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AN ASSESSMENT OF GENE POLYMORPHISMS IN YOUNG SOUTH AFRICAN INDIANS WITH CORONARY ARTERY DISEASE AND THE EFFECT OF ATORVASTATIN *IN VITRO*

BY

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DECLARATION

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DEDICATION

To My Matha, Pitha, Guru and Deivam

These are Sanskrit words which translate to mother, father, teacher and God, respectively represents the hierarchy in which one should respect these entities.

And

To My Dearest Sister,

Nirvana Ishwarlal Munn
(4/11/1979 - 21/8/2012)

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CONTENTS

PUBLICATIONS

1. GSTM1 deletion and GSTP1 A_{105} polymorphisms in young South Africans with cardiovascular disease. Phulukdaree A, Khan S, Moodley D. Chuturgoon AA. South African Medical Journal, Vol. 102 (7) 2012 **(Original article)**

2. The IL-6 -147 G/C polymorphism is associated with increased risk of coronary artery disease. Phulukdaree A, Khan S, Ramkaran P, Govender R, Moodley D, Chuturgoon AA. Journal of Metabolic Disease and Related Disorders, ***In Press***. [Manuscript number MET-2012-0130]

3. Racial variability in telomere length and risk of coronary artery disease. S Khan, AA Chuturgoon, D Moodley and A Phulukdaree SA Heart Congress 2012, Vol 9 (3) 2012 **(Published abstract)**

4. Telomere dynamics in premature coronary artery disease. S Khan, AA Chuturgoon, D Moodley and A Phulukdaree. SA Heart Congress 2011, Vol 8 (4) 2011 **(Published abstract)**

5. Lack of association of -866 G/A uncoupling protein 2 and -55 C/T uncoupling protein 3 polymorphism in young patients with coronary artery disease. Phulukdaree A, Khan S, Moodley D. Chuturgoon AA. South African Journal of Science, **In Review**. [Manuscript number 1418]

6. The -390 CRP C/A/T polymorphism influences the risk of coronary artery disease. Phulukdaree A, Ramkaran P, Khan S, Chuturgoon AA. Journal of Inflammation, **In Review**. [Manuscript number 1521905047367461]

PRESENTATIONS

The paper titled: GSTM1 deletion and GSTP1 A_{105} polymorphisms in young South Africans with coronary artery disease by Phulukdaree A, Khan S, Moodley D, Chuturgoon AA was presented at the following local and international conferences:

1. South African Heart Congress 19th – 21st July 2012, Sun City, South Africa
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2. International Conference on Pure and Applied Chemistry, 2nd – 5th July 2012, Hilton, Mauritius [Published abstract in book of proceedings by Springer]

3. South African Society of Biochemistry and Molecular Biology, 29th January – 1st February 2012, Drakensburg, South Africa

4. College of Health Science Research Symposium 13th – 14th September, University of KwaZulu-Natal, South Africa, 2011

The paper titled: Assessing the interleukin-6 -174 G/C single nucleotide polymorphism and coronary artery disease by Phulukdaree A, Khan S, Moodley D, Chuturgoon AA is accepted for presentation at the following international conference:

1. The 6th World Congress of Paediatric Cardiology and Cardiac Surgery, 17th -22nd February 2013, Cape Town, South Africa

ABSTRACT

The global burden of heart disease increases every year. It has been estimated that by the year 2020, coronary artery disease (CAD) will be the number one cause of death worldwide. Indian populations throughout the world have the highest prevalence of CAD and early onset of the disease compared to other ethnic groups.

Glutathione S-transferases (GSTs) detoxify environmental agents which influence the onset and progression of disease. Dysfunctional detoxification enzymes are responsible for prolonged exposure to reactive molecules and can contribute to endothelial damage, an underlying factor in CAD. Uncoupling proteins (UCPs) 2 and 3 play an important role in the regulation of oxidative stress which contributes to chronic inflammation. Coronary artery disease is a chronic inflammatory disorder characterized by elevated levels of C-reactive protein (CRP) and pro-inflammatory cytokines such as interleukin 6 (IL-6). Polymorphisms of these genes have been linked to CAD and other chronic diseases.

Statins, metabolised in the liver, are the most commonly used drug to control atherosclerosis progression in CAD patients. The pleiotropic effects of statins have been attributed to both favourable and adverse outcomes in CAD patients particularly related to myopathy and hepatotoxicity.

All patients (n=102) recruited into this study were South African Indian males. A corresponding age-, gender- and ethnicity-matched control group (n=100) was also recruited. The frequency of the GSTM1 +/0, GSTP1 A_{105}/G_{105}, IL6 -174G/C and CRP -390C/A/T genotypes was assessed by polymerase chain reaction (PCR) and PCR restriction fragment length polymorphism (PCR-RFLP).

For the *in vitro* study, the biological effect of atorvastatin on HepG2 cells was assessed. The metabolic activity, cytotoxicity, oxidative stress and nitric oxide production was

assessed by the ATP, lactate dehydrogenase (LDH), thiobarbituric acid reactive substance (TBARS) and Griess assays, respectively. The profile of 84 microRNA (miRNA) species was evaluated using the miRNA Pathway Finder PCR SuperArray. The predicted targets of up-regulated miRNAs were determined using the online software, Targetscan. The mRNA levels of guanidinoacetoacetate (GAMT), arginine glycine aminotransferase (AGAT) and spermine oxidase (SMO) were determined using quantitative PCR. Western blotting was used to determine GAMT and phosphorylated p53 levels in treated cells.

The GSTM1 0/0 and GSTP1 A105/A105 genotypes occurred at higher frequencies in CAD patients compared with the control group (36% vs. 18% and 65% vs. 48%, respectively). A significant association with CAD was observed in GSTM1 0/0 (odds ratio (OR)=2.593; 95% confidence interval (CI) 1.353 - 4.971; p=0.0043) and GSTP1 A105/A105 OR=0.6011; 95% CI 0.3803 - 0.9503; p=0.0377). We found a significant association between smoking and CAD; the presence of either of the respective genotypes together with smoking increased the CAD risk (GSTP1 A105 relative risk (RR)=1.382; 95% CI 0.958 - 1.994; p=0.0987 and GSTM1 null RR=1.725; 95% CI 1.044 - 2.851; p=0.0221).

The UCP2 -866G/A and UCP3 -55C/C genotypes occurred at highest frequency in CAD patients (59% vs. 52% and 66% vs. controls: 63% respectively) and did not influence the risk of CAD. Homozygous UCP3 -55T/T genotype was associated with highest fasting glucose (11.87±3.7mmol/L vs. C/C:6.11±0.27mmol/L and C/T:6.48±0.57mmol/L, p=0.0025), HbA1c (10.05±2.57% vs. C/C:6.44±0.21% and C/T:6.76±0.35%, p=0.0006) and triglycerides (6.47±1.7mmol/Lvs. C/C:2.33±0.17mmol/L and C/T:2.06±0.25mmol/L, p<0.0001) in CAD patients.

A significant association between the G allele of the IL6 -174 polymorphism and non-diabetic CAD patients was found (p=0.0431 odds ratio: 1.307, 95% CI: 1.047-1.632). A

significant association with the C allele of the -390 CRP triallelic variants and CAD (p=0.021 odds ratio: 1.75, 95% CI: 1.109-2.778) was also found using a contingency of the C allele vs. the minor A and T allele frequencies. The strength of the association of the C allele with non-diabetic CAD subjects was much higher (p=0.0048 odds ratio: 2.634, 95% CI: 1.350-5.138).

Circulating median levels of IL-6 (0.9 (0.90, 0.91) pg/ml and 0.9 (0.87, 0.92) pg/ml) and CRP (5.65 (1.9, 8.2) mg/l and 2.90 (1.93, 8.35) mg/l) were similar between CAD patients and controls, respectively. A similar finding was observed between controls and non-diabetic CAD subjects.

Levels of IL-6 and CRP in CAD subjects were not significantly influenced by polymorphic variants of IL-6 and CRP. In the control group, the level of IL-6 was significantly influenced by the IL6 -174 G allele (p=0.0002) and the CRP -390 C allele (p=0.0416), where subjects with the homozygous GG (0.9 (0.9, 1,78) pg/ml) and CC (0.9 (0.9, 0.95) pg/ml) genotype had higher levels than the C allele carriers (0.9 (0.64, 0.91) pg/ml) or A and T carriers (0.9 (0.69, 0.91) pg/ml) combined.

The lowest measure of proliferation/metabolism in HepG2 cells was observed at 20µM atorvastatin, with 82±9.8% viability. The level of cytotoxicity was increased in statin treated cells from 0.95±0.02 units to 1.11±0.03 units (p=0.001) and malondialdehyde levels was reduced from 0.133±0.003 units to 0.126±0.005 units (p=0.009) whilst nitrite levels were elevated (0.0312±0.003 units vs. control: 0.027±0.001 units, p=0.044).

MicroRNAs most significantly upregulated by atorvastatin included miR-302a-3p (3.05-fold), miR-302c-3p (3.61-fold), miR-124-3p (3.90-fold) and miR-222-3p (4.4-fold); miR-19a-3p, miR-101-3p and let-7g were downregulated (3.63-fold, 2.92-fold, 2.81-fold, respectively). A list of miRNA targets identified included those with a role in metabolism and inflammation. The miR-124a specifically targets the mRNA of GAMT and SMO.

A 1.8-fold decrease in GAMT, a 1.15-fold decrease in SMO, and a 1.53-fold increase in mRNA levels was observed following atorvastatin treatment. The protein level of GAMT was relatively lower than the untreated control (1.11-fold). The highest level of p53 phosphorylation was observed in atorvastatin treated HepG2 cells (3.2-fold increase).

The finding from this investigation indicates that the genetic polymorphisms in GSTM1, GSTP1 genes and promoter regions (-174 and -390) of IL-6 and CRP, respectively, may influence the risk of young South African Indians to CAD. The *in vitro* investigation provides evidence for the influence of atorvastatin on hepatocytes' metabolism. A mechanism of compromised creatine synthesis by statins has also been described for the first time. Taken together, the genetic analysis result shows the complexity of mechanisms involved in CAD development. The *in vitro* aspect further confirms the multifaceted influence of therapeutics such as statins in controlling CAD progression and the related adverse effects.

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ABCA1	ATP-binding cassette transporter
ADP	Adenosine diphosphate
AGAT	Arginine-glycine amidinotransferase
Ago2	Argonaute-2
AMPK	5’ adenosine monophosphate kinase
Apo	Apolipoprotein
APS	Ammonium phosphate sulfate
Arg	L-arginine
ATP	Adenosine triphosphate
ATPAF1	ATP synthase mitochondrial F1 complex
BCA	Bicinchoninic Acid
BCL6	B-cell lymphoma-6 protein
BHT	Butylated hydroxytoulene solution
BMI	Body mass index
Bp	base pair
BSA	Bovine serum albumin
CAAT	Cytosine-adenine-adenine-thymine
CAD	Coronary artery disease
CCM	Complete culture media
CD	cell death
CD4+	cell death 4 positive
CD8+	cell death 8 positive
cDNA	Complementary DNA
CI	confidence interval
CK	Creatine kinase
CL	Chemiluminescent
CNV	Copy number variation
CO_2	Carbon dioxide
CoA	Coenzyme A
COA5	Cytochrome c assembly factor 5

COQ10B	Coenzyme Q10
CREB	Cyclic adenosine monophosphate responsive element
CRP	C-reactive protein
Cu	Copper
Cu_2SO_4	Copper (II) Sulfate
CYP	Cytochrome P450
CYCS	Cytochrome c
DAG	Diacylglycerol
DC	Dendritic cells
DGCR8	DiGeorge critical region 8
DMSO	Dimethyl sulfoxide
ET1	Endothelin-1
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
FA	Fatty acids
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factors receptor
FRS	Fibroblast growth factors substrate
g	gravitational force
GAA	Guanidinoacetoacetate
GAMT	Guanidinoacetate methyltransferase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GGPP	Geranylgeranylpyrophosphate
Gly	Glycine
GSH	Glutathione
GST	Glutathione-S-transferase
GSTM1	Glutathione-S-transferase Mu
GSTP1	Glutathione-S-transferase Pi
H_3PO_4	Phosphoric acid
HbA1c	Glycated haemoglobin
HDL-C	High density lipoprotein-cholesterol

HMGCR	3-hydroxymethyl-3-glutaryl Coenzyme A reductase
hsCRP	high sensitivity C-reactive protein
HRP	Horseradish peroxidase
IFNγ	interferon-gamma
IL	Interleukin
Ile	Isoleucine
IRAK1	Interleukin-1 receptor associated kinase-1
KH_2PO_4	Potassium dihydrogen phosphate
LDH	Lactate dehydrogenase
LDL-C	Low density lipoprotein- cholesterol
LVAD	Left ventricular assist device
MCP-1	monocyte chemotactic protein-1
MCSF	Macrophage colony stimulating factor
MDA	Malondialdehyde
MDB	Methyl-CpG binding domain protein
MECP2	Methyl-CpG binding protein
$MgCl_2$	Magnesium chloride
MIGC	Myocardial Infarction Genetics Consortium
min	minute
miRNA	MicroRNA
mmHg	millimetre mercury
MMP	Matrix metallopeptidase
mRNA	Messenger RNA
MTT	Methyl thiazol tetrazolium
Mwt	Molecular weight
NADH	Nicotinamide adenine dinucleotide
NAD(P)	Nicotinamide adenine dinucleotide (phosphate)
NCD	Non-communicable diseases
NF-kB	Nuclear factor kappa B
NO	Nitric oxide
Orn	Ornithine
OSB	Oxysterol binding protein

oxLDL	Oxidized lactate dehydrogenase
PAF	Platelet activating factor
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PGM	Phosphoglycerate mutase
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
Pri-miRNAs	Primary MicroRNA transcripts
RFLP	Restriction fragment length polymorphism
RISC	RNA-induced silencing complex
RLU	Relative light units
RNA	Ribose nucleic acid
ROS	Reactive oxygen species
RR	Relative risk
RT	Room temperature
SAM	Significance analysis of microarray
SBP	Systolic blood pressure
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SMO	Spermine oxidase
SNP	Single nucleotide polymorphism
SREBP	Sterol regulatory-element binding protein
ST-	Statin naive
ST+	Statin positive
T2D	Type II diabetes mellitus
TAR	Trans-activation response
Taq	*Thermus aquaticus*
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TBS	Tris buffered saline

TF	Transcription factor
TGFβ	Transforming growth factor beta
Th1	T-helper 1
Th2	T-helper 2
TIGAR	TP53-induced glycolysis and apoptosis regulator
TLR	Toll like receptors
TMB	3,3',5,5'-tetramethylbenzidine
TEMED	Tetramethylethylenediamine
TNFα	Tumour necrosis factor alpha
TRBP	Trans-activation response RNA-binding protein
TTBS	Tris-buffered saline containing 0.5% Tween20
UCP	Uncoupling protein
UV	Ultra violet
Val	Valine
VEGF	Vascular endothelial growth factor
VNTR	Variable number tandem repeat
WHO	World Health Organisation

INTRODUCTION

Cardiovascular disease (CVD) is amongst the major causes of death worldwide (Mathers and Loncar, 2006); (World Health Organisation, 2012). Originally thought to be a disease of the developed countries, the prevalence of heart disease is rapidly increasing in developing countries, particularly within populations of Indian ethnicity (Brooks et al., 2006). This is true for the increase in coronary artery disease (CAD) and type II diabetes mellitus (T2D) morbidity in South Africa (SA). The progression of CAD usually occurs gradually from childhood into the fifth and sixth decades of life. It is only during the late fifties and sixties that the symptoms and clinical indications of fully blown CAD manifest. However, the age of onset is now decreasing by at least 15 to 20 years, raising great concern. This is observed most especially in the SA Indian population as well as Indian populations in the United States of America and the United Kingdom.

Research shows that the onset of CAD is multi-factorial and is influenced by genetic and environmental factors as well as lifestyle. Such genetic changes that contribute to disease susceptibility include gene insertions or deletions, copy number variants (CNVs) and single nucleotide changes (Harris, 1971).

The occurrence and implication of single nucleotide polymorphisms (SNPs) – in CAD and T2D – of the detoxification enzyme glutathione-S-transferase (GST) isozymes (GSTP1 and GSTM1) (Zanobetti et al., 2011), oxidative stress modulating mitochondrial (mt) uncoupling protein 2 (UCP2), uncoupling protein 3 (UCP3) (Hamada et al., 2008) and inflammatory

cytokines interleukin-6 (IL-6) (Tilg and Moschen, 2006) and C-reactive protein (CRP) (Miller et al., 2005) has been well documented.

Oxidative stress, the underlying mechanism for pathology in T2D is a predisposing condition for the development of atherosclerosis and CAD. Hyperglycaemia induces increased superoxide production in mitochondria of endothelial cells that leads to the pathogenesis of T2D complications (Brownlee, 2001). The presence of SNPs in genes such as GST may positively or negatively impact an individual's ability to detoxify reactive oxygen species (ROS) and their products. Consequently, variation in individual specific susceptibility to pathologies associated with disease is frequently observed (Priscilla and Prince, 2009).

Mitochondrial (mt) uncoupling proteins are transmembrane proteins that discharge the proton gradient generated during oxidative phosphorylation. The roles of UCP2 and UCP3 are unclear, though, they have been associated with the protection against oxidative damage, ageing and degenerative diseases (Affourtit et al., 2007). A number of studies have linked UCPs with disease prevalence, particularly diabetes (Hagen and Vidal-Puig, 2002) and obesity (Surwit et al., 1998). These conditions are often associated with patients that suffer from CAD. Interestingly, mt UCPs have also been implicated in heart failure (Laskowski and Russell, 2008). Uncoupling proteins are considered key regulators of energy and glucose metabolism (Lee et al., 2008). As a result, the optimum functioning of UCPs is essential. Studies that investigated the UCP gene polymorphisms UCP2 -866 G/A (Laskowski and Russell, 2008) and UCP3 -55 C/T (Cassell et al., 1999) have implicated a structural and functional link with disease severity, progression and occurrence (Lee et al., 2008).

Interleukin-6, a multi-functional cytokine responsible for acute inflammatory responses, and CRP, an acute phase protein produced by hepatocytes, are implicated in the pathogenesis of CAD. Chronic inflammation in CAD is mediated by IL-6. C-reactive protein expression is primarily induced by IL-6 and synergistically enhanced by IL-1β and to a lesser extent tumour necrosis factor alpha (TNFα) (Rhodes et al., 2011). In CAD, the chronic stimulation of CRP prolongs immune activation and inflammation. Single nucleotide polymorphisms in the promoter regions of the genes for inflammatory cytokines influences cytokine levels and is strongly implicated in the development and progression of inflammatory conditions (Olivieri et al., 2002; Hage and Szalai, 2007; Shanker and Kakkar, 2010; Yeh et al., 2010; Najar et al., 2011; Pereira et al., 2011).

Oxidative stress and inflammation have been investigated individually with respect to the development of CAD and progression to heart failure. The communication between these processes, however, begins from initiation to development and progression of atherosclerotic lesions in CAD. The vicious cycle between oxidative stress and inflammation manifests in shared elements (in circulation, intracellularly and in the plaque core) resulting in a chronic inflammatory response observed in CAD.

An important mechanism of epigenetic control of immunological processes is via miRNA expression. MicroRNAs are small, non-coding RNA species that regulate protein expression through post-transcriptional repression or degradation of messenger RNA (mRNA) species prior to translation (Asirvatham et al., 2008; Kai and Pasquinelli, 2010). The role of miRNAs in plaque development, vascular function (Small et al., 2010), inflammation (Hoekstra et al., 2010) and heart failure (Tijsen et al., 2010, Elton et al., 2011) has recently been investigated.

Epigenetic modifications, especially changes in miRNA expression are affected by therapeutics such as statin therapy. These modifications influence disease progression and improve the prognosis of CAD patients.

The statin family of drugs is widely used for reducing cholesterol levels and decreasing the incidence of CAD (Zhao et al., 2009). The target of statin therapy is the 3-hydroxymethyl-3-glutaryl Coenzyme A reductase (HMGCR) - a key rate-limiting regulatory enzyme in cholesterol synthesis (Baetta and Corsini, 2009) which occurs in the liver. An increase in cholesterol levels in the liver have been shown to induce a change in gene expression and activity of hepatocytes. These changes include switching to a predominant inflammatory state by increasing pro-inflammatory cytokine (IL-1, TNFα and IFNγ) signalling pathways (Kleemann et al., 2007). A range of pleiotropic effects from this family of drugs have been observed. These range from reducing oxidative stress and inflammation (Grothusen et al., 2005) to adverse effects of fatigue and myopathy (Phillips et al., 2002).

To date, there are no reports on the relationship of GSTP1, GSTM1, UCP2, UCP3, IL-6 and CRP polymorphisms in the SA Indian population presenting with CAD. In addition, no epigenetic study (miRNA) has been conducted in this population group. This necessitates studies to be conducted in SA to determine if these polymorphisms or epigenetic changes are linked to the high incidence of CAD. Establishing a link could offer the possibility of genetic screening of patients to determine patient predisposition to CAD and for the early administration of therapeutics.

STUDY RATIONALE AND AIMS

Genetic investigations provide clues on the understanding of the underlying mechanisms of atherosclerosis and CAD. Many of these genetic studies are conducted in the developed world and as such it is difficult to extrapolate this data to diseases in the developing world. Most SA Indians are descendants of indentured labourers who were brought to KwaZulu-Natal from India between 1860 and 1911. In 2006, reports still showed that the highest death rates for CAD in SA occur in Indians (Norman et al., 2006). This suggests that regardless of the 150 years that Indian immigrants have lived in SA, there remains a differing pattern of CAD mortality compared to the natives of the country, despite them sharing the same environment and to some extent similar diets and cultural habits (Lal, 2004).

It has now become necessary to assess these influences in our local Indian population, especially those predisposed to CAD.

The role of the liver as a central metabolic organ was then evaluated. The potential of statins to influence liver function enzymes and play a role in non-alcoholic fatty liver disease has been described. The liver is a rich source of molecules required by peripheral tissue, which include a range of nutrients, lipid transport proteins, and acute phase proteins, to name a few. The effect of atorvastatin, a commonly prescribed drug in CAD patients, was investigated on a human liver cell line (HepG2). We investigated the epigenetic effect of upregulated miRNAs

on selected targets obtained from a list of predicted targets using the TargetScan online software. It was then speculated that from an analysis of miRNAs and their targets, a possible novel mechanism of action of statins may be determined.

The aim of this study was to evaluate why SA Indians are more susceptible to early onset of CAD by assessing selected genetic (SNPs). In addition, the biological and epigenetic effect of atorvastatin on a human liver cell line was evaluated.

