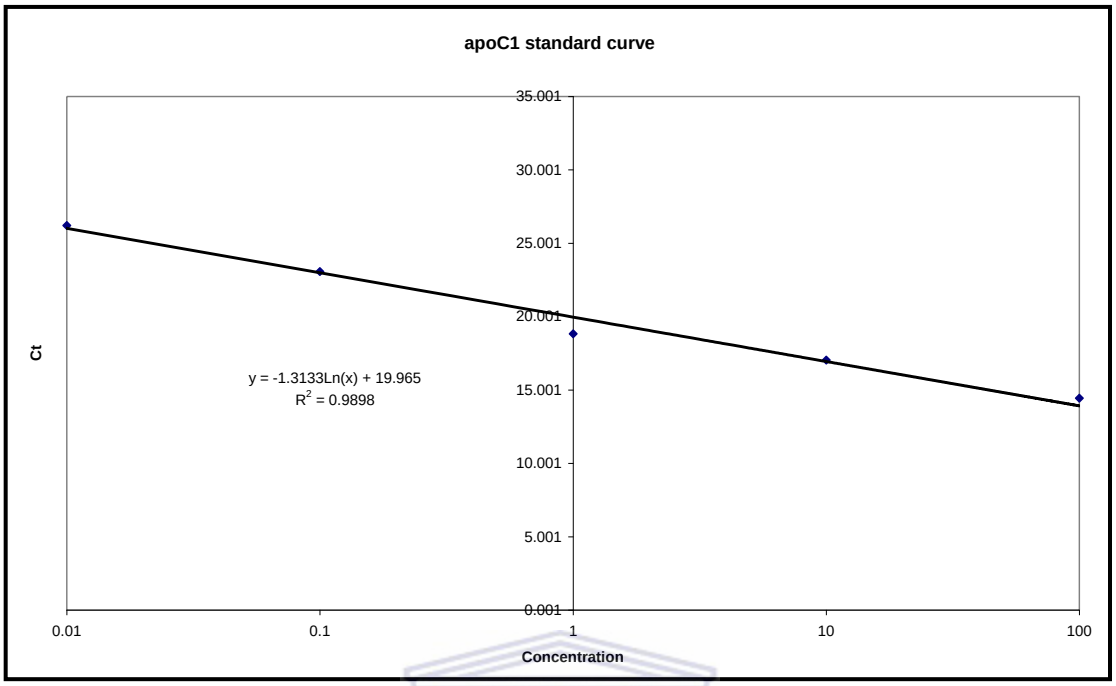

Figure A9: apoC1 Standard curve

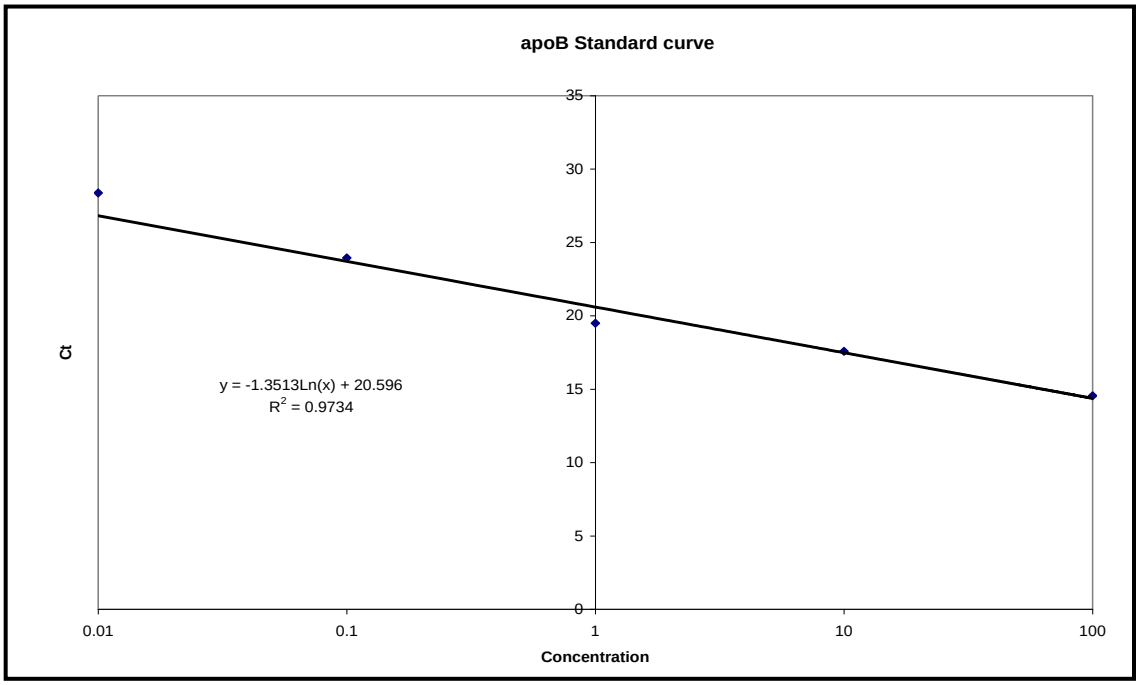

Figure A10: apoB Standard curve

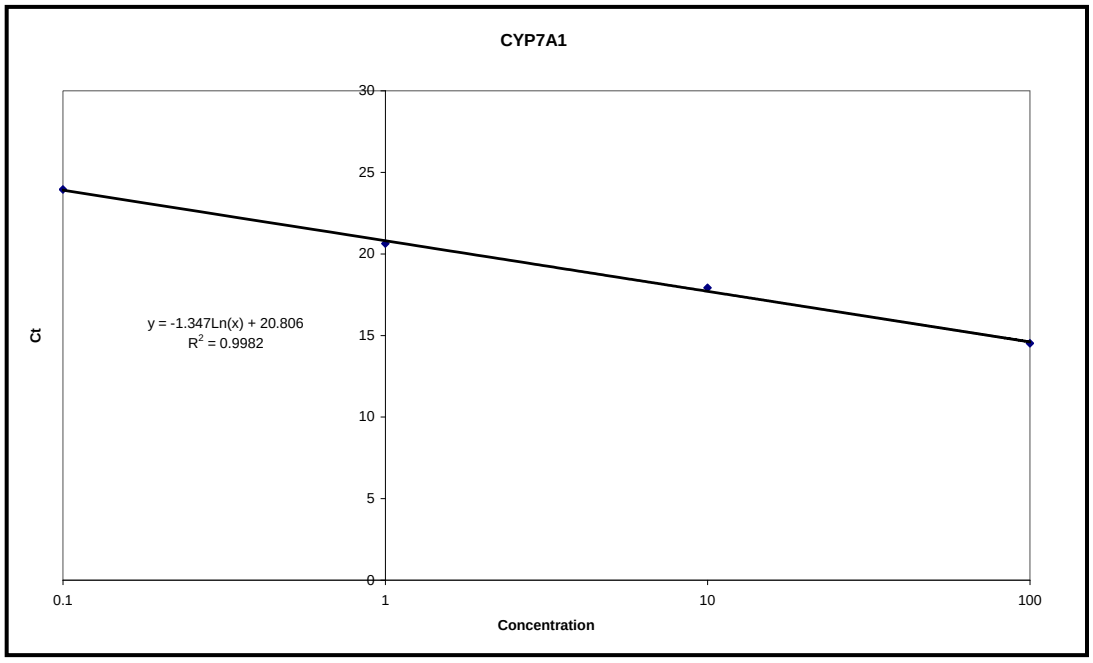

Figure A11: CYP7A1 Standard curve

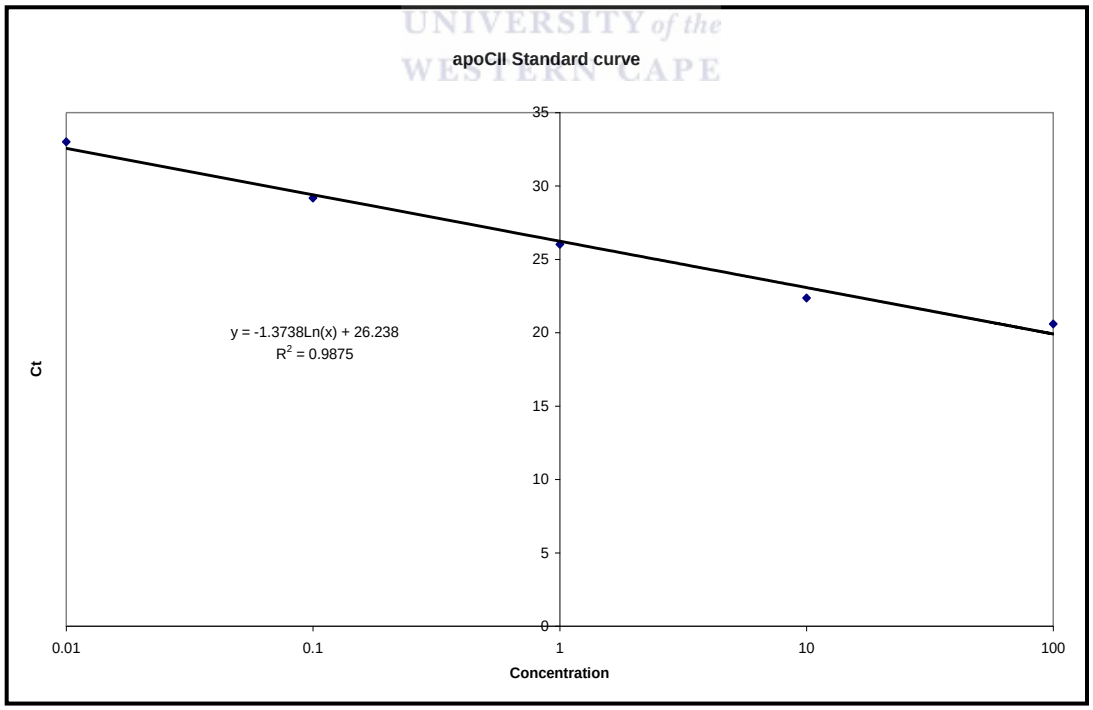

Figure A12: apoC11 Standard curve

9. MATERIALS AND SUPPLIERS

Acetic Acid Glacial	ACE Chemicals
Agarose	Whitehead Scientific
Anaket-V	Norpharm
AxyPrep Blood Genomic DNA Miniprep Kit	Axygen Biosciences
BenchTop 100bp DNA Ladder	Promega
Boric acid	Merck
Bromophenol blue	ACE Chemicals
EDTA	BDH
EDTA blood collection tubes	Pathcare
Ethanol	BDH
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems
Hydrochloric acid	BDH
Hypodermic needles (21 and 23G)	Norpharm
NaOH	Merck
Nicotinic acid (nicin)	Sigma
Paxgene Blood collection tubes	Qiagen
Paxgene Blood RNA Kit	Qiagen
PCR Master mix	Promega
Power SYBR Green PCR Master Mix	Applied Biosystems
RNase Inhibitor	Applied Biosystems
Sodium Hydroxide Pellets	ACE Chemicals
Tris-base	Promega
Turbo Dnase treat	AEC Amersham

Quanti Tech Primer Assays	Qiagen
Wizard SV Gel and PCR Clean-Up System	Promega
Xylene cyanol FF	Sigma