The objectives of this study were designed to assess:-

A: Young SA Indian CAD patients for single nucleotide genetic changes in:-

i) Detoxification enzymes - GSTP1 (codon 105) and GSTM1 (gene deletion)

ii) Oxidative stress modulators - UCP2 -866G/A and UCP3 -55C/T promoter SNPs

iii) Pro-inflammatory markers – IL-6 -174G/C and CRP -390 C/A/T promoter SNPs

B: To assess the effect of atorvastatin in the HepG2 liver cells:-

i) Measure the metabolic activity/cell proliferation

ii) Determine the epigenetic changes in miRNAs in HepG2 cells and their potential targets using a miRNA pathway finder array.

CHAPTER 1

LITERATURE REVIEW

1.1 Overview of Coronary Artery Disease

Atherosclerosis and thrombosis are the two major contributing mechanisms in CAD, a major health problem and one of the leading causes of global death (World Health Organisation, 2012).

Atherosclerosis is a chronic inflammatory vessel disorder characterised by lipid deposition and formation of fibrous plaques on the arterial wall, which over a lengthy period of time becomes unstable as a result of multiple factors leading to clinical symptoms of heart disease (Figure 1.1) (Libby, 2002; Ross, 2005; Watkins and Farrall, 2006). The process of atherogenesis activates the immune response that involves a host of inflammatory cells and cytokines (Libby, 2002). The development of atherosclerotic lesions is amplified by chronically high levels of ROS (McGill et al., 2000; Watkins and Farrall, 2006).

In the past centuries, CAD has undeniably been under-estimated with respect to the huge impact it has on the global economy. The exponential increase in the global burden of CAD and T2D are two examples of non-communicable diseases (NCDs) that have been neglected. Both diseases occur as a result of multiple factors ranging from genes to environmental interactions, but most importantly due to poor lifestyle choices (Tyroler, 2000).

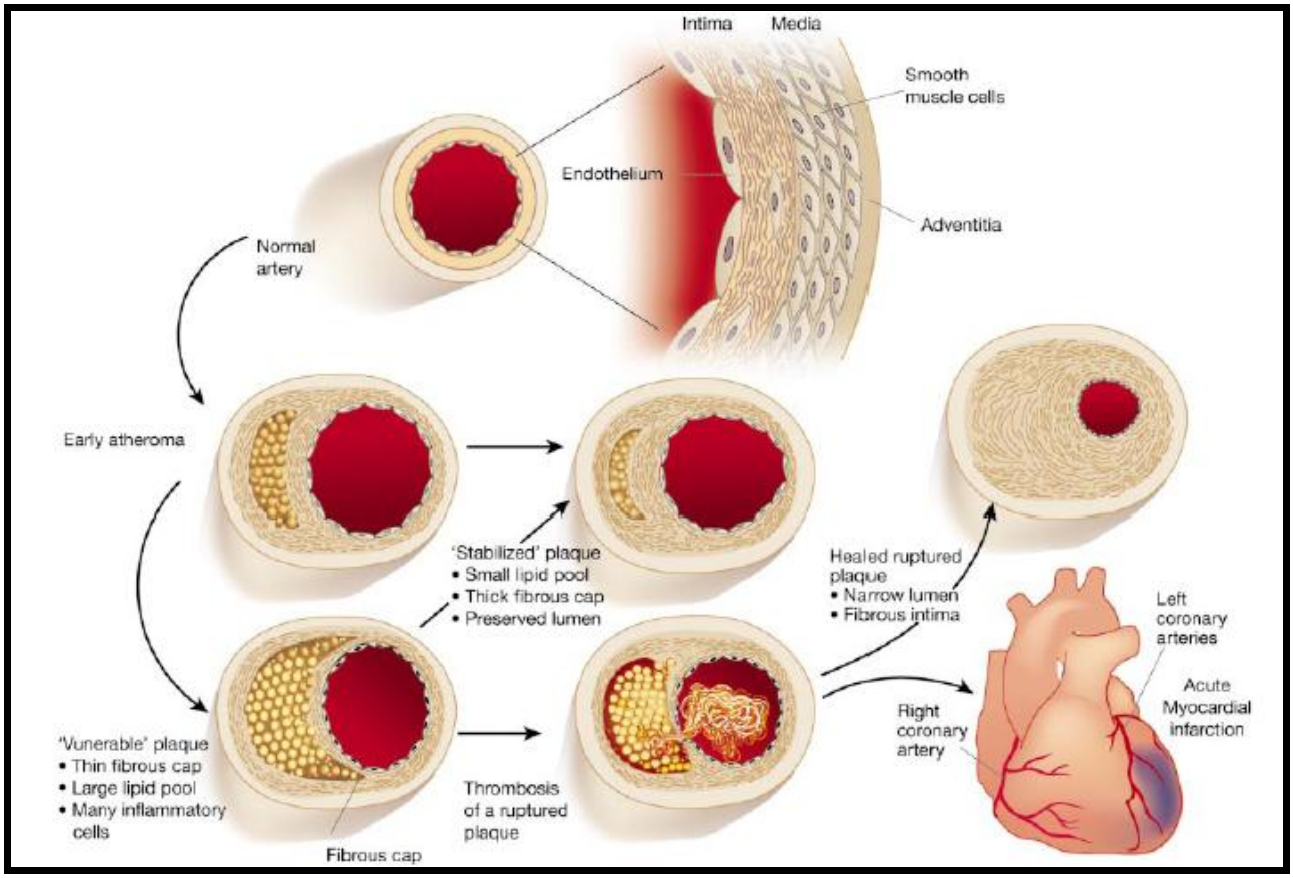


Figure 1.1 Schematic development and progression of an atherosclerotic plaque and clinical manifestation of acute myocardial infarction (Libby, 2002).

1.2 Epidemiology of CAD

The World Health Statistics of 2012 estimates that “57 million global deaths in 2008, 36 million (63%) were due to NCDs. Population growth and increased longevity are leading to a rapid increase in the total number of middle-aged and older adults, with a corresponding increase in the number of deaths caused by NCDs. It is projected that the annual number of deaths due to CVD will increase from 17 million in 2008 to 25 million in 2030” (World Health Organisation, 2012).

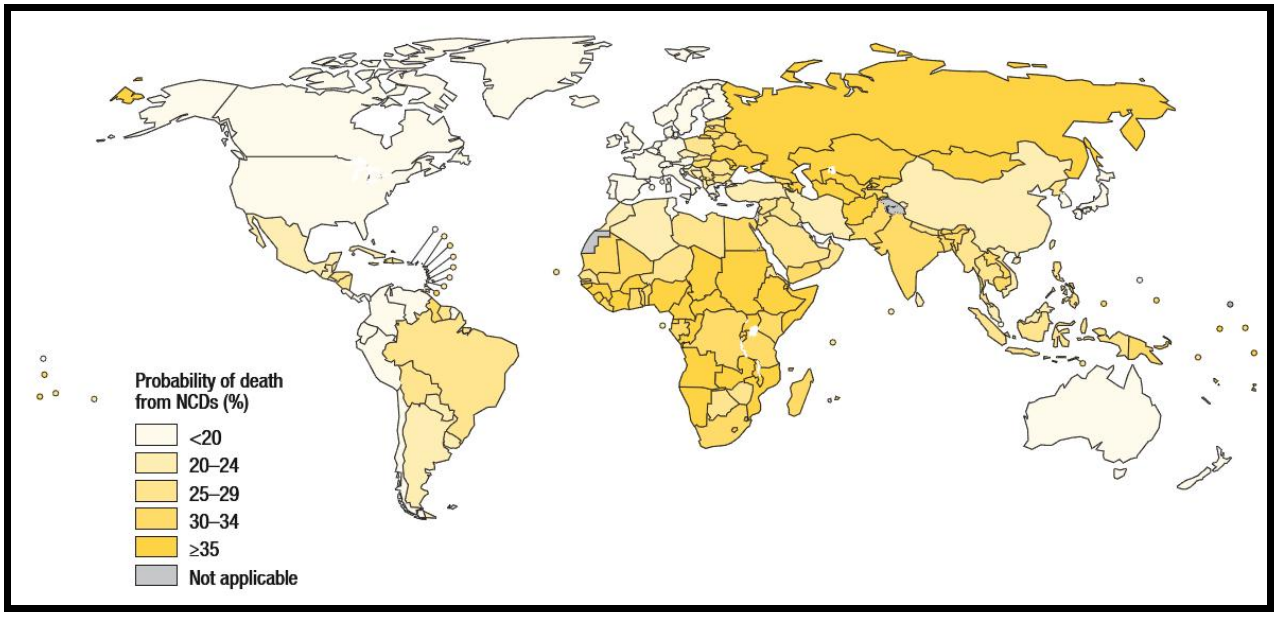


Figure 1.2: Global view for the probability of death (%) from non-communicable diseases between the ages of 30 and 70 as at 2008 (World Health Organisation, 2012).

Non-communicable disease risk factors are known; the largest proportion of NCD deaths is caused by CVD (48%), followed by cancers (21%) and chronic respiratory diseases (12%). Diabetes is directly responsible for 3.5% of NCD deaths (Figure 1.2) (World Health Organisation, 2012).

1.3 Classification of CAD risk factors

Risk factors associated with cardiac failure are classified as modifiable and non-modifiable risk factors. Modifiable risk factors are risk factors that occurs as a result of circumstance and are under the control of an individual, and this includes behavioural risk factors. Non-modifiable risk factors fall beyond the control of an individual that include hereditary genetic factors.

1.3.1 Non-Modifiable risk factors

1.3.1.1 Age

Symptomatic CAD is usually prevalent in individuals over the age of 60 and was considered a disease of the aged/elderly. This, however, is changing as individuals are presenting with coronary artery syndromes as early as mid-twenties, a statistic that is notable amongst Indian patients in SA. In a study on CAD in Indians, approximately half of all myocardial infarctions occurred in individuals under the age of 50, with 25% being under 40 years of age (Enas et al., 2001). A recent study found no relationship between obesity-related polymorphisms and the metabolic syndrome in 485 young (≤ 45 years) SA Indian patients with acute myocardial infarction ((Ranjith et al., 2011).

1.3.1.2 Genetic/Hereditary

- **Diabetes**

Type II Diabetes mellitus is classified as a disease that can either be inherited or acquired, in which the likelihood of acquisition is much higher if both parents have the disease. The mechanisms by which diabetic complications influence atherosclerotic development range from microvascular complications of retinopathy and nephropathy to neuropathy. This is a result of hyperglycaemia induced oxidative stress, pro-inflammatory cytokine production and activation of protein kinase C (PKC) (Figure 1.3)(Wagner et al., 2002).

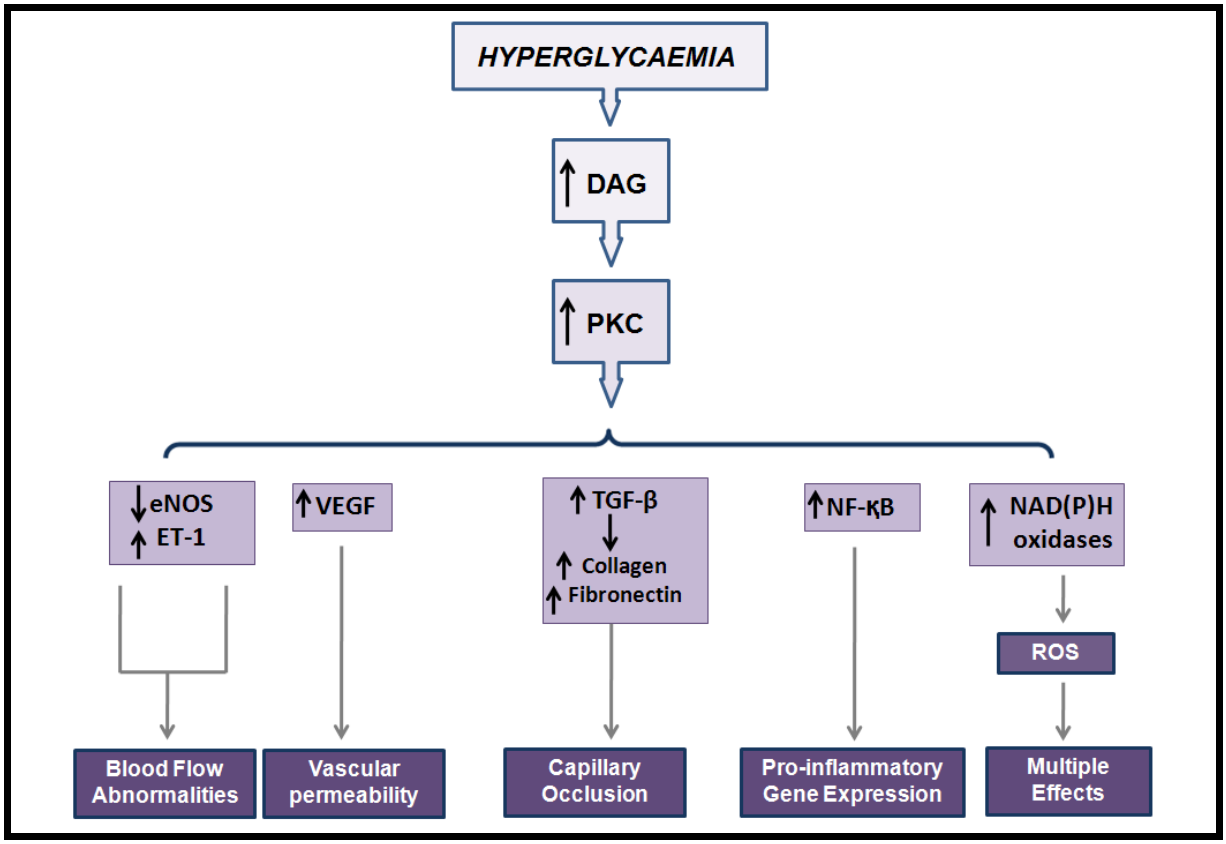


Figure 1.3: Hyperglycaemia-induced activation of protein kinase C (PKC) which influences cardiovascular function, increases diacylglycerol (DAG), and nitric oxide production. Activated PKC also affects endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), transforming growth factor-b (TGF-b) and plasminogen activator inhibitor-1 (PAI-1), NF-kB and NAD(P)H oxidases (Brownlee, 2001).

In addition, the chronic hyperglycaemia associated with diabetes modifies protein structure and function due to glycosylation of amino acid residues. Glycated proteins and growth factors such as insulin stimulate the proliferation of smooth muscle cells. Type 2 diabetes mellitus is also associated with elevations of PAI-1 and alterations in the lipid profile such as elevated triglycerides and low density lipoprotein cholesterol (LDL-C) and reductions in high density lipoprotein cholesterol (HDL-C) (Brownlee, 2001; Wagner et al., 2002). Taken

together, these hyperglycaemic factors influence the development and progression of atherosclerosis.

- **Gender**

Adult women are less likely to develop heart disease compared to males due to the cardio-protective effects of oestrogen. Other factors such as oral contraceptives and smoking, however, alter this protection and the risk of developing coronary syndromes increases (Tanis et al., 2001).

A recent study published in the New England Journal of Medicine evaluated the role of the Y chromosome in CAD (Charchar et al., 2012). Three study cohorts of 3233 men of British origin were included in the study and their Y chromosomes were tracked back to 9 different lineages, which they defined as haplogroups.

It was shown that British men who inherited haplogroup I had an approximately 50% higher risk of CAD than men with other types of the Y chromosome, irrespective of age, body-mass index, blood pressure, lipids, diabetes, smoking, alcohol consumption, socioeconomic status, or circulating concentrations of CRP. A further transcriptomic analysis of men with haplogroup I presented with a reduced adaptive immunity and increased inflammatory response pathways in their macrophages compared with carriers of other Y chromosome types. This indicates that predisposition to CAD is “most likely mediated through immune response” (Charchar et al., 2012).

- **Ethnicity**

The high incidence of CAD in SA Indians in Durban was first reported in 1969 (Wainwright, 1969). More than 40 years later, epidemiological data in SA shows that Indians have the highest mortality (the rate of deaths in a population) for CAD, followed by mixed ethnicity, white and black (Norman et al., 2006).

A global case-control study of the risk factors of acute myocardial infarction called the INTERHEART study investigated the association of risk factors for acute myocardial infarctions in relatively young native South Asians (mean age of 53 years), compared with individuals from other countries (mean age of 58 years). They found that apolipoproteins: (apo) apoB/apoA-1 ratios were found to have the highest attributable risk among South Asians (n=10 728) compared to individuals from other countries (n=12 431) (Joshi et al., 2007).

The Framingham Heart Study: Impact on the Prevention and Control of Cardiovascular Diseases in India reported in 2010, reported their assessment of the changing disease profile in India. The results from this study indicate that the classical risk factors that determine the CVD play a role in all populations and ethnic groups (Reddy and Satija, 2010).

It was recommended, however, that Indians be classified as a high risk group, and the classical ranges for risk factors be evaluated when calculating the Framingham score (a risk score for cardiovascular disease) (Reddy and Satija, 2010).

1.3.2 Modifiable risk factors

1.3.2.1 Behavioural risk factors

Behavioural risk factors contribute to 80% of coronary heart disease and cerebrovascular disease. These include tobacco use, physical inactivity, unhealthy diet and the consumption of alcohol. These risk factors are associated with four key changes in metabolism and physiology i.e. elevated blood pressure, overweight leading to obesity, hyperglycaemia and hyperlipidaemia.

These changes can have multiple effects. “For example, in addition to its direct role in diabetes, raised fasting blood glucose also increases the risk of cardiovascular deaths, and was estimated to cause 22% of coronary heart disease-related deaths and 16% of stroke-related deaths. In terms of attributable deaths, the leading behavioural and physiological risk factors globally are raised blood pressure (to which 13% of global deaths are attributed), followed by tobacco use (9%), raised blood glucose (6%), physical inactivity (6%) and being overweight or obese (5%)” (World Health Organisation, 2012).

1.3.2.1.1 Tobacco Use

Cigarette smoking is associated with adverse changes in several components of cardiovascular physiology, including endothelial cell dysfunction, and leukocyte and platelet activation. Cigarette smoking is associated with first and recurrent coronary events, a lower

HDL-C, endothelial cell dysfunction, platelet aggregation, plaque rupture, oxidative stress and mt DNA damage.

Smoking may increase acute coronary events and strokes by increasing inflammation and thrombosis (Bazzano et al., 2003). Free oxygen radicals derived from cigarette smoke lead to increased oxidative stress and decreased nitric oxide production (Blann et al., 1998). Antioxidant capacity is compromised as a result of smoke-derived free radical scavenging, losing the capacity to protect against endogenous free radicals produced through normal respiration. This process contributes further to levels of oxidized-low density lipoprotein – cholesterol (oxLDL-C) and atherosclerotic lesion progression. In addition, nicotine is a strong activator of the sympathetic nervous system, which releases catecholamines that interfere with haemodynamics, resulting in increased myocardial oxygen demand (Yusuf et al., 2004).

1.3.2.2 Hypertension

Hypertension is a very common disease experienced by approximately 30% of all adults. Commonly referred to as the ‘silent killer’, hypertension is largely asymptomatic and puts strain on the heart and arterial system resulting in mechanical damage to the endothelium lining, promoting the onset of atherosclerosis.

Approximately 51% of stroke-related deaths and 45% of coronary heart disease-related deaths occur as a consequence of elevated blood pressure. Reports from the World Health statistics from 2012 showed that the mean blood pressure decreased dramatically in nearly all high-income countries (World Health Organisation, 2012).

In the United States of America, the mean age-standardized male systolic blood pressure (SBP) decreased from 131 mm Hg (95% uncertainty interval 127–135) in 1980 to 123 mm Hg (120–127) in 2008, while mean age-standardized female SBP decreased from 125 mm Hg (121–130) to 118 mm Hg (115–122) mm Hg. In contrast, mean blood pressure has been increasing in most African countries (World Health Organisation, 2012).

The major cause of elevated blood pressure is due to dietary intake of salt but more importantly, psychological stress. Both diet and stress factors have been addressed in the developed world countries. Experts believe that developing countries can reduce the mortality of CAD by learning from the lessons in the developed countries (World Health Organisation, 2012).

1.3.2.3 Diet and Physical Inactivity

A high-fat and carbohydrate diet with high glycaemic index impairs endothelial function directly and indirectly. A cholesterol-rich diet directly increases in LDL-C concentrations. A high carbohydrate diet can lead to hyperglycaemia which indirectly influences oxidative stress, inflammation and PKC activation (as described under hereditary factors: ‘Diabetes’). Epidemiologic studies show an inverse relationship between dietary intake of antioxidant vitamins and CAD (Stampfer et al., 1993; Nunes et al., 1997).

According to the World Health statistics, 2.8 million people die yearly as a result of being overweight or obese. “Being overweight or obese can lead to adverse metabolic effects on blood pressure, cholesterol and triglyceride levels, and can result in T2D. Being overweight or obese thus increases the risks of CAD, ischaemic stroke and T2D. Between 1980 and 2008, the worldwide prevalence of obesity (body mass index (BMI) ≥30 kg/m2) almost doubled. By 2008, 10% of men and 14% of women in the world were obese, compared with 5% of men and 8% of women in 1980. As a result, an estimated half a billion men and women over the age of 20 were estimated to be obese in 2008. In all World Health Organisation regions, women were more likely to be obese than men” (World Health Organisation, 2012).

In contrast, regular exercise has been shown to consistently improve endothelial function, improve insulin sensitivity and reduce the risk for development of coronary heart disease.

1.4 The pathophysiology of coronary artery disease

The architecture of the normal human coronary artery comprises a tri-laminar structure (Libby, 2002). The layer in direct contact with circulating molecules is the endothelial cell layer which rests on a basement membrane. The endothelium is regarded as an endocrine organ as it releases one or more factors involved in local vasodilation (Vogel, 1997). The intimal layer lies below the endothelium and consists of smooth muscle cells in an intimal extracellular matrix. The internal elastic lamina creates a barrier between the tunica intima and media. The media is made up of tightly packed multiple layers of smooth muscle cells embedded in a matrix of elastin and collagen (Figure 1.4).

Endothelial cell injury is initiated by increased shear stress caused by the combination of high blood viscosity, hypertension and vasoconstriction - providing the opportunity for the formation of atherosclerotic lesions or fatty streaks.

During the early stages of the development of an atherosclerotic plaque, endothelial cell injury occurs. This leads to endothelial cell dysfunction and expression of adhesion molecules which recruits inflammatory cells via leukocyte adhesion and infiltration. A damaged endothelial layer also promotes the entry and accumulation of lipids, of which apo B in LDL-C in particular undergoes oxidative modification to form extensively oxLDL-C (Navab et al., 1996; Witztum and Palinski, 1999).

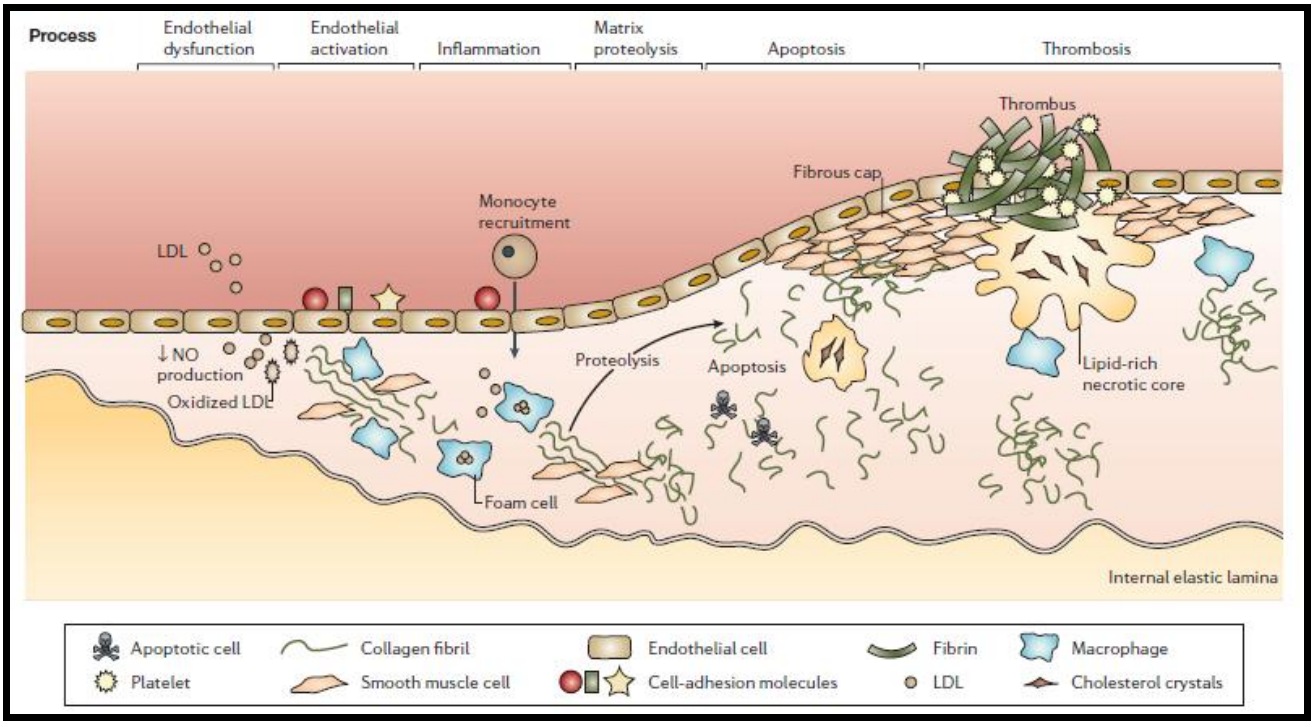


Figure 1.4 Molecular and cellular processes from initiation to rupture of an atheroma (Watkins and Farrall, 2006).

The monocytes, which have migrated into the subendothelial space, differentiate into macrophages and take up oxLDL-C by phagocytosis. This leads to foam cell formation. Oxidized LDL-C taken up by scavenger receptors undergo esterification and are stored in lipid droplets. The more soluble forms are exported to extracellular HDL-C acceptors via cholesterol transporters (Glass and Witztum, 2001).

Macrophages take on a characteristic foamy appearance, and become activated which leads to a state of chronic inflammation. This results in a lipid-rich core in the intima (Glass and Witztum, 2001; Libby, 2002) that causes the artery to enlarge in an outward direction (Libby, 2002; Ross, 2005; Watkins and Farrall, 2006).

The lipid-rich core and dysfunctional endothelium induces the recruitment of inflammatory cells. This further contributes to an inflammatory state. Inflammation and persistent dyslipidaemia are factors that promote the growth of the lipid core. As a result, activated leukocytes release proteinases which degrade the extracellular matrix components. These changes render the plaque fragile and susceptible to rupture (Figure 1.4) (Watkins and Farrall, 2006).

A ruptured plaque interacts with tissue factor in circulation and induces the coagulation cascade and thrombus formation. If the thrombus occludes the vessel persistently, it results in an acute myocardial infarction (Libby, 2002; Ross, 2005; Watkins and Farrall, 2006).

1.4.1 The role of lipids in plaque development

The source of serum cholesterol stems from endogenous production in the liver or from the diet. Serum cholesterol is transported by low density and high density lipoprotein molecules. Although LDL-C has an essential function of cholesterol delivery to peripheral tissue, excessive amounts of LDL-C in circulation increases the risk of CAD (Libby, 2002). LDL-C is recognized by LDL-C receptors of cells due to the N-terminal domain of the apo-B100 component. LDL-C receptor expression is controlled by the transcription factor sterol regulatory-element binding proteins (SREBPs), which are activated when low intracellular levels of cholesterol are detected.

Statins, the most prescribed drug in the world, are used in the management of CAD patients to reduce total cholesterol and LDL-C levels and improve HDL-C levels (Dirks and Jones, 2006). Statins inhibit endogenously synthesized LDL-C by competitive inhibition of HMGCR (the rate-limiting enzyme of cholesterol synthesis), enhancing the clearance of LDL-C from circulation and reducing the availability of LDL-C to contribute to atherosclerosis.

Statins have been shown to possess anti-inflammatory properties by reducing the activation of TNF-α-induced hypoxia-inducible factor-1α and inhibiting platelet aggregation (Chandrasekar et al., 2006). The antioxidant effects of statins were demonstrated in a monocyte cell line via the inhibition of superoxide radical production by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Delbosc et al., 2002).

In a clinical setting, the dose of statin prescribed is dependent on the statin type. The dose range falls between 20mg to 80mg per day, with a plasma half-life of between 2-4 hours, with the exception of atorvastatin which has a longer half-life (14-20 hours) (Chong et al., 2001; Manzoni and Rollini, 2002). The statin family of drugs are generally well tolerated in patients, but approximately 11% of cases present with skeletal muscle myopathy that include muscle pain, stiffness, cramp and fatigue, and rare cases of severe rhabdomyolysis (Chong et al., 2001; Bruckert, 2005).

1.4.2 Inflammation and atherosclerosis

At the site of the atherosclerotic lesion the initiating cytokine, macrophage-colony stimulating factor (M-CSF) promotes monocyte differentiation into macrophages (Figure 1.5). An investigation on mutated mice lacking M-CSF showed a drastic reduction in macrophage accumulation and lesion development (Smith et al., 1995).

The differentiation of monocytes to macrophages also induces expression of toll like receptors (TLRs) and pattern-recognition receptors for innate immune activation (Janeway and Medzhitov, 2002; Peiser et al., 2002). Cell particles from apoptosis as well as oxLDL-C are internalized by macrophages as they contain pathogen-like molecular patterns, which are recognized by scavenger receptors (Peiser et al., 2002). It is this source of oxLDL-C that accumulates and results in foam cell formation.

Toll-like receptors are also expressed on mast cells and endothelial cells. The binding of the oxLDL recognised as 'foreign' molecules to TLRs can initiate a signal cascade that induces cell activation (Janeway and Medzhitov, 2002). Once activated macrophages produce a range of molecules that promote atherosclerosis. Reactive oxygen species oxidise LDL molecules in the lesion, whilst proteases (matrix metallopeptidases) cleave matrix proteins and components of the sub-endothelial membrane causing plaque weakening (Xu et al., 1999).

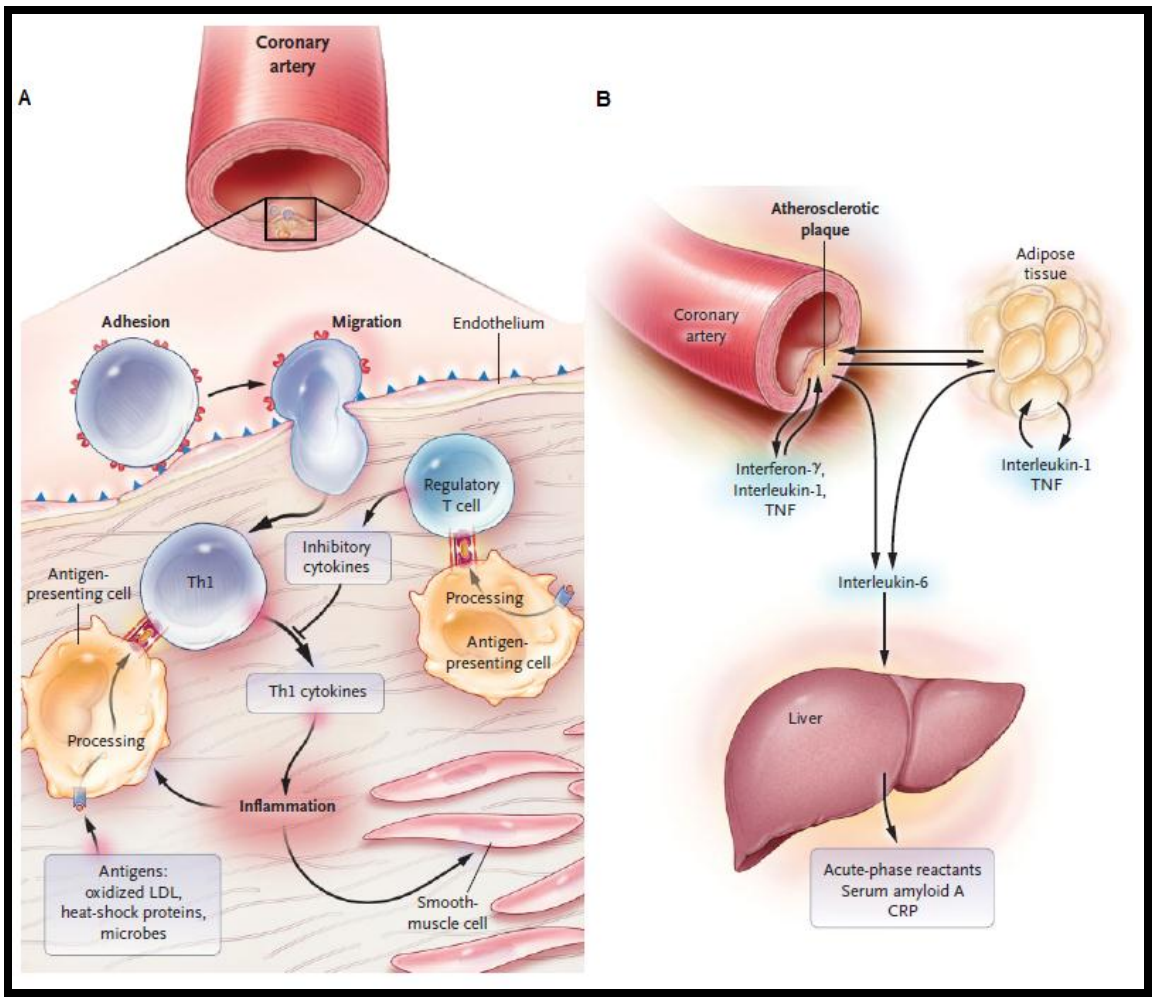


Figure 1.5 The (A) activation of inflammatory T-cells and (B) cytokine cascade in atherosclerotic plaques (Adapted from (Hansson, 2001).

Activated macrophages also release chemokines (monocyte chemoattractant protein-1 (MCP-1), IL-8, interferon-gamma (IFNγ), and eotaxin) which promote adhesion molecule expression (vascular cell adhesion molecule-1 (VCAM-1), P- and E-selectin). *In vivo* studies on mice lacking MCP-1 or its receptor CCR2 have lower levels of mononuclear cells at atherosclerotic lesions (Boring et al., 1998; Gu et al., 1998). The expression of VCAM-1 is initiated by pro-atherogenic factors and the initial accumulation of LDL-C in the intima. The expression of VCAM-1 is further potentiated by pro-inflammatory cytokines IL-1 and TNFα (Libby, 2002).

The release of these chemokines attracts immune and inflammatory cells (monocytes, leukocytes, B and T lymphocytes, and mast cells) to the atherosclerotic lesion. The T cells together with macrophages then further contribute to the cytokine milieu by producing IL-1, IL-6, IFNγ, TNFα, and angiotensin II (Glass and Witztum, 2001; Hansson, 2001) (Figure 1.5).

Atherosclerotic lesions always contain an infiltrate of cells, predominantly cell death (CD) 4 positive (CD4+) T cells and to a lesser extent CD8+ B cells. Once activated T-cells differentiate into T-helper 1 (Th1) and 2 (Th2) cells, they contribute to the cytokine milieu in the plaque, particularly IFNγ. Interferon gamma induces synthesis of the pro-inflammatory cytokines, IL-1β and IL-6, which are released into circulation. Both IL-1β and IL-6 then stimulate the synthesis of acute phase proteins in the liver. This results in a chronic inflammatory response that promotes atherosclerosis (Figure 1.5) (Hansson, 2001).

Single nucleotide polymorphisms and mutations in genes of detoxification enzymes, modulators of oxidative stress as well as inflammatory cytokines have been shown to

influence mRNA levels and protein expression. These genetic changes have thus been associated with complex diseases and have become an area of interest for pharmacogenomic research.

1.5 The significance of genetic variation in CAD

The decrease in the age for onset of the ‘chronically inflammatory disease’ has been attributed to hereditary factors associated with diabetes and obesity and familial-linked CAD. This has prompted researchers to assess a possible genetically-related (SNPs, CNVs, insertions/deletions) or epigenetic (DNA methylation, chromatin and histone modification and miRNA expression) link.

Epigenetic modification by DNA methylation is well understood. This process entails a covalent modification of cytosine maintained at CpG islands by methylation catalysed by DNA methyl transferases. This methylation then induces gene silencing and chromatin modification (Bjornsson et al., 2004). These are stable, heritable changes that do not affect the DNA sequence (Li et al., 2009). Abnormal regulation of epigenetic processes, have been linked to the onset and progression of several diseases, including CAD (Hmadcha et al., 1999; Bjornsson et al., 2004; Elton et al., 2011).

1.5.1 Single nucleotide polymorphisms and gene deletions

Multiple genetic and environmental factors influence an individuals' susceptibility to disease. Unlike inherited monogenic disorders where the presence or absence of a specific allele dictates the presence or absence of disease; in complex diseases, the presence or absence of risk alleles, in more than 1% of the population, may only mildly increase the probability of disease onset (Lander and Schork, 1994; Chakravarti, 1999; Risch, 2000).

In 1971, Harris hypothesized that common DNA variants provide the basis for variation of a single enzyme and "are quite possibly the underlying biochemical cause of much of the inherited diversity in the physical and physiological characteristics of individuals, and also in relative susceptibilities to various diseases and other disorders" (Harris, 1971). The first genetic variants tested were usually restriction fragment length polymorphisms (RFLPs), but with advancing technology such as the development of the polymerase chain reaction (PCR), microsatellites, variable number tandem repeats (VNTRs), insertion/ deletion polymorphisms, and SNPs can all be analysed.

Association studies, usually have a case-control design, and compares genotypic variants in patients to healthy controls. These types of studies are used to assess the presence of a gene variant and relate the frequency at which they occur between groups to susceptibility of various diseases. The conclusion of such studies is that the polymorphism being tested either affects risk of disease directly or is a marker for nearby genetic variants that affects disease risk (Hirschhorn et al., 2002; Samani et al., 2007).

The Myocardial Infarction Genetics Consortium (MIGC) has classified early onset myocardial infarction as 'a promising phenotype for genetic mapping' (Kathiresan et al., 2009). The MICG assessed SNPs at nine loci which reached genome-wide significance, three of which were newly identified and six that were replicated; and five hundred and fifty four copy number variations (CNVs), none of which were significant (Kathiresan et al., 2009). The observations in these studies show associations of SNPs with myocardial infarction lending credence to investigations of genetic changes linked to disease risk.

1.5.2 The role of microRNAs in coronary artery disease

MicroRNAs are a group of small non-coding RNA molecules which were discovered almost twenty years ago, but only over the past 5 years has there been an exponential increase of research in this field (Bushati and Cohen, 2007; Bartel, 2009). The miRNA registry has therefore created a set of standard nomenclature for this set of molecules (Table 1.1, (Griffiths-Jones, 2004).

Table 1.1 Nomenclature of miRNAs (Griffiths-Jones, 2004)

miRNA	Nomenclature	Example
miRNA	The prefix "mir" is followed by a dash and a number, which is attributed sequentially	mir-123 was likely discovered before mir-423
Pre-miRNA	Indicated by the uncapitalised prefix "mir"	mir-155
Mature form of miRNA	Indicated by the capitalised prefix "miR"	miR-155
miRNAs with nearly identical sequences (i.e., one or two nucleotides different)	Denoted with an additional lowercase letter	miR-125a miR-125b
Pre-miRNAs that are located at distinct genomic loci but that leaad to identical mature miRNA	Denoted with an additional dash-number suffix	mir-125b-1 miR125b-2
Two mature miRNAs that have originated from opposite arms of the same pre-miRNA, when relative expression levels are known	The predominant miR is named as described above and the other miR expressed at lower levels is followed by an asterisk	miR-155 miR-155*
When the relative expression levels of the two mature miRNAs originating from opposite arms of the same pre-miRNA are not known	The suffix -5p is added to the miR originating form the 5' arm and -3p is added to the miR from the 3' arm	miR-423-5p miR-423-3p
miRNA that originates from different species	The species of origin is designated by a three letter prefix	hsa-miR-155 (Homo sapiens) mmu-miR-155 (*Mus musculus*)

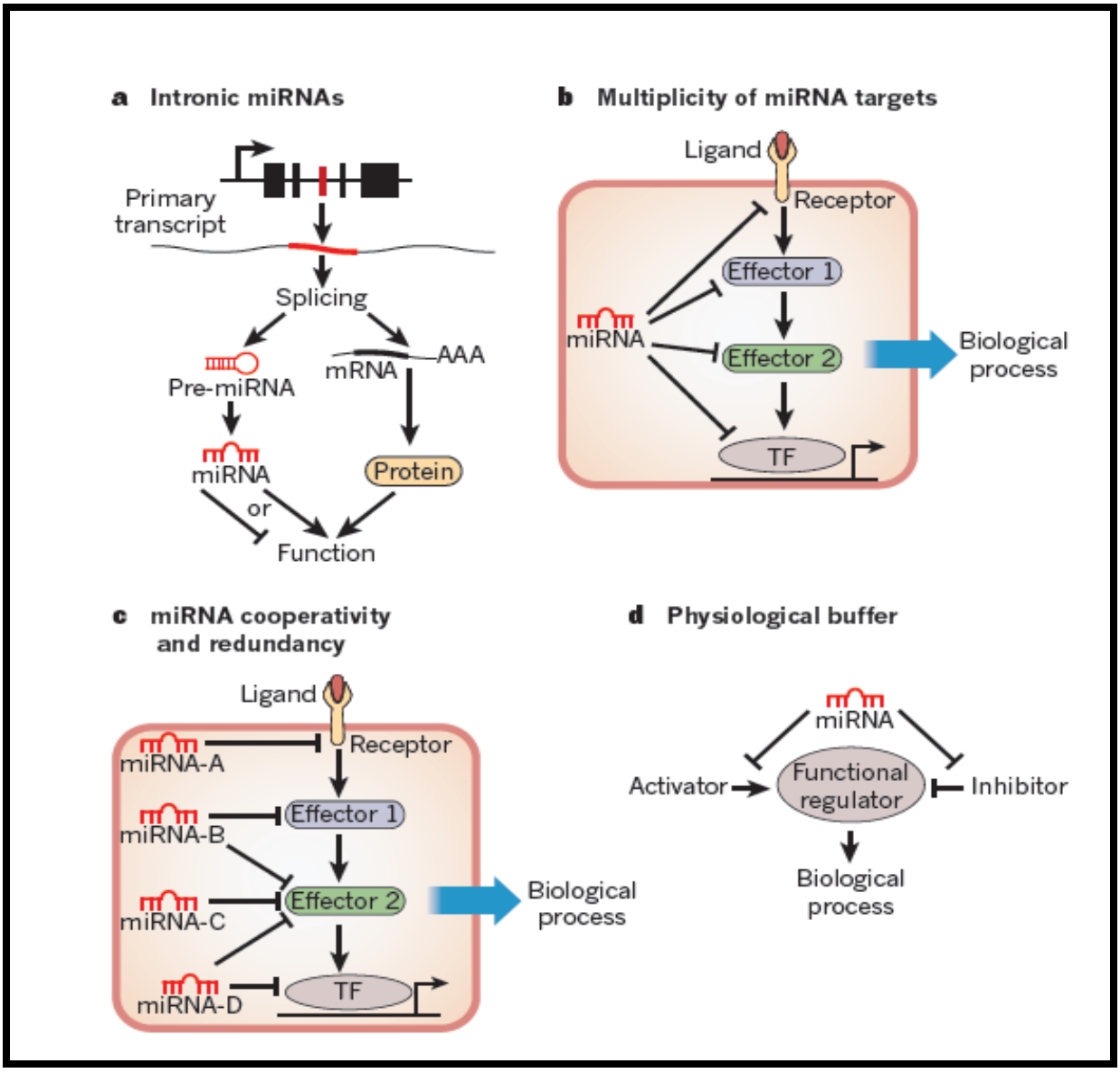


Figure 1.6 Concepts of miRNA functions. The potential sites of regulation of gene expression by miRNAs are represented in (a) intronic miRNAs encoded within the same protein code transcript that regulates similar processes of that protein; (b) a single miRNA targets several mRNA targets involved in the same biological process, TF, transcription factor; (c) several miRNAs regulate the same biological process by targeting individual components of a pathway; (d) miRNAs targets molecules that are involved in the regulation of a particular process (Small et al., 2010).

The newly found interest in miRNAs and their function (Figure 1.6) has been attributed to the critical role of these molecules in controlling several biological processes ranging from cell differentiation and proliferation to development and apoptosis. MicroRNAs regulate gene expression by acting as RNA silencers.

Most miRNA genes are generated from independent transcription units located in regions of the genome distant from protein-coding genes but at least 25% of human miRNAs are found in the introns of protein-coding genes. The primary transcripts (pri-miRNAs) are normally a few kilobases long and are characterized by the presence of stem loop structures (Figure 1.7).

The maturation of pri-miRNAs occurs via a cascade of reactions. The first reaction is catalysed by an enzymatic complex: RNAse III-type protein Drosha and the DiGeorge critical region 8 (DGCR8) proteins (Winter et al., 2009). Drosha and DGCR8 catalyse the cleavage of pri-miRNAs at the stem of the hairpin resulting in pre-miRNAs. Pre-microRNAs are approximately 80 nucleotide bases in length. Not all miRNAs require this type of processing as some miRNAs are generated from mirtrons (short intron sequences) with the potential to form hairpin loops.

A nuclear transport protein, exportin-5 which functions with GTPase Ran, then transports pre-miRNAs into the cytoplasm where further processing occurs (Winter et al., 2009). Cytoplasmic RNase Dicer, theRNA-binding protein: trans-activation response (TAR) RNA-binding protein (TRBP) and Argonaute-2 (Ago2) forms part of a multiprotein complex which

cleaves off the loop of pre-miRNAs generating 22-nucleotide miRNA duplexes with two nucleotides protruding at the 3 prime ends.

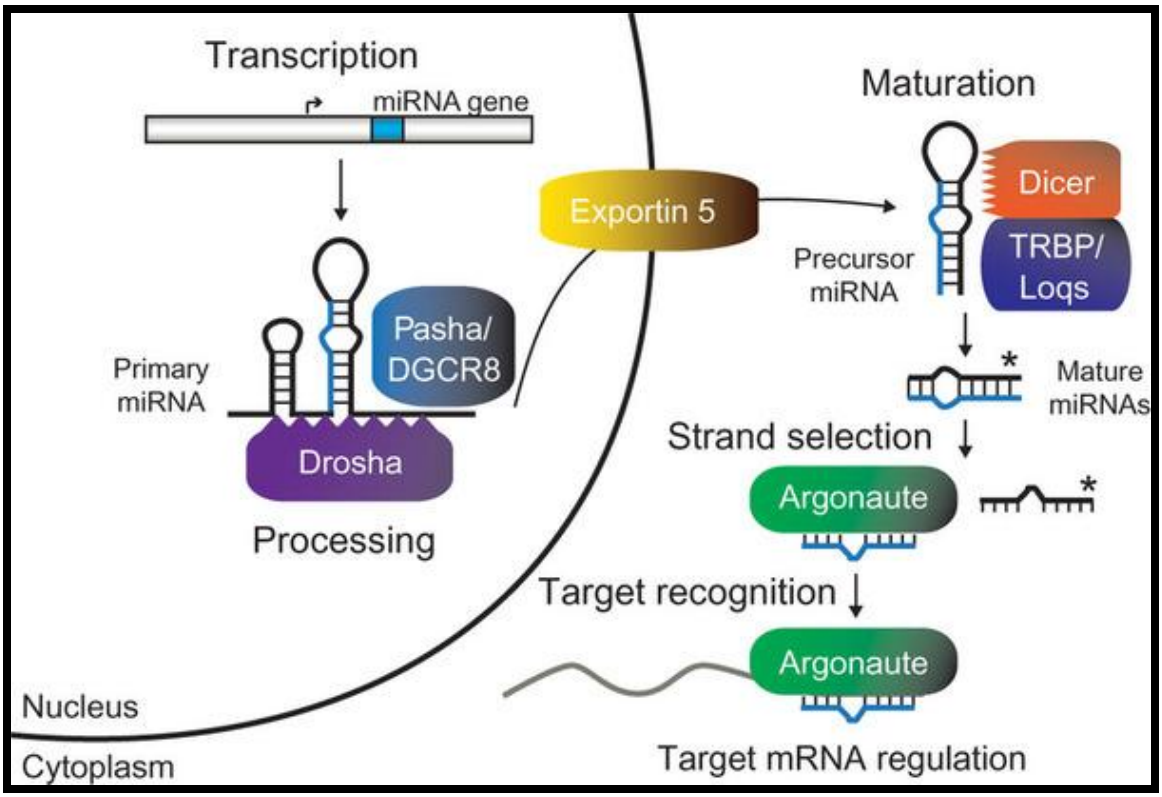


Figure 1.7 The process of miRNA maturation and regulation (Kai and Pasquinelli, 2010).

Once cleaved and after dissociation from the Dicer and TRBP, the miRNA is unwound and the functional strand remains bound to Ago2. The functional strand is complementary to the mRNA target associated with Ago2 guides the silencing activity of the RNA-induced silencing complex (RISC) effecter complex while the other strand is usually degraded (Figure 1.7, (Stefani and Slack, 2006; Mattes et al., 2007; Farazi et al., 2008; Winter et al., 2009; Kai and Pasquinelli, 2010).

Similar to other cellular processes, aberrant regulation of the expression of miRNAs is linked to the onset and progression of several diseases. By regulating gene expression post-transcriptionally by inhibiting translation and/or induction of protein degradation of specific RNAs (Lee et al., 2004), miRNAs have been implicated in contributing to CVDs such as acute myocardial infarction (Meder et al., 2011), heart failure (Tijsen et al., 2010), CAD (Fichtlscherer et al., 2010; Hoekstra et al., 2010) and stroke (Laterza et al., 2009; Saenger and Christenson, 2010).

Research into the modulation of miRNAs has therefore become an appealing idea for the treatment of heart disease (van Rooij et al., 2008). Several miRNA mimics and antagonists of miRNAs have already advanced to clinical trials (Turchinovich et al., 2011). The role of miRNAs in the regulation of inflammatory gene expression in immune cells has been well-investigated. The liver, however, also plays an integral role in the development of atherosclerosis being the source of cholesterol and lipoprotein synthesis, lipid metabolism and hepatic inflammation (Kleemann et al., 2007). Therapeutics (such as statins) is able to influence these processes and have also been implicated in affecting miRNA expression (Takwi et al., 2012; Wang et al., 2012). The significance of miRNAs undoubtedly requires further investigation to provide new insights into the pathogenic mechanisms and applications for CAD.

CHAPTER 2

GLUTATHIONE-S-TRANSFERASE POLYMORPHISMS AND EARLY-ONSET CORONARY ARTERY DISEASE IN YOUNG SOUTH AFRICAN INDIANS

2.1 Introduction

Atherosclerosis and thrombosis are the two major contributing mechanisms in CAD. The formation of atherosclerotic lesions is augmented by elevated levels of ROS (McGill et al., 2000). Endothelial cell injury is initiated by increased shear stress caused by the combination of high blood viscosity, hypertension and vasoconstriction that may be induced by smoking (Nagy et al., 1997; Blann et al., 1998).

Cigarette smoke contains a complex mixture of approximately four thousand different chemical species. In addition, cigarette smoke contains plentiful oxygen centred, nitrogen centred and carbon centred free radicals which increase ROS generation and promote oxidative damage (Koul et al., 2001).

Reactive oxygen species are produced by cells through normal metabolic processes and disruption of the mt electron transport chain (Hulsmans and Holvoet, 2009). Cell-intrinsic mechanisms, such as the glutathione (GSH) antioxidant defence system act to combat ROS. Disturbances in the endogenous antioxidant defence mechanism or ROS overproduction

however, leads to accumulation of free radicals which induce oxidative damage to biomolecules by lipid peroxidation, protein nitration and DNA adduct formation.

Cellular detoxification systems protect against both endogenous and exogenous harmful substances. Of particular interest is the super family of glutathione S-transferase (GST) enzymes, which modulate prostaglandin signalling pathways and oxidative stress (Hayes and McLellan, 1999).

Human GSTs exist in at least eight different classes (Alpha, Mu, Pi, Theta, Kappa, Zeta, Omega, and Sigma), which are assigned based on the similarity of their sequences. The metabolic role of GSTs is integral as they function to detoxify electrophilic substrates by catalysing their reactions with GSH (Figure 2.1), thereby reducing the interactions between reactive electrophiles and DNA, protein and lipids that impairs cellular function (Townsend and Tew, 2003).

Glutathione-S-transferases function to detoxify toxicants, including those found in cigarette smoke, by facilitating their conjugation to GSH (Sies, 1999). However, GST function is influenced by genotypic differences arising from SNPs (Hu et al., 1997).

The GST genes contain several polymorphic variants that occur at high frequency. In the GSTP1 variant, there is a single nucleotide change from A to G at codon 105, resulting in an isoleucine (Ile) to valine (Val) amino acid change. The GSTM1 variant is the complete deletion of the gene coding for this isoform and diminished enzymatic activity (Gong et al., 2006).

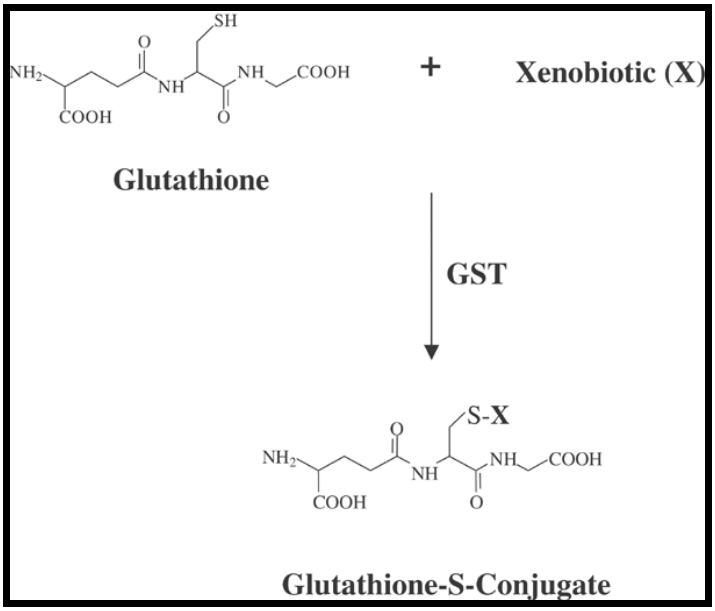


Figure 2.1 The detoxification reaction catalysed by glutathione-S-transferase (Townsend and Tew, 2003).

The genetic susceptibility in response to air pollution and cardiovascular outcomes showed that GSTP1 and GSTM1 played a role in ROS modulation (Zanobetti et al., 2011). Other studies also found an association of the GSTM1 null genotype with heart failure (Chahine et al., 2007; Madrigano et al., 2010).

A study by Madrigano et al. (2010) found that black carbon and particulate matter exposure was associated with increased intracellular and vascular cell adhesion molecules. These molecules promote atherosclerosis. The association they found was modified by the presence of the GSTM1 polymorphism (Madrigano et al., 2010). GSTP1 and GSTM1 polymorphisms have been associated with the biomarker of oxidative DNA-damage, 8-hydroxy-2'-deoxyguanosine (Ren et al., 2010) and increased susceptibility to smoking-related CAD (Palmer et al., 2003; Park et al., 2004; Singh et al., 2011).

The high incidence of CAD in SA Indians in Durban was first reported in 1969 (Wainwright, 1969) and decades later Indians in SA have the highest mortality for CAD (Norman et al., 2006). The age of onset of CAD, in this ethnic group has decreased in the past decades. Recent studies show a strong familial link with the history of T2D hypertension and CAD, supporting a genetic basis for the development of the disease (Ranjith et al., 2004; Ranjith et al., 2005).

In light of the evidence that variants of GSTP1 and GSTM1 influenced disease risk, this study aimed to assess the association of GST polymorphisms in young smoking and non-smoking SA Indian CAD patients.

2.2 Materials and Methods

Molecular grade reagents were purchased from Promega, United States, Fermentas, South Africa, Qiagen, United States and Biorad, SA, unless otherwise stated. All other reagents were purchased from Merck (SA).

2.2.1 Patient recruitment

One hundred and two CAD patients and 100 age, ethnicity and gender matched controls were enrolled in the study following institutional ethical approval (BE154/010). A full pathology report clinical markers was assessed by routine laboratory testing at the South African

National Accredited System Global Clinical and Viral Laboratory (Amanzimtoti, South Africa). The following parameters were tested: Haematology (Roche Sysmex 1800XT), Chemistry (Beckman Coulter DXC600), Endocrinology (Siemens Centaur XP), Serology (BD Biosciences FACS Calibur) as per international standards to obtain levels of total cholesterol, HDL, LDL, triglycerides, fasting glucose, 2h glucose, fasting insulin, glycosylated haemoglobin, sodium, potassium, bicarbonate, chloride, urea, creatinine, glomerular filtration rate, cell death (CD) 4 count, CD8 count, CD45 count and CD3 count. The physical measurements of weight, height, abdominal circumference, waist circumference and patient history were conducted by the clinician. The inclusion criterion for CAD patients were: Indian ancestry and unrelated, adults below the age of 45 years with stable CAD confirmed at angiography. The exclusion criteria for controls were: an acute coronary syndrome/revascularization procedure in the preceding 3 months, chronic renal or liver disease, malignancy and known active inflammatory or infectious disease.

2.2.2 DNA isolation

Genomic DNA was extracted from 300µl whole blood. A 1ml aliquot of 0.1M PBS (pH 7.4) was added to the whole blood. Tubes were inverted on a rotor for 10min and centrifuged (5 000xg, 3min, room temperature (RT)). The supernatant was decanted and 1ml Tris-Cl (20mM, pH 8.0) solution was added to the pellet. The resuspended pellet was then inverted on a rotor for 10min and centrifuged (5 000xg, 3min, RT). The supernatant was discarded and 900µl of Tris-Cl (20mM, pH 8.0) solution was added to the pellet, inverted for 8min and centrifuged at 5 000xg for 3min. The supernatant was decanted and 500µL lysis buffer (0.5%sodium dodecyl sulphate (SDS), 150mM NaCl, 10mM ethylenediaminetetraacetic acid (EDTA), 10mM Tris–

HCl (pH 8.0) was added to the pellet of white blood cells. To this, RNase A (100µg/ml; DNase-free) was added and incubated (37°C, 1h). Subsequently, proteinase K (200µg/ml) was added and incubated (3h, 50°C) and a 0.1% volume 5mM potassium acetate was added before centrifugation (5 000xg; 15 min). Supernatants containing genomic DNA were transferred to fresh tubes, extracted with 100% isopropanol and washed with 70% ethanol. DNA samples were solubilised in 10mM Tris and 0.1mM EDTA (pH 7.4, 4°C). Concentrations of DNA were determined spectrophotometrically.

2.2.3 Polymerase Chain Reaction

The principle of PCR is based on the amplification of a segment of DNA using specifically designed primer pairs, DNA monomers, the enzyme DNA polymerase, the relevant co-factors (magnesium chloride ($MgCl_2$)) and a DNA template. There are three major steps involved in the PCR amplification reaction viz. denaturation, annealing and extension under thermocycling conditions (Figure 2.2).

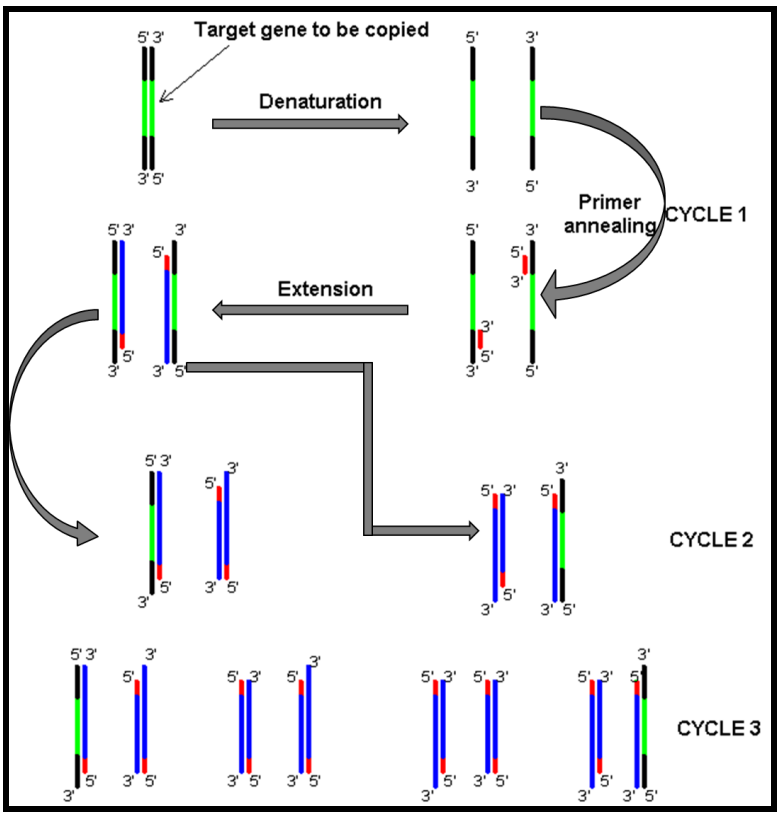


Figure 2.2 The polymerase chain reaction.

DNA denaturation occurs at approximately 95^{o}C during which double stranded DNA forms single stranded templates. The process of annealing involves the primer sets to anneal to the 3' ends of each DNA template, a process which occurs at a lower temperature (between 54^{o}C and 60^{o}C) and is dependent on the guanine and cytosine content of the primer sequence. The extension of DNA occurs at 72^{o}C, the optimal temperature at which DNA polymerase (derived from *Thermus aquaticus* (Taq)) functions to extend the target DNA sequence from the 3' end of the annealed primer (Ishmael and Stellato, 2008).

2.2.3.1 Optimisation of polymerase chain reaction

To ascertain the effective concentrations required for an efficient and specific amplification process, optimization of the major components of the PCR mastermix was conducted. Briefly, the primer sets, $MgCl_2$ and GoTaq® DNA polymerase were titrated over a range of concentrations. Primer sets were optimised first by varying the concentrations between 20 and 130 ρmol, while all other components were kept at a standard concentration. Once the PCR reactions were completed, they were verified by electrophoresis (150 V; 50 min) on a 1.8% agarose gel containing 0.5 mg/ml ethidium bromide and visualized by UV light and digitally photographed using a gel documentation system (Uvitech Alliance 2.7). The reaction containing minimal primer dimers and a specific, pristine band of the expected size was selected. This concentration was then used in the next set of reactions where $MgCl_2$ concentrations were varied between 0.5 and 3.5 mM. This was then repeated for the *Taq* polymerase (0.5 and 1.1U).

2.2.3.2 PCR-Restriction fragment length polymorphism

A 176 base-pair PCR product was amplified using 15pmol of forward and reverse primers in a 25µL reaction containing 200µM of each dNTP, 2.5mM $MgCl_2$, 1XGreen GoTaq® Flexi buffer (Promega, United States), 0.5U GoTaq® DNA polymerase (Promega, United States) and 100ng genomic DNA template. Primer sequences were:

Forward: 5'-ACCCCAGGGCTCTATGGGAA-3';

Reverse: 5'-TGAGGGCACAAGAAGCCCCT-3'

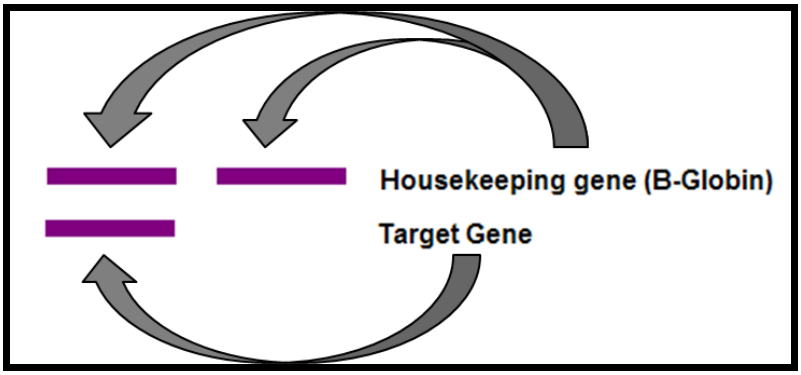
Following initial denaturation (96°C, 5min), amplification was carried out by 30 cycles of denaturation (96°C, 30s), annealing (55°C, 30s) and extension (72°C, 30s). This was followed by a final extension (72°C, 5min).

Presence of the polymorphic restriction site was analyzed by restriction endonuclease digestion of the PCR amplicon. Restriction endonucleases cleave DNA at specific consensus sequences along the strand in order to determine the presence of the polymorphism (change in nucleotide base). The consensus sequences and cleavage sites for each enzyme is unique.

Overnight digestion (37°C) was performed in 25µL (15µL PCR product, 4.5µL Buffer-R and 0.5µL (5U) BsmAI (Fermentas, South Africa)). Amplicons homozygous for the G_{105} allele were completely digested and resulted in two restriction fragments (91bp and 85bp). Restriction fragments were electrophoresced on an agarose gel (3%, 0.5mg/ml ethidium bromide) and visualized (Appendix 6).

2.2.3.3 Differential PCR

To assess the GSTM1 polymorphism, a differential PCR was performed. Differential PCR is referred to as a semi-quantitative technique in which more than one gene is amplified in a single PCR reaction mixture. In this case, the co-amplification of a house-keeping gene, β-globin, is necessary to prove the efficiency of the PCR reaction and an absence of the target gene of interest (Figure 2.3).



2.3 Schematic representing amplicons following differential polymerase chain reaction.

Two PCR products (a 268 base-pair (β-globin) and 215 base-pair (GSTM1) product) were amplified using 30pmol of primers for the GSTM1 gene and 10pmol primers for the β-globin gene in a 25μL reaction (200μM each dNTP, 3.3mM $MgCl_2$, 1XGreen GoTaq Flexi buffer, 1U Taq polymerase, 100ng genomic DNA template). Primer sequences were:

GSTM1 Forward: 5'-GAACTCCCTGAAAAGCTAAAGC-3';
Reverse: 5'-GTTGGGCTCAAATATACGGTGG-3'

β-globin Forward: 5'-CAACTTCATCCACGTTCACC-3';
Reverse: 5'-GAAGAGCCAAGGACAGGTAC-3'

Following initial denaturation (96°C, 5min) amplification was carried out by 25 cycles of denaturation (96°C, 30s), annealing (57°C, 30s), extension (72°C, 30s) and a final extension (72°C, 5min). Amplification products were electrophoresced on an agarose gel (4%, 0.5mg/ml ethidium bromide) and visualized. Presence of a 268bp product only is indicative of the homozygous null genotype. The amplification of a 215bp product is indicative of the presence

of the gene in either a homozygous positive for the presence of the gene or heterozygote state.

2.2.4 Statistical Analysis

Hardy-Weinberg statistics were calculated to assess the distribution of genotypes in our sample population. Graphpad Prism Software was used for the Fisher's exact test to assess the contingency of alleles. The D'Agostino and Pearson normality tested column statistics followed by the students' t test or Mann Whitney test for differences between groups.

2.3 Results

All clinical parameters assessed were similar between the groups with the exception of hypertension, with a larger percentage of patients presenting with higher systolic/diastolic pressure (Table 2.1). Single nucleotide changes at codon105 of the GSTP1 gene were investigated using RFLP-PCR. The genotype frequencies observed did not deviate from those predicted by Hardy-Weinberg statistics (GSTP1: p=0.294, CAD patients, p=0.413 controls; GSTM1 p=0.083, CAD patients, p=0.64; chi-square test).

Table 2.1 Demographics and clinical parameters

	Control (n=100)	CAD patients (n=102)	p value
Age (years)	37±4.31	37±4.45	0.953
BMI (kg/m^2)	27.8±0.5	27.93±0.4	0.888
Hypertensive (%)	22 *(22)*	44 *(43)*	0.0016*
Diabetic (%)	39 *(39)*	59 *(58)*	0.982
Total Cholesterol (mmol/L)	5.41±0.101	5.37±0.182	0.537
LDL (mmol/L)	3.70±0.091	3.39±0.170	0.104
HDL (mmol/L)	0.96±0.031	0.93±0.027	0.414
Triglycerides (mmol/L)	1.90±0.184	2.26±0.137	0.115

*Clinical parameters represented as mean ± standard error of the mean. *P<0.01*

A significant skew toward the A_{105}/A_{105} genotype was observed in CAD patients with a higher frequency compared to controls. In the control group the homozygous A_{105}/A_{105} and heterozygous A_{105}/G_{105} genotypes were observed at frequencies of 48% and 45%, respectively. The frequency of the A_{105} allele was significantly higher in CAD patients (80% vs. 70.5%; odds ratio=0.6011 95% CI=0.3803-0.9503, p=0.0377; Table 2.2). The GSTM1 0/0 genotype was also significantly more frequent in CAD patients (36% vs. 18%; odds ratio=2.593, p=0.0043; Table 2.3).

Table 2.2: Genotype and Allelotype GSTP1 frequency in CAD patients and controls

	Control, *n (%)*	CAD patients, *n(%)*	p value
Genotype frequency			0.797#
GSTP1 A_{105}/A_{105}	48 *(48)*	66 *(65)*	
GSTP1 A_{105}/G_{105}	45 *(45)*	31 *(30)*	

GSTP1 G_{105}/G_{105}	7 *(7)*	5 *(5)*	
Allelotype frequency			0.0377
GSTP1 A_{105}	141 *(70.5)*	163 *(80)*	
GSTP1 G_{105}	59 *(29.5)*	41 *(20)*	

[#] *Chi-square test for heterogeneity between CAD patient and control genotype distribution. Chi-square statistic = 0.587, 1 degree of freedom.*

Since a higher frequency of the GSTP1 A_{105} and GSTM1 null alleles were observed in patients, this study investigated whether there were genotypic differences in relative CAD risk with respect to these loci. Table 2.2 shows that the presence of the GSTP1 A_{105} and GSTM1 null genotype confers a significant risk for CAD. The groups were then stratified according to smoking history and the number of smokers in the patient group was much higher than the controls and significant association between smoking and CAD was found ($p<0.0001$, odds ratio=0.2245, 95% CI=0.1062-0.4746).

Table 2.3: Genotypic frequency of GSTM1 in CAD patients and controls

	Control, *n (%)*	CAD patients, *n (%)*	p value
Genotype frequency			0.83[#]
GSTM1 +/+ or +/0	82 *(82)*	65 *(64)*	
GSTM1 0/0	18 *(18)*	37 *(36)*	0.0043

[#]*Chi-square test for heterogeneity between CAD patients and controls allele frequency. Chi-square statistic = 4.463, 1 degree of freedom.*

The majority of patients who smoked presented with the GSTP1 A/A genotype (p=0.0987, odds ratio=0.5667 95% CI=0.2952-1.088; Table 2.4) and the GSTM1 0/0 (p=0.0221, odds ratio=2.386 95% CI=1.137-5.009; Table 2.4). Taken together the data shows that there is a

higher relative risk of CAD associated with GSTP1 A_{105}/A_{105} and GSTM1 null genotypes and this risk is increased with smoking.

Table 2.4: Summary of smokers stratified by genotypes for CAD risk identification

	Control, *n (%)*	CAD patients, *n (%)*	
Non-smokers	35 *(35)*	11 *(10)*	p<0.0001
Smokers/Ex-smokers	65 (65)	91 *(90)*	[a]RR=1.826 [b]95% CI(1.427-2.336)
Smokers/Ex-smokers GSTP1 A_{105}/G_{105} & G_{105}/G_{105}	31 (*31*)	31 *(30)*	p=0.0987 RR=1.382
GSTP1 A_{105}/A_{105}	34 (*34*)	60 (*59*)	95% CI(0.958-1.994)
Smokers/Ex-smokers GSTM1 +/+ & +/0	52 (*52*)	57 (*56*)	p=0.0221 RR=1.725
GSTM1 0/0	13 (*13*)	34 (*33*)	95% CI(1.044-2.851)

[a]RR: relative risk, [b]95% CI: 95% confidence interval

2.4 Discussion

The pathogenic processes of atherosclerosis and thrombosis leading to the development of CAD are closely related to genetic and environmental influences.

The INTERHEART study placed smoking second on the list of risk factors for myocardial infarctions (OR: 2.87, 99% CI) (Yusuf et al., 2004). The Systemic Coronary Risk Evaluation project also estimated the 10-year fatal cardiovascular risk to be twice as high for smokers vs. non-smokers for any given age, systolic blood pressure and cholesterol level (Conroy et al., 2003). The number of smokers worldwide is expected to reach 1.7 billion by 2025 (Mathers and Loncar, 2006; World Health Organisation, 2012). According to the World Health

Organisation, smoking related deaths is expected to rise from 4.8 million in the year 2000 to 8 million in 2030 if current trends are maintained.

Endothelial cell damage in vasculature is caused by changes in haemodynamics and oxidative stress, leading to thrombosis and atherosclerosis. The continuous cycle of inflammation from atherosclerotic plaques and arterial wall lesions contributes to elevated levels of CRP in CAD patients. The low grade inflammation, recruitment and activation of leucocytes to the atherosclerotic lesion plays an important role in the generation of ROS and RNS derived from the myeloperoxidase system and macrophage respiratory burst in an attempt to circumvent the spread of foreign material, while simultaneously exposing biomolecules to oxidative stress (Hulsmans and Holvoet, 2009).

The data in this study shows an association of the GSTP1 wild-type variant A_{105} and the GSTM1 0/0 with CAD. GST acts as a general base catalyst by increasing the rate of GSH conjugation to hydrophobic substrates by deprotonation of GSH to GS^- by an active tyrosinase. The change in codon105 of the GST gene causes an amino acid substitution from Ile to Val, resulting in a change in enzyme activity. Two previous studies showed the change from Ile to Val caused deviations in the atomic coordinates of the side chain of the key H-site residues that affected the enzyme's catalytic activity and increased susceptibility to smoking-related CAD (Palmer et al., 2003; Park et al., 2004; Singh et al., 2011). Earlier literature, however, indicates a 7-times more active conjugation of GSTP1 with the Val variant to diol epoxides as well as increased catalytic efficiency to aromatic epoxides (Hu et al., 1997).

These observations suggest the presence of the GSTP1 G allele in healthy controls enhances the efficacy of antioxidant mechanisms. The null GSTM1 genotype, however, results in the complete absence of that specific isoform of the enzyme, hence affecting the detoxification capacity. The strong association of the null genotype in CAD patients indicates a key role of this enzyme in the pathogenesis of the disease.

2.5 Conclusion

The association between smoking and CAD seen in this cohort is in agreement with previous studies (Palmer et al., 2003; Park et al., 2004; Singh et al., 2011). The high percentage of patients who smoked and presented with the GSTP1 A_{105}/A_{105} and GSTM1 0/0 genotypes supports the hypothesis that GSTs play an important role in CAD.

CHAPTER 3

THE ROLE OF UNCOUPLING PROTEIN 2 -866 G/A AND UNCOUPLING PROTEIN 3 -55 C/T POLYMORPHISMS IN YOUNG SOUTH AFRICAN INDIANS WITH CORONARY ARTERY DISEASE

3.1 Introduction

Uncoupling proteins are members of the super family of anion carrier proteins present in the inner mt membrane. The roles of UCPs 2 and 3 are not clearly defined but have been linked in the protection against oxidative damage; ageing and degenerative diseases (Affourtit et al., 2007). They regulate the mt membrane potential by the discharge of the proton gradient generated during oxidative phosphorylation and negatively regulate mt ATP synthesis. UCP2 and UCP3 can reduce the production of superoxide radicals at complex I, II and III of the mt respiratory chain by reducing the electrical potential across the inner mt membrane (Laskowski and Russell, 2008).

The function of UCP2 and UCP3 is dependent on specific activators such as hydroxynonenal, a by-product of phospholipid peroxidation, as well as fatty acids (FAs) (Echtay et al., 2003; Rial et al., 2004). Once activated, UCP2 and UCP3 increase the net proton conductance and the export of fatty acids and other anions from the mt matrix (Figure 3.1) (Brand and Esteves, 2005). In pancreatic beta cells, UCPs are thought to regulate insulin secretion by discharge of the proton motive force, decreasing ATP production and accumulation of ADP in the cytoplasm, thereby decreasing insulin secretion (Chan et al., 2001).

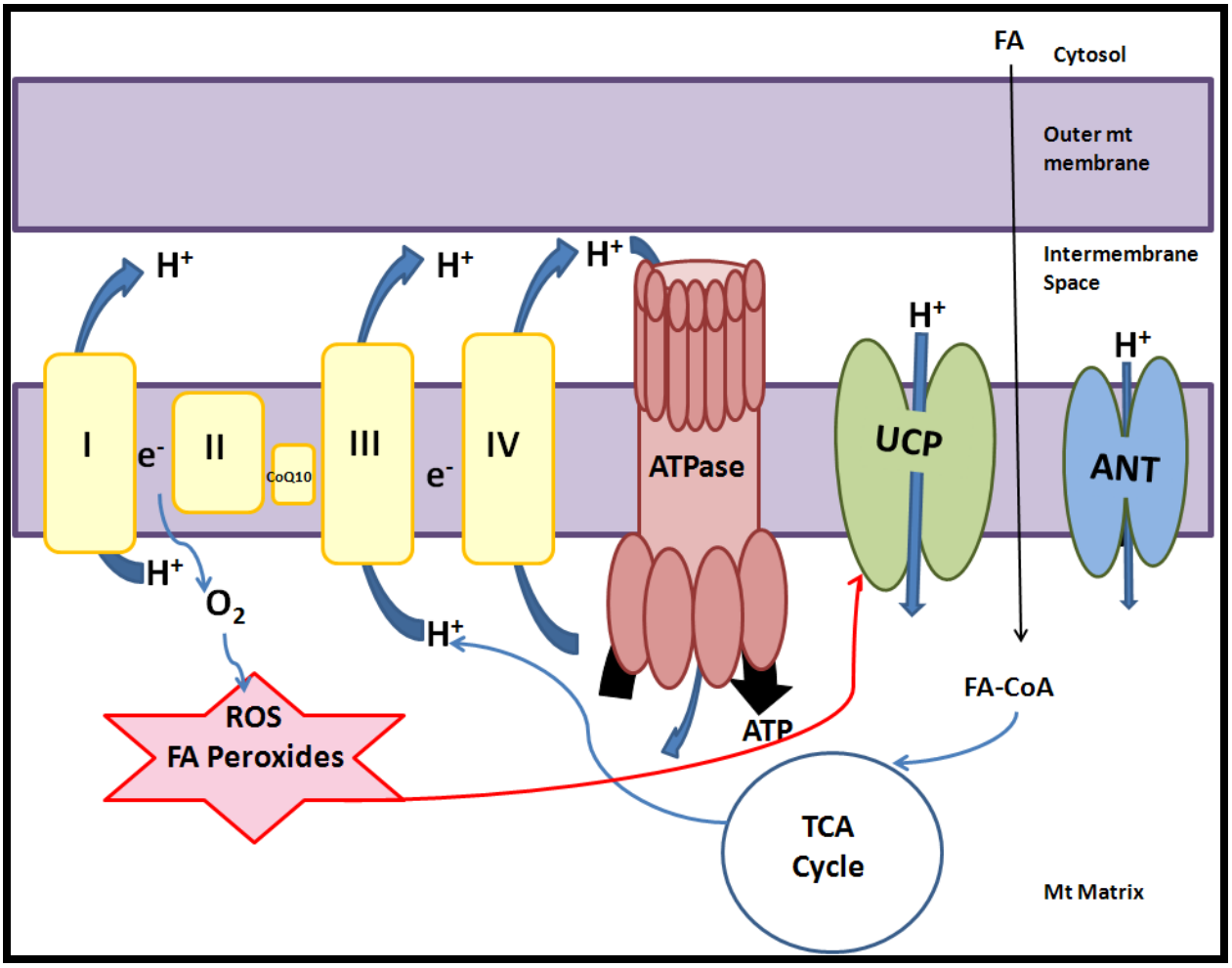


Figure 3.1 A model of the physiological functions catalyzed by UCP2 and UCP3. The complexes (I, III and IV) of the electron transport chain on the inner mitochondrial (mt) membrane transfers protons (H+) from reducing equivalents (NADH, $FADH_2$) in the mt matrix into the intermembrane space creating a proton gradient for the synthesis. Protons are pumped back into the mt membrane space by ATPase, uncoupling proteins (UCPs) or via an ATP dependant anion transporter (ANT). Electron leakage from the electron transport chain reacts with molecular oxygen to form reactive oxygen species (ROS). An excess of ROS causes peroxidation of lipids resulting in fatty acid (FA) peroxides.

In Figure 3.1, the double purple structures represent the inner and outer mt membrane. FAs are converted to fatty acyl CoA in the matrix, then reducing equivalents (H^+) are removed by β-oxidation and passed to the electron transport chain (Complexes I, II, III & IV represented by yellow boxes) where they are oxidized, causing proton pumping into the inter-membrane space. The resultant proton motive force drives ATP synthesis (at ATP synthase) or is dissipated by UCPs. During ROS production, UCPs are activated which lower the proton motive force to protect the cell against oxidative stress (Echtay et al., 2003). Another function of UCPs is to export FA anions generated when FA CoA is hydrolyzed and CoA is released for β-oxidation of FA (Rial et al., 2004). If ROS induces peroxidation of membrane phospholipids forming FA peroxide anions, these may also be exported by UCPs.

The role of UCPs in regulating oxidant stress and implications with respect to the pathogenesis of heart failure was reviewed (Laskowski and Russell, 2008). UCP2 in macrophages is necessary for efficient mt oxidation of glutamine, a strong inducer of UCP2 expression (Hurtaud et al., 2007; Nubel et al., 2008). It was also shown that UCP2 increased FA oxidation and promoted the metabolic shift from glucose oxidation to FA oxidation in mouse embryonic fibroblasts (Pecqueur et al., 2008). Several studies showed that UCP2- and UCP3-deficient mice overproduce ROS and hyper-secrete insulin (Vidal-Puig et al., 2000; Zhang et al., 2001; Brand et al., 2002; Blanc et al., 2003). Other studies have linked UCPs with disease prevalence such as diabetes (Xu et al., 2011) and obesity (Salopuro et al., 2009), the major predisposing factors leading to the development of CAD.

Compromised mt function, especially skeletal muscle FA oxidation, leads to increased triglyceride synthesis and ectopic lipid deposits (Roberts and Sindhu, 2009; Rogge, 2009).

The accumulation of cellular triglycerides has been implicated in increased lipid peroxidation, nitric oxide synthase (NOS) activity and pro-inflammatory cytokine production due to oxidative stress (Roberts and Sindhu, 2009). Furthermore, oxidative stress has emerged as the underlying mechanism for pathology in T2D and CAD. The distribution of UCP SNPs has been identified in several ethnic groups at different frequencies (Xu et al., 2011).

A few studies assessed the UCP2 -866 G/A SNP (located in the *cis*-regulatory site of the promoter region) and the UCP3 -55 C/T SNP (located 6 base-pairs upstream from the TATA box in the core promoter region) and found a functional link with CAD severity, progression and occurrence (Salopuro et al., 2009; Srivastava et al., 2010). In a group of diabetic patients there was a significant association of CRP levels with the UCP2 -866G/A SNP (Lapice et al., 2010).

The aim of this study was to explore the relationship between UCP2 -866G/A and -UCP3 55C/T SNPs and the risk of CAD in young SA Indians.

3.2 Materials and Methods

3.2.1 Patient recruitment

As described in Section 2.2.1.

3.2.2 DNA isolation

As described in Section 2.2.2

3.2.3 PCR-RFLP

Evaluation of SNPs in the UCP2 -866(G/A) (rs659366) and the UCP3 -55(C/T) (rs1800849) promoter region was done using PCR-RFLP. The UCP2 promoter region was amplified using 10*p*mol of primers:

Forward: 5'CACGCTGCTTCTGCCAGGAC'3

Reverse: 5'AGGCGTCAGGAGATGGACCG'3,

resulting in a 363bp PCR product.

The UCP3 region was amplified using 20*p*mol of primers:

Forward: 5'GGATAAGGTTTCAGGTCAGGC'3

Reverse: 5'AAGGGATGAGGGAGGAGAA'3,

resulting in a 194bp PCR product.

The PCR reaction volume for each sample was 40µL, comprising of: GoTaq® Flexi Buffer, 25mM $MgCl_2$, 200µM of each dNTP, 0.5U Taq polymerase (Promega, United States), and 10ng genomic DNA template. Amplification conditions: initial denaturation (95°C, 5min), followed by 35 cycles of denaturation (95°C, 30 sec), annealing (UCP2: 68°C and UCP3: 54°C) for 30 sec and extension (72°C, 30 sec). Final extension was achieved at 72°C for 7 min.

Following PCR, the amplicons were subjected to cleavage by restriction enzymes to determine the presence of polymorphisms at the designated sites. For digestion, a 31µL

reaction volume containing 10µL of PCR amplicon, 1µL specific RE and 2µL appropriate buffer as specified by the manufacturer (Fermentas, South Africa) was prepared. Digestion proceeded for 16 h at 37°C. Restriction products were analyzed on a 3% agarose gel stained with 0.5mg/ml ethidium bromide and visualized under UV light. *Mlu* I was used to restrict the UCP2 amplicon, *Hae* III was used for the UCP3 amplicon (Appendix 6).

3.3 Results

Clinical and laboratory parameters were measured in CAD patients and controls (See Table 2.1 in Section 2.3.1).

Single nucleotide changes at positions -866 in the UCP2 and -55 in the UCP3 promoter regions were investigated using RFLP-PCR. The genotype frequencies for UCP2 -866G/A and UCP3 -55C/T polymorphism observed did not deviate with those predicted by Hardy-Weinberg statistics (UCP2: p=0.319 controls, chi-square statistic=0.99; p=0.8487, chi-square statistic=0.036 CAD patients; UCP3: p=0.16 controls, chi-square statistic=1.984; p=1, chi-square statistic=0.0005 CAD patients).

Table 3.1: Frequency of genotypes and allelotypes in control and CAD patients

		Control (n=100)	CAD Patients (n=100)	Odds Ratio (95% CI)
UCP2 -866 Genotype	AA	35	29	
	AG	52	59	
	GG	13	12	
Alleles	A	40	42	1.110 (0.744-1.655)
	G	60	59	
UCP3 -55 Genotype	CC	63	66	
	CT	30	32	
	TT	7	2	
Alleles	C	78	82	0.788 (0.482-1.289)
	T	22	18	

No significant association between UCP2 -866G/A and UCP3 -55C/T genotypes was observed in CAD patients compared to controls (Table 3.1). For the UCP2 -866G/A SNP

(control group) the homozygous GG, AA and heterozygous GA genotypes were observed at frequencies of 13%, 35% and 52%, respectively compared to cases with 12%, 29% and 59%, respectively (Table 3.1). The homozygous CC and TT genotypes were observed at 63% vs. 66% and 7% vs. 2% between controls and CAD patients, respectively, with C/T presenting at 30% in controls and 32% in patients.

Table 3.2: Summary of elevated laboratory parameters in CAD patients stratified by -866G/A UCP2 and -55C/T

		Triglycerides (mmol/L)	Fasting glucose (mmol/L)	HbA1c (%)	hsCRP (mg/L)
UCP2 -866	AA	2.04±0.43	6.31±0.54	6.31±0.54	4.43±0.79
	AG	2.53±0.23	6.06±0.36	6.48±0.26	10.1±1.93
	GG	2.15±0.19	6.66±0.54	6.76±0.36	5.45±0.10
UCP3 -55	CC	2.33±0.17	6.11±0.27	6.44±0.21	8.13±1.47
	CT	2.06±0.25	6.48±0.57	6.76±0.35	7.44±1.09
	TT	6.47±1.70$^{*\#}$	11.87±3.74$^{*\#}$	10.1±2.57$^{*\#}$	10.23±4.14

*Parameters represented as mean (standard error of the mean) P<0.05compared to -55UCP3 CC*or CT$^{\#}$*

When comparing the presence of elevated fasting glucose, HbA1c and triglyceride levels in relation to the UCP2 -866 AA, AG and GG genotypes and UCP3 -55CC, CT and TT genotypes, a significantly higher level of fasting glucose (p=0.0006), HbA1c (p=0.0025) and triglycerides (p<0.0001) in patients with the UCP3 -55 TT genotype was observed (Table 3.2). No other associations between the investigated polymorphisms and laboratory parameters were observed.

3.3 Discussion

This study showed that no correlation between SNPs in the -866G/A UCP2 and -55C/T UCP3 promoter region and CAD in young South Africans of Indian descent but stratification of clinical parameters such as triglycerides, fasting glucose and percentage HbA1c, higher in CAD patients, was elevated in the presence of the UCP3 -55T/T genotype. A larger sample size in this genotypic group, however, is required to make meaningful conclusions.

Inflammation plays an integral role in initiation, progression and rupture of atherosclerotic plaques, and CRP is widely recognized as a marker for CVD (Hulsmans and Holvoet, 2009; Kengne et al., 2011). Oxidative stress, another major contributing factor to chronic inflammation, is prominent in atherosclerotic plaques. The regulatory function of UCPs in ROS generation has important implications for a role in inflammation (Palmer et al., 2009).

The UCP2 -866G/A polymorphism has been associated with CAD and T2D in several population groups (Salopuro et al., 2009; Srivastava et al., 2010). The overproduction of ROS and hyper-secretion of insulin in UCP2- and UCP3-deficient mice models indicated a functional role of UCPs in the development of T2D (Zhang et al., 2001). Recent meta-analysis on the UCP2 -866 and UCP3 -55 SNPs in Indian subjects with T2D or obesity was conducted (Dalgaard, 2011; Xu et al., 2011). The UCP2 -866 A allele was associated with increased risk of obesity and hyperinsulinaemia between 200 patients and 290 controls (Srivastava et al., 2010); a lack of association of UCP2 -866 and reduced risk UCP -55 with diabetics (n=487)

compared to controls (n=385) (Vimaleswaran et al., 2011); and an association of the UCP2 -866 G allele with diabetes mellitus (n=762 vs. controls n=924) (Gable et al., 2006).

The UCP3-55 T allele was shown previously to increase the onset of T2D in middle-aged men (Gable et al., 2006), was associated with abdominal fat distribution (Cassell et al., 1999) and obesity (Otabe et al., 2000; Halsall et al., 2001). The influence of UCP3-55T/T genotype on clinical markers of obesity and T2D is an important finding based on the premise that T2D and obesity are predisposing factors for CAD development.

A significantly high number of CAD patients were overweight with a mean BMI of 27.93kg/m^2. This is important as adipose tissue, and the macrophages found within, is a source of IL-6 and adipokines that contribute to atherosclerosis. Based on an immune-modulatory role of UCP2 due to its expression in macrophages and T cells, Lapice et al. (2010) reported a significant association of elevated hsCRP levels with the UCP2 -866 GG genotype (Lapice et al., 2010). Elevated levels of pro-inflammatory cytokines are found in inflammatory disease conditions (Murray and Freeman, 2003). This is evident in CAD patients with higher levels of CRP, its levels are not susceptible to circadian variation (Szalai et al., 2005) and its plasma half-life of 19h is nearly constant between normal subjects and patients with inflammatory conditions (Otabe et al., 2000).

In the present study, hsCRP levels were elevated in CAD patients presenting with the UCP2 -866 homozygous G genotype as well as the UCP3 -55 homozygous T genotype supporting the hypothesis of increased ROS activating nuclear factor kappa B (NF-$_K$ B) pathway which induces transcription of IL-6 and hence CRP production (Lapice et al., 2010).

The association of UCP2 polymorphisms with disease risk has been addressed in several studies. However, the genetic studies on polymorphisms on CAD, especially in Indians in SA are limited. Genetic association studies provide a potentially powerful tool for identifying genetic variations that influence susceptibility to common diseases. It is well-known that ethnicity is one of the most important factors for evaluating genetic effects on common complex traits.

3.4 Conclusion

The presence of the UCP -866 G/A and UCP -55 C/T SNPs does not directly influence CAD susceptibility in SA Indians. The association between UCP3 -55T/T genotype with elevated triglycerides, fasting glucose, HbA1c and CRP is in agreement with other studies (Lapice et al., 2010; Dalgaard, 2011; Xu et al., 2011) and may therefore play an important role in CAD.

CHAPTER 4

PROMOTOR POLYMORPHISMS IN THE INTERLEUKIN-6 (-174 G/C) AND C-REACTIVE PROTEIN (-390 C/A/T) GENES INFLUENCE THE RISK OF CORONARY ARTERY DISEASE

4.1 Introduction

Pro-inflammatory factors play a major role in the development of CAD, and elevated levels are used as diagnostic markers and risk factors (Pearson et al., 2003; Lubrano et al., 2009). Interleukin 6, a pro-inflammatory cytokine, has recently become a focus of interest in CAD development and is produced by an array of immune cells including activated macrophages, lymphocytes and endothelial cells (Figure 4.1) (Yudkin et al., 2000; Lubrano et al., 2009).

Interleukin 6 is one of the principle mediators of the hepatic acute phase response due to its ability to promote inflammation through the activation and proliferation of lymphocytes, differentiation of B cells and recruitment of immune cells (Stenvinkel et al., 2005). Adipose tissue is considered as one of the most significant sources of IL-6 due to stimulation from adipokines. It is for this reason that obesity is linked to low grade inflammation as a potential trigger for CVDs (Tilg and Moschen, 2006).

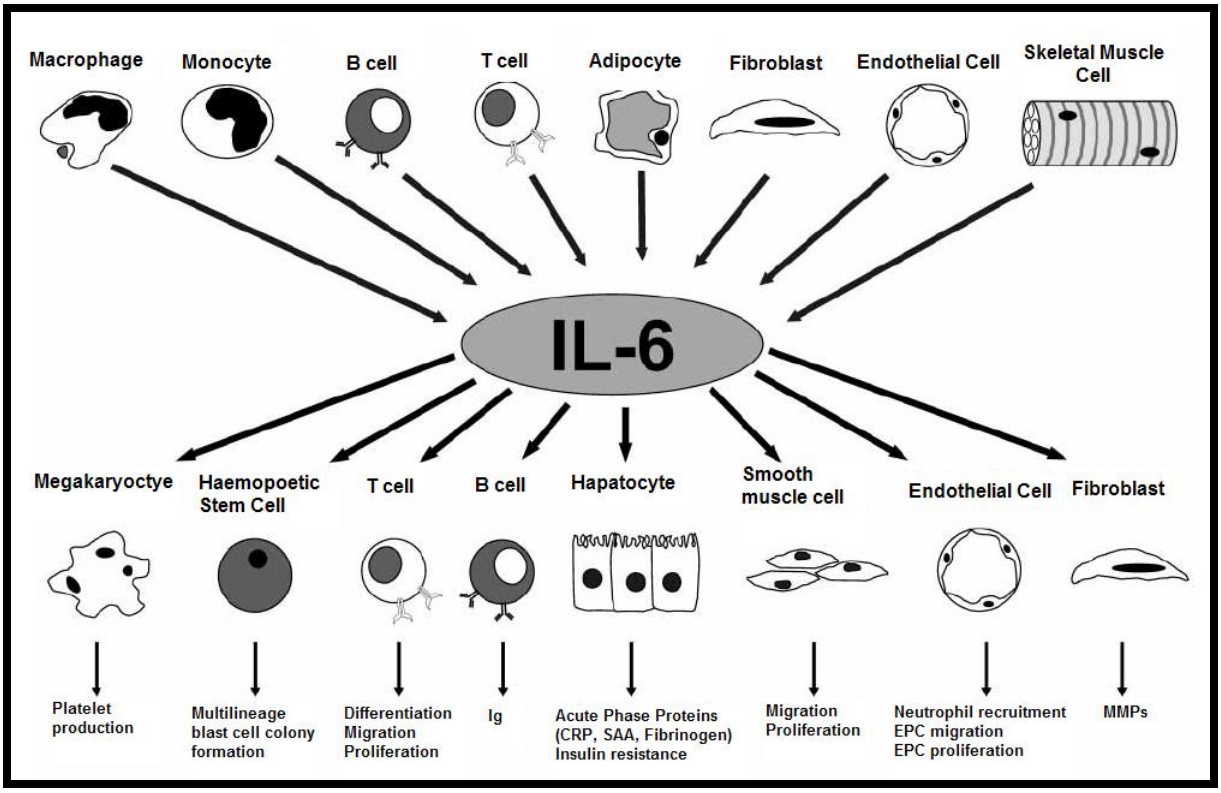


Figure 4.1 Interleukin 6 is produced from a range of cell types and also has several targets (Schuett et al., 2009).

C-reactive protein expression is primarily induced by IL-6 and synergistically enhanced by IL-1β and to a lesser extent by TNF-α (Rhodes et al., 2011). The biological roles of CRP are attributed to its ligand binding properties, functional in the clearance of apoptotic, damaged host cells as well as pathogenic agents such as bacteria via the formation of immune complexes as illustrated in Fig. 4.2, thus contributing to inflammation.

The baseline level of CRP amongst individuals varies by up to 40% due to genetic factors (Hage and Szalai, 2007; Cordeiro et al., 2008). As a result, there is a prominent individual-specific inflammatory response to injury between individuals (Terry et al., 2000; Aker et al., 2009).

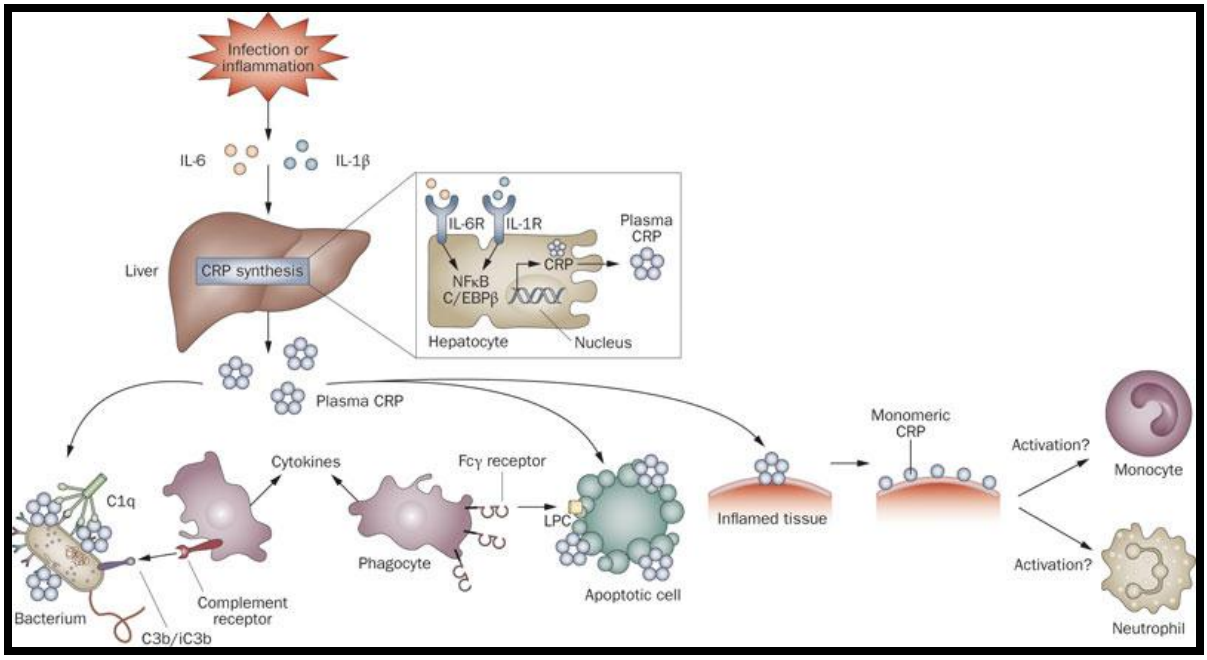


Figure 4.2 CRP synthesized in response to pro-inflammatory cytokines is released into circulation and interacts with several target molecules (Rhodes et al., 2011).

Single nucleotide polymorphisms in the promoter regions of the genes of inflammatory cytokines have been shown to influence mRNA and cytokine levels in circulation and are implicated in the development and progression of inflammatory conditions (Olivieri et al., 2002; Hage and Szalai, 2007; Shanker and Kakkar, 2010; Yeh et al., 2010; Najar et al., 2011; Pereira et al., 2011). The promoter region of IL-6 is very sensitive to environmental stress (as a result of oxidative stress, hypoxia or inflammation activated molecules such as the mitogen activated protein kinases - JNK and p38 - both of which have recently been targeted by therapeutics to reduce inflammation) and is thought to be highly conserved in the regions susceptible to transcription factor binding (Schuett et al., 2009). The IL-6 SNP at position 174 in the promoter region is of particular importance in chronic inflammatory diseases (Fishman et al., 1998).

The IL-6 gene is located at chromosome 7 position p21, (Ray et al., 1990; Kiszel et al., 2007). The IL-6 -174 SNP is situated near a transcription factor 'hotspot area' that is closely related to transcriptional regulation of IL-6 (Ray et al., 1990). Another important factor is the that two multiple responsive elements as well as additional transcription factor binding sites, namely cyclic adenosine monophosphate responsive element (CRE) and cytidine-cytidine-adenine-adenine-thymine (CCAAT)/enhancer binding sites, lie in the IL-6 gene region between positions -173 and -145. It has therefore been speculated that a single nucleotide change at position -174 may interfere with transcription factor binding in a positive or negative manner. This has been observed in several population genetic studies which revealed conflicting results on IL-6 production and the presence of the SNP (Fishman et al., 1998; Brull et al., 2001; Sie et al., 2006).

Several studies have assessed the IL-6 -174 G/C promoter polymorphism in different populations, including subjects of Indian ethnicity. The presence of the SNP was observed at varying frequencies in these populations (Vickers et al., 2002; Ivanova et al., 2011; Ujcic-Voortman et al., 2011). A recent meta-analysis study on Caucasian, Turkish, Tunisian and Chinese populations with CAD showed a high frequency of the IL-6 -174 GC genotype compared to the CC genotype with only a few studies showing different outcomes (Niu et al., 2012). A study on Indians (from India) showed a high frequency of GG genotypes with higher frequencies of the G allele (Maitra et al., 2008). To date, however, no studies have investigated the distribution of this SNP in the SA population.

Single nucleotide polymorphisms in the promoter region of CRP have been shown to influence circulating levels of CRP and are strongly associated with inflammatory conditions

(Hage and Szalai, 2007; Shanker and Kakkar, 2010; Najar et al., 2011). Distribution of SNPs in the CRP gene and promoter region have been identified in various ethnic groups (Vickers et al., 2002; Szalai et al., 2005; Hage and Szalai, 2007; Paik et al., 2007; Ivanova et al., 2011; Ujcic-Voortman et al., 2011).

The influence of promoter SNPs on gene expression and protein levels has been observed in several studies (Carlson et al., 2005; Miller et al., 2005; Szalai et al., 2005). A recent study on a group of diabetic North Indians found that the rs3093059 CRP polymorphism is a *cis*-acting contributor to the variance in hsCRP levels (Mahajan et al., 2011).

Promoter polymorphisms in the CRP gene have been assessed. One such study evaluated the bi-allelic SNP at position -409 and tri-allelic SNP at position -390 resides within the sequences -412CACGTG-407 (E-box 1) and -394CACTTG-389 (E-box 2) respectively (Szalai et al., 2005). The SNP at position -390 is interesting as the nucleotide cytosine can either be replaced with adenine or thymine. The A allele has been observed at very low frequencies. Both E-box 1 and E-box 2 contain DNA sequences that support transcription factor (such as upstream stimulatory factor-1) binding. In the presence of the -409 G-allele and the -390 T-allele, transcription factor binding is enhanced (Szalai et al., 2005).

In this study the frequency of promoter polymorphisms in IL-6 (-174G/C) and CRP (-390C/A/T) genes of young SA Indian CAD patients was evaluated. In addition, the effect of the genotypic variants on the circulating levels of IL-6 and CRP was also investigated.

4.2 Materials and Methods

4.2.1 Patient recruitment

As described in Section 2.2.1.

4.2.2 DNA isolation

As described in 2.2.2

4.2.3 PCR-RFLP

Evaluation of SNP at position 174 in the IL-6 promoter region (G/C) (rs1800795) and the tri-allelic SNP in the CRP promoter region (-390 C/A/T) was done using PCR-RFLP. The IL-6 and CRP promoter region was amplified using 40*p*mol of both forward and reverse primers. PCR reaction volume for each sample was 40µl, comprising of: GoTaq® Flexi Buffer, 25mM $MgCl_2$, 200µM of each dNTP, 0.5U GoTaq®DNA polymerase (Promega, United States), and 10ng genomic DNA template. Amplification conditions: initial denaturation (95°C, 5min), followed by 35 cycles of denaturation (95°C, 30s), annealing (55°C, 30s) and extension (72°C, 30s). Final extension was achieved at 72°C for 7 minutes.

The following primers were used:

IL-6 Forward: 5'TGACTTCAGCTTTACTCTTTG3';

IL-6 Reverse: 5'CTGATTGGAAACCTTATTAAG3'

CRP Forward: 5'AAGGGGTTAGTGATAATGTCC3'

CRP Reverse: 5'GGGAAATGGTAACATATTAATC3

The PCR amplified a 168bp product of the IL-6 promoter region and 141bp product of the CRP promoter region. DNA bands were visualized by UV light and captured using a gel documentation system (Uvitech Alliance 2.7). To determine the -174G/C genotype, the PCR product was restricted with the restriction enzyme *Nla* III (5u/µl) (Fermentas, South Africa), which cuts in the presence of the -174 C-allele, resulting in two fragments of 119bp and a smaller fragment (migrates off the gel). No cleavage occurs in the presence of the -174 G-allele. For the tri-allelic SNP, *Bfa* I and *Taq* I restriction enzymes were used to detect the CRP -390 SNP. Note that provision for *Taq* I restriction site was made by the underlined nucleotide in the reverse primer, which indicates the mismatch (T/A) (Appendix 6).

4.2.4 IL-6 Enzyme-linked immunosorbent assay (ELISA)

Human IL-6 ELISA Kit II (BD OptEIA™, Figure 4.3) was used to measure the IL-6 serum levels of patients and controls. As per user manual, ELISA diluent (12ml buffered protein base, 0.09% sodium azide) was added into a 96 well microtitre plate coated with anti-human IL-6 monoclonal antibody. A serial two fold dilution ranging from 0*p*g/ml to 250*p*g/ml was prepared. Standards and samples were added into appropriate wells. Following incubation (2h, RT) wells were rinsed with wash buffer (1× detergent solution with ProClin™-150). Working detector (containing Streptavidin-horseradish peroxide and Biotinylated anti-human IL-6 monoclonal antibody) was added into each well and incubated (1h, RT, dark). Wells were rinsed with wash buffer, seven times, followed by the addition of TMB One-Step Substrate Reagent (3,3',5,5'-tetramethylbenzidine) and incubated (30min, RT, dark). Following incubation, stop solution (1M phosphoric acid) was added, and absorbance measured at

450nm (reference: 570nm) with a microplate reader (Bio-Tek µQuant, United States). Serum concentrations of IL-6 were calculated by extrapolation from the standard curve (Appendix 1).

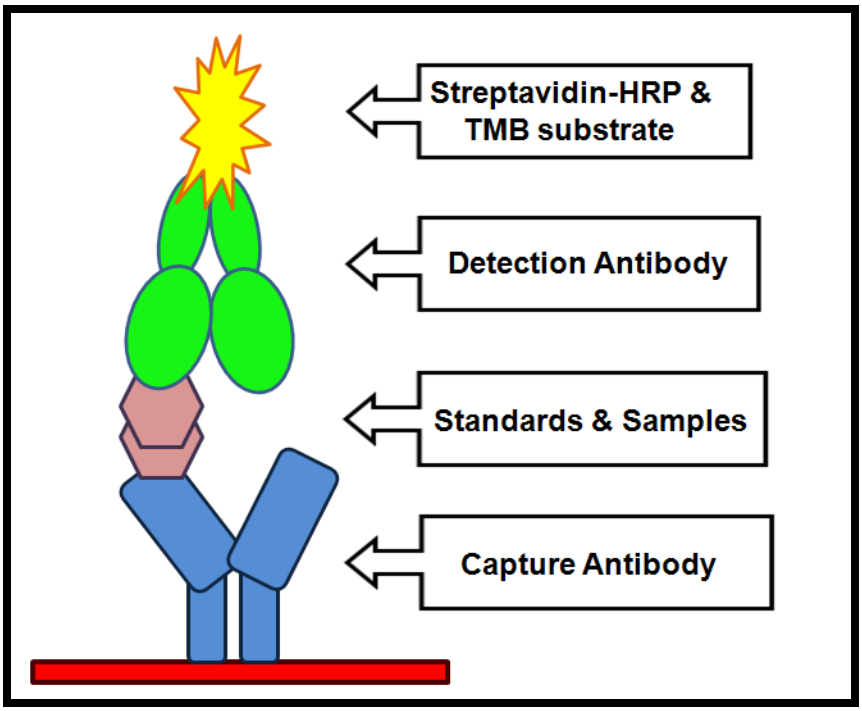


Figure 4.3 Representation of the ELISA antibody configuration.

4.3 Results

The single nucleotide change at positions -174 in the IL-6 promoter region and -390 in the CRP promoter region was investigated using PCR-RFLP.

Table 4.1 Frequency of genotypes and alleles in IC, CAD, non-diabetic IC and CAD.

		IC	CAD	OR (95% CI)	IC (non T2D)	CAD (non T2D)	OR (95% CI)
IL-6 -174 G/C	Genotype frequency						
	GG	58	67		34	29	
	GC	32	27		19	11	
	CC	10	6		8	1	
	Allelotype frequency						
	G	148	161	0.689	87	69	1.307
	C	52	39	(0.430-1.105)	35	13	(1.047-1.632)
CRP -390 C/A/T	Genotype frequency						
	AC	3	3		3	2	
	AT	7	4		5	2	
	CC	56	74		29	30	
	CT	24	9		15	5	
	TT	10	10		9	2	
	Allelotype frequency						
	C	139	160	1.755	78	67	2.634
	A/T	61	40	(1.090-2.778)	46	15	(1.350-5.138)

Fischer's exact test for heterogeneity between alleles of the IL-6 -174G/C SNP where p=0.152 for Indian control (IC) vs. CAD; and p=0.0431 for Control and CAD patients who were not diabetic (non T2D). Fischer's exact test for heterogeneity between alleles of the CRP -390C/A/T SNP where p=0.0211 for IC vs. CAD; and p=0.0048 for non T2D IC and non T2D CAD patients. (Percentages not shown as n=100 for each group).

The genotypic frequencies for the -174 IL-6 G/A polymorphism conform to the Hardy-Weinberg equilibrium (IC: X^2=2.84, p=0.09; CAD: X^2=1.96, p=0.16, Table 4.1). No association was found between CAD and the IL-6 -174G/A SNP (p=0.152, OR: 1.54 95% CI: 0.905-

2.324). A high percentage of the IC (39%) and CAD patients (59%) were pre-diabetic/ diabetic. We assessed genotypic frequencies between the IC and CAD following exclusion of diabetic subjects in each group and found a weak association of the IL-6 -174 G allele with CAD (p=0.0431, OR: 2.135 95% CI: 1.049-4.347). Interestingly, the non-diabetic IC presented with a significantly higher frequency of the C allele (71% -G vs. 29% -C) compared to non-diabetic CAD patients (84% -G vs. 16% -C; Table 4.1).

For the tri-allelic -390 C/A/T CRP polymorphism, the control group presented with 56% and 10% homozygous CC and TT genotypes and 7%, 3% and 24% heterozygous AT, AC and CT genotypes, respectively. The frequency of -390 CRP genotypes in CAD patients were 4% AT, 3% AC, 74% CC, 9% CT and 10% TT (Table 4.2). A weak association (p=0.0211, OR 1.299 95% CI: 1.064-1.586) with CRP -390 C allele and CAD patients was observed compared to controls (Table 4.1). This strength of the association, however, increased (p=0.0048, OR 2.634 95% CI: 1.350-5.138) when diabetic subjects were excluded from the analysis of both the IC and CAD groups (Table 4.1).

The levels of IL-6 and CRP between IC and CAD patient groups and non-diabetic IC with non-diabetic CAD patients were then compared (Table 4.2). The median levels of IL-6 were similar in CAD patients and non-diabetic CAD patients compared to respective control groups. CRP levels were higher in CAD patients and non-diabetic CAD patients compared to respective control groups (Table 4.2). These differences, however, did not reach statistical significance.

Table 4.2 IL-6 and CRP levels between IC, CAD, non-diabetic IC and CAD groups.

	IL-6 (pg/mL)	p value	CRP (mg/L)	p value
IC	0.9 (0.87, 0.92)	0.963	2.9 (1.93, 8.35)	0.466
CAD	0.9 (0.90, 0.91)		5.65 (1.9, 8.2)	
IC (non-T2D)	0.9 (0.74, 0.91)	0.558	2.4 (1.90, 7.80)	0.157
CAD (non-T2D)	0.9 (0.90, 0.91)		6.0 (1.9, 9.4)	

Data represented as the median (25th percentiles, 75th percentiles).

Table 4.3 IL-6 and CRP in IC, CAD, non-diabetic IC and CAD stratified according to homozygous wild-type and variants of the IL-6 -174G/A polymorphism.

	Wildtype vs. Variants	IL-6 (pg/mL)	p value	CRP (mg/L)	p value
IC	GG	0.9 (0.90, 1.78)	0.0002[a]	3.2 (1.9, 7.65)	0.3111
	GC/CC	0.9 (0.64, 0.91)		2.8 (2.0, 9.50)	
CAD	GG	0.9 (0.90, 0.91)	0.774	5.8 (1.90, 8.5)	0.385
	GC/CC	0.9 (0.90, 0.92)		5.4 (1.87, 6.8)	
IC (non-T2D)	GG	0.9 (0.9, 4.295)	0.0001[b]	2.2 (1.8, 7.35)	0.209
	GC/CC	0.75 (0.64, 0.9)		2.8 (2.0, 9.50)	
CAD (non-T2D)	GG	0.9 (0.90, 0.91)	0.544	7.4 (1.9, 10.45)	0.125
	GC/CC	0.9 (0.67, 0.93)		3.9 (1.86, 6.78)	

[a]p<0.05 IL-6 levels higher in the GG group than the GC/CC group in IC, [b]p<0.05 IL-6 levels higher in the homozygous G group than the GC/CC group in non-diabetic Indian controls (IC). Data represented as the median (25th percentiles, 75th percentiles).

IL-6 levels were then stratified according to the homozygous wild-type genotypes of the IL-6 -174G/C and CRP -390C/A/T polymorphisms and a combined group of the variant alleles (Table 4.3, Table 4.4).

Table 4.4 Levels of IL-6 and CRP between IC, CAD patients, and non-diabetic IC and CAD patient stratified according to homozygous wild-type and variants of the CRP -390C/A/T polymorphism.

	Wildtype vs. Variants	IL-6 (pg/mL)	p value	CRP (mg/L)	p value
IC	CC	0.9 (0.90, 0.95)	0.0416[a]	5.0 (1.9, 7.78)	0.713
	AC/AT/CT/TT	0.9 (0.69, 0.91)		2.5 (2.03, 8.88)	
CAD	CC	0.9 (0.9, 0.91)	0.328	5.5 (1.89, 7.83)	0.346
	AC/AT/CT/TT	0.9 (0.9, 0.91)		6.3 (2.34, 9.35)	
IC (non-T2D)	CC	0.9 (0.89, 0.92)	0.438	2.4 (1.85, 7.75)	0.209
	AC/AT/CT/TT	0.9 (0.70, 0.92)		2.75 (2.0, 7.88)	
CAD (non-T2D)	CC	0.9 (0.9, 0.913)	0.394	5.8 (1.89, 8.85)	0.303
	AC/AT/CT/TT	0.9 (0.87, 0.91)		7.4 (5.2, 9.8)	

[a]*p<0.05 IL-6 levels significantly higher in the CC group than the group of combined variant alleles in indian controls (IC) (Mann-Whitney Test). Data represented as the median (25th percentiles, 75th percentiles).*

A consistent trend amongst the control and CAD groups was observed. The group of subjects that presented with the homozygous IL-6 -174 GG and those which had the CRP-390 CC genotype had the higher median levels of IL-6 and CRP compared to the variant allele carriers.

4.4 Discussion

Modification and entry of LDL into the endothelium promotes an inflammatory response to vascular injury (Schuett et al., 2009). This stimulates the recruitment of inflammatory cells such as monocyte-derived macrophages and lymphocytes, and subsequent release of pro-inflammatory cytokines. Both CRP and IL-6 are implicated in the pathogenesis of atherogenesis and have clinical significance as risk factors for CVD (Kiszel et al., 2007; Shanker and Kakkar, 2010).

Interleukin 6 and its signalling effects have been shown to contribute to both atherosclerotic plaque formation and its eventual destabilization through several mechanisms (Schuett et al., 2009). These include endothelial activation, smooth muscle cell proliferation, lymphocyte recruitment, and mediation of the acute phase response (Brull et al., 2001). In addition, IL-6 affects expression of scavenger receptors SR-A and CD-36 which are involved in the uptake of modified LDL and therefore promotes foam cell production (Schuett et al., 2009).

The IL-6 -174 SNP location near the 'hotspot vicinity' for transcription factor NF-1 binding, acts as a repressor of gene expression (Liu et al., 1997). Several population genetic studies revealed conflicting results regarding the influence of the IL-6 -174 SNP on IL-6 production (Sie et al., 2006). A study of IL-6 levels in a group of healthy men and women (adjusted for age, body mass index, gender, and smoking) found low levels, similar to the findings in this study, in the presence of the CC genotype: 1.63 (95% CI 1.44–1.86) compared to GG: 2.74 (95% CI 2.43–3.10) and GC: 2.64 (95% CI 2.35–2.97), (P=0.02 by ANOVA), (Fishman et al., 1998). Another study conducted on 127 patients (following coronary artery bypass graft

surgery) showed a recessive effect of the C-allele resulting in an increase IL-6 levels (Brull et al., 2001).

In this study, the highest levels of IL-6 were detected in non-diabetic CAD subjects with the homozygous G allele (p=0.0001, Figure 4.2). The transcriptional regulation of IL-6 is highly complex and nearby genetic variants may influence transcription of the IL-6 gene (Shanker and Kakkar, 2010). The data from this study indicates the CAD subjects grouped according to genotype presented with highest IL-6 levels had corresponding elevated levels of CRP. A study in an Italian group of 88 patients assessed the prognostic significance of IL-6 in the diagnosis of acute myocardial infarctions. They found that elevated creatine kinase-MB ($p<0.05$) and IL-6 levels ($p<0.01$) were independently associated with a final diagnosis of acute myocardial infarctions. Elevated IL-6 levels also significantly predicted the risk of AMI (OR=2.47, p=0.006) (Ferroni et al., 2007).

In 2005 the Physicians Health Study reported that the T-allele was associated with increased risk of myocardial infarction, although associated with lower CRP levels (Miller et al., 2005); which suggested that this may be due to the complex regulatory mechanisms involved in CRP production. These SNPs in the CRP promoter region viz., the rs3093032 and 3872C/T and 5237A/G affect transcription factor binding, transcriptional activity and CRP levels (Carlson et al., 2005; Miller et al., 2005). In this investigation of the tri-allelic CRP -390C/A/T promoter polymorphism, we found an increased risk of CAD in the presence of the homozygous wild-type CC genotype. The Indian control group (who were diabetic and non-diabetic) with the homozygous CRP -390 C-allele, though not statistically significant, also presented with the higher levels of CRP compared to the group of controls that presented with other variants.

Binding of transcription factors regulate CRP gene expression and the -390 T-allele was associated with higher promoter activity (Szalai et al., 2005). This finding is in agreement with data from participants in the Framingham heart study which showed an association between the minor A and T alleles of the CRP -390 C/A/T polymorphism and higher CRP levels (Kathiresan et al., 2006). The current data, however, also shows highest levels of CRP in the presence of the T-allele in CAD patients, but the frequency of patients presenting with this allele is too small to draw any conclusions. In the presence of either the variant A or T alleles grouped together (in order to increase statistical power) compared to the homozygous wild-type C genotype, median levels were higher.

It has been well established that pro-inflammatory markers could serve as biomarkers for risk assessment of CAD. A relevant study, described above, concluded that IL-6 may behave as an adjunctive diagnostic tool to assist in the risk assessment of acute myocardial infarctions. The presence of polymorphic variants in genes of these molecules, however, should be taken into consideration as it may influence levels detected in circulation.

4.5 Conclusion

Single nucleotide polymorphisms in the promoter region of IL-6 and CRP genes may contribute to levels of these pro-inflammatory factors which play a role in the pathogenesis of atherosclerosis. The present study indicates a potential protective effect of the IL-6 -174 C-allele and an increase risk with the CRP -390 C-allele with respect to CAD independent of diabetic status.

CHAPTER 5

THE EFFECTS OF ATORVASTATIN ON OXIDATIVE STRESS, METABOLIC ACTIVITY AND MICRORNA PROFILE IN A LIVER CELL LINE

5.1 Introduction

Heart disease is a global health problem that claims millions of lives annually. The target of drug intervention is the underlying cause of vascular disease - atherosclerosis. Statins are very important drugs in cardiovascular medicine. Large clinical trials have shown a significant reduction in the proportion of cardiovascular deaths in patients on statin therapy (Wright et al., 1994; Shepherd et al., 1995; Schwartz et al., 2001). It is well known that statins reduce the risk of myocardial infarction, stroke, and death, primarily via the competitive inhibition of HMGCR, the rate-limiting enzyme of cholesterol synthesis (Strandberg et al., 2004). Other factors which play a key role in the development and progression of heart disease are nitric oxide and acute-phase CRP (Rao and Milbrandt, 2010). The synthesis of both cholesterol and CRP occur in the liver. The first pass metabolism of statins also occurs in the liver, via the cytochrome P450 (CYP) group of enzymes.

Atorvastatin (Figure 5.1) is a frequently prescribed HMGCR inhibitor for patients with CAD. The differences between HMGCR inhibitors are their pharmacokinetic properties (Igel et al., 2001; McTaggart et al., 2001). Atorvastatin is a synthetic statin derived from mevalonate and contains three main groups which include the HMGC analogue and two hydrophobic hydroxy-hexahydro naphthalene ring structures (Schachter, 2005). During metabolism of atorvastatin,

the acid form is biotransformed to a lipophilic lactone, by coenzyme A-dependent or an acyl glucuronide intermediate pathway. Both atorvastatin and its lactone form are further metabolized by *CYP3A4* and to a lesser extent *CYP2C8*. The atorvastatin lactone is hydrolyzed to an acid form nonenzymatically or by esterases and paraoxonases (Lennernas, 2003; Neuvonen et al., 2006).

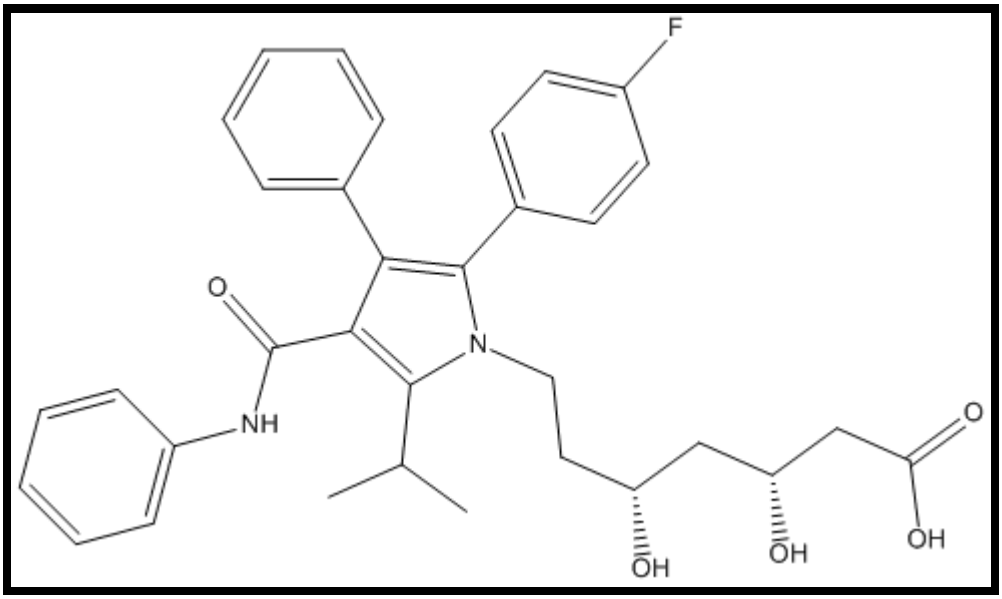


Figure 5.1 Chemical structure of atorvastatin (Bracha et al., 2010).

The pharmacological properties of statins include anti-inflammatory effects, vasodilation, and antithrombotic activity (Boyle, 2005; Mangat et al., 2007). Amongst these pleiotropic effects, statins have also been reported to influence the progression of atherosclerosis (Grothusen et al., 2005). The antioxidant effects of statins is achieved by reducing oxLDL (Li et al., 2002), ROS and NADPH oxidase activity (Figure 5.2) (Delbosc et al., 2002; Wassmann et al., 2002; Yu et al., 2005; Forstermann, 2008; Guasti et al., 2008; Deo et al., 2012) increasing endothelial NOS (eNOS) activity (Figure 5.2)(Laufs and Erdmann, 1998).

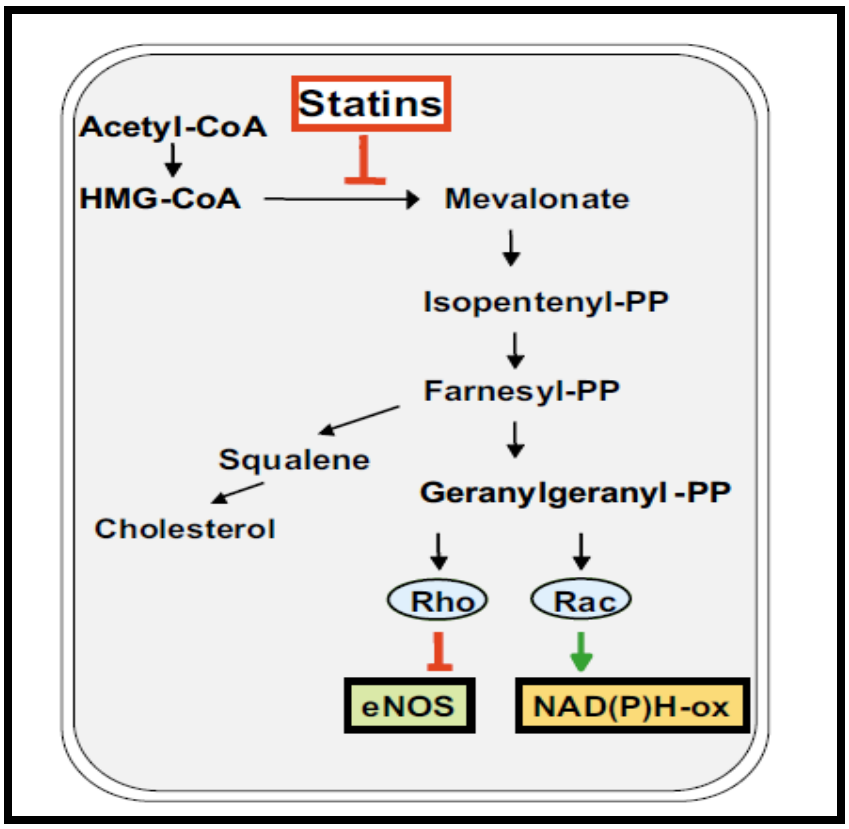


Figure 5.2 Inhibition of the mevalonate pathway by statins (Endres, 2005).

A study assessing the hepatotoxicity of statins concluded that "statins are remarkably safe from a hepatic standpoint, but there are several issues related to their usage in humans that require further research and scrutiny" (Chalasani, 2005). A recent review on the epigenetic effects of common pharmaceuticals assessed possible mechanisms of drug-induced epigenetic changes (Csoka and Szyf, 2009). Three main epigenetic processes exist in a normally regulated genome, i.e., DNA methylation, histone modification and miR interactions.

MicroRNAs control gene expression and modulate multiple target transcripts in eukaryotic cells (Leung et al., 2006; Asirvatham et al., 2008). The role of miRs in coordinating immune responses is by regulating a repertoire of genes in various immune cells (Chen et al., 2004; Cobb et al., 2006; O'Connell et al., 2007; Welker et al., 2007; Asirvatham et al., 2008;

Baltimore et al., 2008). A recent study identified 275 predicted immune gene-miRNA interactions which included transcription factors, inflammatory cytokines and signalling molecules (Asirvatham et al., 2008). This has important implications for CAD as chronic inflammation is a critical role-player in atherosclerotic development and progression.

One of the first studies to assess a differential effect of statins on miRNA expression examined the physiological role of miR-221／222 on the proliferation of endothelial cells obtained from patients with CAD. In the study, atorvastatin increased endothelial progenitor cell numbers and decreased miR-221／222 levels in patients with CAD, possibly contributing to the beneficial effects of the lipid lowering therapy of statins (Minami et al., 2009).

As mentioned in chapter 1, the primary function of statins is to inhibit cholesterol biogenesis in the liver but other metabolic products such as creatine also enter peripheral circulation. This is evidenced by the fact that statin therapy is associated with myopathy (Phillips et al., 2002). It has been established that miRNAs play a crucial role in coordinating the inflammatory response by directly affecting the transcriptome and thus heavily influence gene expression patterns (Asirvatham et al., 2008). There is emerging evidence for modulation of the transcriptome by statins (Wang et al., 2011). Limited data is available on the effects of these drugs on miRNAs involved in the regulation of specific biological pathways.

The miRFinder miR PCR Array provides an ideal platform to assess at least 84 of the most abundantly expressed and best characterised miRNAs in the miRNA database. These miRNAs can regulate one or more mRNA transcripts, and although well-characterised, the complex role of each miRNA is not completely understood. The liver plays an integral role in

the metabolism of statins and the effects thereof. In this arm of the study the effect of atorvastatin on the metabolism (ATP, LDH), oxidative damage (ROS, RNS) and miRNA profile in a liver cell line, $HepG_2$ cells, was assessed. A pathway finder based miRNA PCR array panel was used to profile 84 miRNAs and identify possible targets from which a molecular mechanism of statin-induced hepatic changes could be investigated.

5.2 Materials & Methods

HepG2 cells were purchased from Highveld Biologicals (Johannesburg, SA). Cell culture reagents were purchased from Whitehead Scientific (Johannesburg, SA). ECL-LumiGlo chemiluminescent substrate kit was purchased from Gaithersburg (USA) and western blot reagents were purchased from Biorad (SA). All other reagents and consumables were purchased from Merck (SA), unless otherwise stated.

5.2.1 Maintenance of HepG2 cells in culture

The liver is the most important drug metabolising organ in mammals. Liver cell lines are generally used for the assessment of metabolism or bioactivation of xenobiotics. The commonly used human HepG2 cells have an epithelial like morphology which resembles liver parenchymal cells and they have retained most of the functions of normal liver cells.

The best environment for growing cells *in vitro* should be matched as close as possible to the natural physiological conditions. The essential requirements are an optimum environment of temperature, pH, gas phases, growth substrate and media containing necessary nutrients. The optimal temperature is provided by the use of a humidified incubator supplied with 5% carbon dioxide (CO_2). The gas phases supplied to the culture includes oxygen which is maintained at atmospheric pressure, and CO_2 to ensure that the bicarbonate and CO_2 tension is in equilibrium.

$HepG_2$ cells were cultured (37^oC, 5% CO_2) to 90% confluency in 25cm^3 flasks in complete culture media (CCM) [Eagle's minimum essential medium, 10% foetal calf serum, 1% L-Glutamine and 1% penstrepfungizone]. The culture medium is by far the most important single factor in culturing cells. The extracellular medium must meet the essential requirements (nutritional, hormonal and stromal factors) for survival and growth (appendix 2).

5.2.1.1 Trypsinisation

In order to sub-culture and plate cells for the various experimental assays, the process of trypsinisation was used to detach cells once 90% confluency was reached. The process of trypsinisation involved the critical step of rinsing the cells with 3ml aliquots of warm 0.1M PBS and incubating the cells with 1ml of trypsin-EDTA (Lonza) for 1min.

The cells were monitored using an inverted light microscope (Olympus IXSI; 20x magnification) and once rounded the trypsin was discarded and CCM was added to the flask

of cells. The flask was agitated to detached cells and the cell suspension was then enumerated by dye exclusion using a haemocytometer. Trypan blue (0.4%) was utilised in a dye exclusion procedure for cell counting.

The principle of dye exclusion using trypan blue is based on compromised cell membranes in dead/damaged cells which readily allow entry of the dye into the cells and are stained blue whereas viable cells remain unstained.

5.2.2 Cell proliferation and metabolic activity assay

The effect of atorvastatin in $HepG_2$ cells was measured using a methyl tetrazolium dye reduction assay, the [3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) assay. This assay measures cell proliferation/metabolic activity *in vitro*. This technique is particularly useful for cells that are metabolically active based on their redox potential and capacity of dehydrogenase enzymes to convert yellow water-soluble salt into a purple water-insoluble formazan product. The insoluble crystals are then dissolved in dimethyl sulfoxide (DMSO) and the absorbance is read on a spectrophotometer. The amount of formazan produced is directly proportional to cell number thus allowing for the determination of cell viability and proliferation (Supino, 1995).

HepG2 cells (15,000/well) were incubated for 24h with a range of concentrations of atorvastatin (Sigma Aldrich, SA) (1µM, 10µM and 20µM) in triplicate in a microtitre plate together with an untreated control (cells incubated with CCM only). The concentration and

time used for this experiment was based on the half-life of atorvastatin (20h-30h) and the recommended physiological dose between 10mg and 40mg/day, the bioavailability of which is 14%. Each experiment was conducted twice on separate occasions in order to confirm the data results from the first set matched the repeated experiment. The cells were then incubated ($37^{o}C$, 5%CO_2) with the MTT substrate (5 mg/ml in PBS) for 4h. Thereafter all supernatants were aspirated, and DMSO (100 µl/well) was added to the wells. Finally the optical density was measured at 570 nm and a reference wavelength of 690 nm with an ELISA plate reader (Bio-Tek µQuant).

The net MTT-dependant absorbance of each sample was calculated by subtracting the average absorbance of the blank from the average absorbance of each sample. Data are represented as % cell viability plus or minus the standard deviations. Percentage cell viability was calculated by utilising the following equation:

$$\% \text{ Cell viability} = \frac{\text{mean absorbance of treated cells}}{\text{mean absorbance of control cells}} \times 100$$

5.2.3 HepG2 cell culture treatment for subsequent assays

HepG2 cells were seeded (200,000cells/flask) in $25cm^3$ flasks and grown to approximately 90% confluency. For each assay, three $25cm^3$ flasks of untreated cells and three $25cm^3$ flasks of 20µM atorvastatin treated cells were incubated for 24h ($37^{o}C$, 5% CO_2). Following

incubation, the supernatant was aspirated and stored at -80^{o}C; and cells were rinsed thrice with 2ml of 0.1M PBS to remove any residual media.

5.2.4 The ATP quantification assay

The level of intracellular ATP was measured using a CellTiter-Glo® kit (Promega, United States, Madison, WI, USA). The ATP quantification assay utilises bioluminescence to ascertain ATP levels in cells. The assay is based on the conversion of a luciferase-inactive derivative by ATP in the presence of magnesium ions to D-luciferin (Figure 5.3).

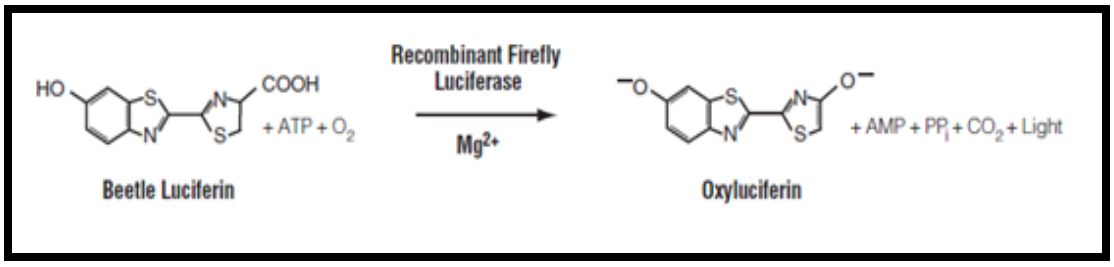


Figure 5.3 The luciferase reaction where mono-oxygenation of luciferase occurs in the presence of magnesium, ATP and molecular oxygen.

D-luciferin, a luciferase substrate, reacts with the enzyme luciferase to produce oxyluciferin and release energy in the form of luminescence. This luminescent signal is directly proportional to the concentration of ATP present in the cells.

For the procedure, treated cells were seeded into a white luminometer plate (20,000 cells/well) in triplicate followed by the addition of 10μl/well of the reagent. The plate was agitated and incubated in the dark (30min; RT) to allow for cell lysis and the luciferace-based

reaction to occur (Figure 5.3). Thereafter, the luminescent signal was measured on a microplate luminometer (Turner Biosystems, USA). The ATP concentration was expressed as Relative Light Units (RLU).

5.2.5 The lactate-dehydrogenase (LDH) assay

The LDH cytotoxicity detection kit (Roche) was used to measure cell death/damage of atorvastatin treated HepG2 cells. Lactate dehydrogenase is a stable cytosolic enzyme which is released from cells due to compromised cell membrane integrity. A colorimetric assay was used for the quantification of LDH activity in the supernatant of atorvastatin treated HepG2 samples. The assay is a two-step enzymatic reaction where NAD+ is reduced to NADH/H+ by the conversion of lactate to pyruvate. Thereafter, a diaphorase catalyst transfers H/H+ from NADH/H+ to a tetrazolium salt to yield a formazan product. To measure LDH activity, supernatant (100µl) was transferred into microtitre plates in triplicate. Thereafter, substrate mixture (100µl) containing catalyst (diaphorase/NAD+) and dye solution (INT/sodium lactate) from the kit was added to the supernatant and allowed to react at ambient temperature for 25 minutes. Optical density of the resulting formazan product was measured at 500nm with an ELISA plate reader (Bio-Tek uQuant). Results are represented as mean optical density.

5.2.6 The thiobarbituric acid reactive substance assay

One of the most commonly applied assays is the thiobarbituric acid (TBA) reactive substance (TBARS) assay, which measures malondialdehyde (MDA) formed in peroxidising lipid

systems (Halliwell and Chirico, 1993). This method is based on the condensation of two molecules of TBA with one molecule of MDA to form a pink chromagen (TBA pigment; Figure 5.4), the intensity of which is a measure of MDA concentration (lipid peroxidation) and can be measured by spectrophotometry at 532nm.

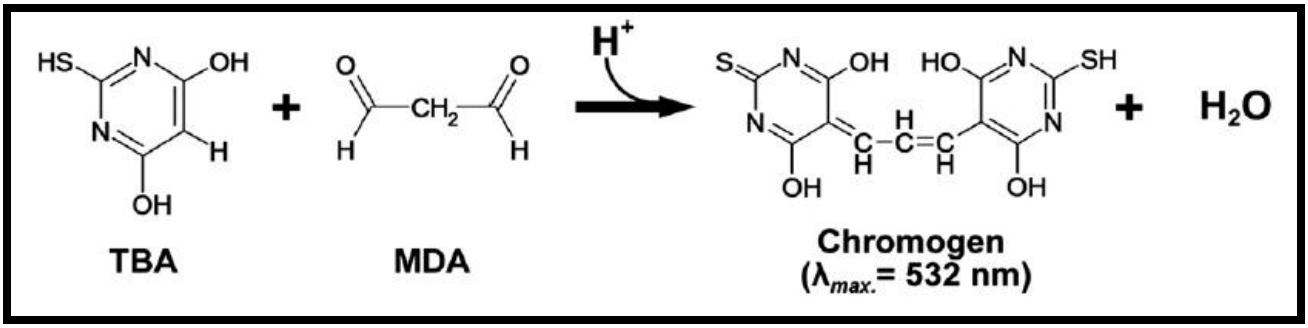


Figure 5.4 Reaction between thiobarbituric acid (TBA) and malondialdehyde (MDA) to form the thiobarbituric acid pigment (Bastos et al., 2012).

Approximately 500µl of supernatant per treatment was transferred to appropriately labelled glass test tubes containing 400µl 2% phosphoric acid (H_3PO_4). A blank sample (negative control) containing homogenate from the control was also included in order to correct background absorbance. A 200µl aliquot of 7% H_3PO_4 was then dispensed into each test tube followed by the addition of 400µL TBA/Butylated hydroxytoluene (BHT: Merck, SA) solution (BHT is added to the TBA solution to prevent oxidation in the reaction during heating) to each sample, and 400µL of 3mM HCl to the blank.

All test tubes were briefly vortexed and the pH of each solution was subsequently adjusted to 1.5 using 1M HCl. The test tubes were thereafter placed in a water bath (100ºC). After 15min,

the tubes were removed and allowed to cool to room temperature before pipetting 1.5ml of butanol into each tube.

All test tubes were then vortexed (1 min each) and the samples were allowed to separate. Approximately 500µl of the butanol (which comprised the upper phase of the solution present in the test tube) was then transferred to sterile 1.5ml tubes and centrifuged (2 500xg, 6min, 24ºC).

A 100µl aliquot of each sample was then pipetted in triplicate to wells of a 96-well microtiter plate. The absorbance of the butanol phase was measured at 532nm with a reference wavelength of 600nm using a Bio-Tek µQuant microplate spectrophotometer.

5.2.7 The Griess assay

Nitric oxide production was measured by the standard Griess assay described by (Miranda et al., 2001). This assay relies on a diazotization reaction that detects NO_2^- in solution as shown in Figure 5.5.

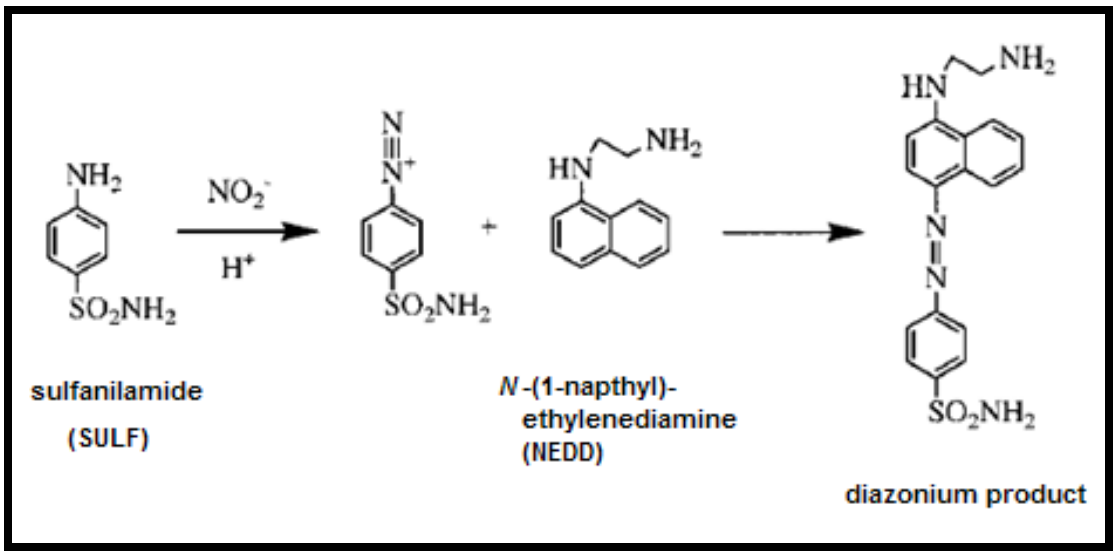


Figure 5.5 Principle reaction of the Griess assay.

The concentration of nitrites in supernatant was determined as described by Miranda et al. (2001). A positive control of 100µM nitrate solution was prepared in 0.1M PBS, and a blank was prepared (0.1M PBS only). Each sample was aliquoted into wells of a 96-well microtitre plate (100µl, in triplicate). Vanadium (III) chloride (8mg/ml, 100µl) was then added to each well followed by 50µl of sulfanilamide (2%) and 50µl of N-(1-Naphthyl) ethylenediamine dihydrochloride (0.1% in 5% HCl).

The plate was then incubated (37^{o}C, in the dark, 45min). The absorbance was measured on a Biotek® µquant spectrophotometer (540nm, reference 690nm). The net absorbance was calculated by subtracting the absorbance of the blank from the treated samples. The data is represented as mean absorbance.

5.2.8 Isolation of RNA from atorvastatin treated HepG2 cells

The TRI Reagent® solution was used for the isolation of total RNA. The TRI Reagent solution combines phenol and guanidine thiocyanate in a monophasic solution to rapidly inhibit RNase activity. The serum samples were homogenized in TRI Reagent solution, and the homogenate was then separated into an aqueous and organic phase by adding bromochloropropane and centrifuging. Total RNA (containing ribosomal RNA, mRNA and transfer RNA) then partitions into the aqueous phase. The RNA was precipitated with isopropanol, and finally washed with ethanol and solubilised.

An aliquot of 500µl Tri Reagent® Solution (Ambion) was added to 500µl of for treated and untreated $HepG_2$ cells isolation of total RNA and incubated (5min, RT). The lysed cells were transferred to 500µl of 0.1M PBS in sterile 1.5ml tubes. An aliquot of chloroform (100µl) was then added to each sample and vortexed vigorously for 15 sec and incubated for 3min at RT before centrifugation (12,000xg, 4^{o}C). The resulting aqueous phase was carefully transferred to a fresh tube to which 250µl of isopropanol was added to each sample and incubated for 1h at -80^{o}C. Following incubation, samples were thawed at RT and then centrifuged (20min, 12,000xg, 4^{o}C). The supernatant was removed and the pellet was washed with 75% cold ethanol (500µl) and centrifuged (15min, 7,400xg, 4^{o}C). The ethanol was removed and the pellet was allowed to air dry before being resuspended in 15µl nuclease free water and quantified spectrophotometrically using the Nanodrop 2000.

5.2.9 cDNA synthesis

For each sample, cDNA was synthesised using the First-Strand Kit (Qiagen, United States). Approximately 100ng of RNA was added to a sterile PCR tube with 1µl miR reverse transcription primer & ERC Mix (M1), 2µl 5X miR reverse transcription Buffer 2 (M5), 1µl miR RT enzyme Mix (M3), 1µl nucleotide mix (M4) and 10µl RNase-free H_2O per sample. The contents were mixed gently and incubated at 37^oC for 2h. Samples were then heated at 95^oC for 5min to degrade RNA & inactivate reverse transcriptase and then placed on ice for at least 1min before 90µl of RNase-free H_2O was added to each 10µl cDNA synthesis reaction.

5.2.10 MicroRNA SuperArray Analysis by quantitative PCR, analysis and selection of significant microRNAs

The Human miFinder miRNA PCR Array (MIHS-001Z, Qiagen, United States) was used to profile the expression of 84 miR species (Table 5.1). Each sample was prepared for analysis by adding 1275µl 2x RT^2 SYBR Green PCR Mastermix to 100µl of the diluted cDNA reaction product described in section 5.2.4, and 1175µl nuclease free water. The PCR array plates were then carefully removed from sealed bags and the experimental cocktail was loaded into each well (25µl). The plates were sealed with optically thin wall cap strips and inspected from the bottom to ensure that no bubbles were formed. Plates were centrifuged at 250xg for 2min at RT to remove bubbles and then transferred to Real-time Chromo4 thermocycler (Biorad, SA) pre-set with appropriate thermocycling conditions. The miScript Primer Assays (Qiagen, United States) were used to validate highly expressed miR species.

Data was normalized using U6 small RNA expression. Differences in miR expression were determined by fold change analysis using the $2^{-\Delta\Delta Ct}$ method (Appendix 3). To stringently select miRs which were significantly up- or down-regulated in experimental groups, a significance analysis of microarray (SAM) strategy was used. Significant ($p<0.05$) deviations of residuals from normal distribution was used to identify miRs of interest. Data was analysed with Microsoft excel (2011) Statplus plug-in and R.

MicroRNA target analysis was conducted using the Targetscan algorithm.

5.2.11 Transfection of HepG2 cells with miR-124a-3p mimics and inhibitors

In order to directly assess the effect of miR-124a-3p on mRNA and protein expression, the mimic as well as the inhibitor to this miR was purchased (Qiagen, United States). Transient transfection of adherent cells involves the introduction of nucleic acids into a cell that does not become permanently integrated into the cellular genome, and the effect only lasts a period of time. Attractene Reagent is a non-liposomal lipid which enables highly efficient nucleic acid transfection with minimal cytotoxicity.

To determine the effect of elevated miR-124a-3p on the mRNA expression of guanidinoacetate methyltransferase (GAMT), spermine oxidase (SMO) and arginine-glycine amidinotransferase (AGAT) and protein expression of GAMT, HMGCR and p53; cells were transfected with the relevant mimic (Syn-hsa-miR-124a-3, MYS0000422, Qiagen, United

States) and inhibitor (Anti-hsa-miR-124a-3p, MYS0000422, Qiagen, United States) of miR-124a-3p.

For the transfection procedure, HepG2 cells were seeded at a density of 400,000 cells/well in a 6-well plate and allowed to adhere for 24h (37^{o}C, 5% CO_2) and monitored until 80% confluency was reached. Lyophilised miR mimics (5nmol) and inhibitors (5nmol) were reconstituted with 250µl nuclease free water to obtain a stock concentration of 20uM. On the day of transfection, 10µl miR mimic/inhibitor was added to with medium without serum, proteins, or antibiotics to a total volume of 60µl to which 2µl of Attractene Transfection Reagent was added and mixed by reverse-pipetting. Samples were then incubated for 15min (RT) to allow complex formation. During this time CCM was gently aspirated from the cells and 1940µl fresh CCM was added such that the final concentration of mimic/inhibitor per well was 50nM. The transfection complexes were added in a drop-wise fashion into the appropriate well with gentle swirling of the plate to ensure uniform distribution of the transfection complexes. An untreated control and a 20µM atorvastatin treatment well were included before the cells were incubated under normal growth conditions for 24h and utilized for protein extraction.

5.2.12 Gene Expression Analyses of specific targets of highly expressed microRNAs

The targets of the most up-regulated miRs were assessed using TargetScan. A few targets of interest involved in the synthesis of creatine and polyamines were identified and assessed by quantitative PCR.

Total RNA was extracted as described in section 5.2.8. RNA was reverse transcribed using the iScript cDNA synthesis kit (Biorad, SA). Real-time PCR was performed using the iQ Superscript reagent (Biorad, SA). Levels of mRNA for SMO, AGAT and GAMT were quantified with standardization of levels to the average of a housekeeping gene: GAPDH.

The primers utilized were as follows:

GAMT:	sense	5'-TGGCACACTCACCAGTTCA-3'
	antisense	5'-AAGGCATAGTAGCGGCAGTC-3'
AGAT:	sense	5'-TCACGCTTCTTTGAGTACCG-3'
	antisense	5'-TCAGTCGTCACGAACTTTCC-3'
SMO:	sense	3'-GGATGAGGATGAGCAGTGGTC-5',
	antisense	3'-CGACACGGTCACAATCACATG -5'
GAPDH:	sense	3'-CAACAGCCTCAAGATCATCAGC-5',
	antisense	3'-TGAGTCCTTCCACGATACCAAAG-5'

The PCR conditions were as follows: 40 cycles of a denaturation step (95^{o}C, for 30sec), an annealing step (56^{o}C, for 15s), a plate read and an elongation step at 72^{o}C. This was followed by a melt curve from 60^{o}C to 95^{o}C, with a plate read every 2^{o}C held for 30s. For GAMT, AGAT SMO and GAPDH, the final concentrations of primers used were 300nM and 100nM, respectively. The method described by Livak and Schmittgen (2001) was used to calculate a relative fold change of each gene from cycle threshold values (Livak and Schmittgen, 2001). All experiments were conducted in triplicate and repeated at least once.

5.2.13 Protein Extraction, Quantification and Standardisation

HepG2 cells were treated for protein isolation as described in section 6.2.8. Approximately 200µl of the CytoBuster™ protein extraction reagent (a formulation of detergents optimised for efficient extraction of soluble proteins from mammalian cells) was added to a 25cm^3 flask of treated HepG2 cells; placed on ice for 10 min before the cells were harvested using a cell scraper. The cell solution was then centrifuged (600xg, 10 min, 24ºC) and the resulting supernatants (450µl) served as the crude protein extract. The supernatants were transferred to appropriately labelled 1.5ml microcentrifuge tubes and kept on ice until utilised.

Crude protein extracts were quantified using the bicinchoninic acid (BCA) assay. The BCA assay is a colorimetric assay which relies on two reactions. First, the peptide bonds in protein reduce Cu^{2+} ions to $Cu^{1+.}$ The amount of Cu^{2+} reduced is proportional to the amount of protein present in the solution and second, two molecules of BCA chelates with each Cu^{1+} ion, forming a purple-coloured product that strongly absorbs light at a wavelength of 562nm.

A standard curve of serially diluted bovine serum albumin (BSA: 0, 0.2, 0.4, 0.6, 0.8 and 1.0mg/ml) was used to construct a standard curve (Appendix 4). An aliquot of 25µl of each sample and the relevant standards (BSA) were added to appropriately labelled wells.

The BCA working solution (202µl, 4µl Cu_2SO_4 and 198µl BCA) was then transferred into each well and incubated (37ºC, 30 min) and the absorbance was measured at 562nm using a spectrophotometer (BioTek µQuant). All samples were then diluted using storage buffer [0.1M

KH_2PO_4 (pH 7.4), 0.5mM K_2EDTA, 0.1mM DTT and 0.25M sucrose] and standardised to 1mg/ml (Appendix 4).

5.2.14 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is based on the separation of proteins according to size. Sample preparation prior to being resolved by SDS-PAGE is accomplished by denaturation with sample buffer containing β-mercaptoethanol and SDS. The β-mercaptoethanol reduces disulphide bridges holding together the protein tertiary structure, whereas SDS, an anionic detergent, binds strongly to and denatures the protein. This results in the proteins becoming linear and with a uniform charge (Wilson and Walker, 2005).

Once loaded, the negatively charged protein-SDS complexes move toward the anode and as they pass through the resolving gel, the proteins separate due to the molecular sieving properties of the gel (Wilson and Walker, 2005).

The Mini-PROTEAN 3 SDS-PAGE apparatus (Biorad, SA) was assembled according to manufacturer's instructions. A 7.5% resolving gel [dH_2O, 1.5M Tris-HCl (pH 8.8), 10% (w/v) SDS, 30% Acrylamide/bis, 10% APS, TEMED] was prepared and added in-between the cleaned glass plates. A 4% stacking gel [dH_2O, 0.5M Tris-HCl (pH 6.8), 10% (w/v) SDS, 30% Acrylamide/bis, 10% APS, TEMED] was then added in-between the glass plates. A 1.5mm plastic 10-well comb was placed into the stacking gel between the glass plates. Following polymerization, samples prepared in Laemmli buffer [dH2O, 0.5M Tris-HCl (pH 6.8), glycerol,

10% SDS, β-mercaptoethanol, 1% bromophenol blue, (1:1)] were loaded into appropriate wells. A molecular weight marker (Mwt) was loaded into a separate well in order to identify the weight of the migrated proteins. A 1x electrode buffer (dH_2O, Tris, glycine, SDS; pH 8.3) was then added to the tank and samples were electrophoresced (150V, 1h, on ice). After electrophoresis, gels were allowed to equilibrate in transfer buffer (25mM Tris, 192mM glycine, 20% v/v methanol; pH 8.3) (10min, RT).

5.2.15 Western blotting

Western blotting is a technique that allows for the electrophoretic protein transfer from a SDS-PAGE gel to a nitrocellulose membrane, which can then be probed with antibodies. Primary polyclonal or monoclonal antibodies react specifically with antigenic epitopes presented by target proteins attached to the membrane. A secondary antibody, specific to the primary antibody conjugated with horseradish peroxidase (HRP) allows for the detection of the bound antibody. The HRP-conjugated secondary antibodies are then able to catalyse a reaction between hydrogen peroxide and a chemiluminescent substrate, luminol. This is a light-emitting reaction which is detected a chemiluminescence detection imaging system and captured as an image which can be quantified.

For the procedure, a set of fibre pads and nitrocellulose were pre-soaked in transfer buffer. A gel sandwich was assembled by first placing a fibre pad on the area of the gel holder cassette located closest to the cathode, followed by filter paper, the equilibrated gel, the nitrocellulose membrane, a second filter paper and a final fibre pad. The gel holder cassette was then tightly

closed and placed into the transfer module and mini tank with transfer buffer. Proteins were then transferred to the nitrocellulose membranes at a constant current of 400mA for 1 h.

After the transfer, the nitrocellulose membranes were blocked (1h, RT) with 5% BSA in TTBS [100ml; Tris-buffered saline (TBS) containing 0.5% Tween20] after which, each membrane was incubated for 1h with primary antibody (anti-mouse GAMT. ab119269 (Abcam); anti-rabbit phosphoP53 (ser15) 9284S (Cell Signalling)) diluted 1:1 000 in 1% BSA in TTBS. After incubation, the primary antibody was discarded and the membranes were washed thrice (10min, 10ml TTBS). The membranes were subsequently incubated with HRP-conjugated goat anti-mouse IgG secondary antibody (ab7023, diluted 1:2 000 in 1% BSA in TTBS, RT) and anti-rabbit IgG secondary antibody (ab9746, diluted 1:2 000 in 1% BSA in TTBS, RT) for 1h. The secondary antibody was then discarded and the membrane was washed thrice (10min) with 10ml TTBS.

Development of each membrane was facilitated by chemiluminescence detection. Briefly, the two chemiluminscence reagents (luminol/enhancer and peroxide buffer) comprising the KPL Lumiglo HRP substrate kit were mixed in a 1:1 ratio. Approximately 1ml of this mixture was dispensed onto the nitrocellulose membrane already placed into the chamber of an Alliance 2.7 Gel Documentation System (UViTech). The images of the protein bands on the membrane became visible following exposure. The captured image was assessed using the Alliance Analysis Software (UViTech) to determine the relative intensity of each band.

5.2.16 Statistical analysis

Statistical analysis for differences between groups was calculated using the Graphpad Prism Software. The Mann Whitney test was used for differences between two groups and the Kruskal-Wallis Multiple comparisons test was used to test for differences between more than two groups.

5.3 Results

5.3.1 Cell proliferation, metabolic activity and cytotoxicity of atorvastatin treated HepG2 cells

The MTT assay was used as a measure of cell proliferation/metabolic activity of atorvastatin in HepG2 cells. Following a 24h incubation with atorvastatin at the low concentrations of 1µM (104±0.5%) and 10µM (102±8%), no toxicity was observed (Figure 5.6).

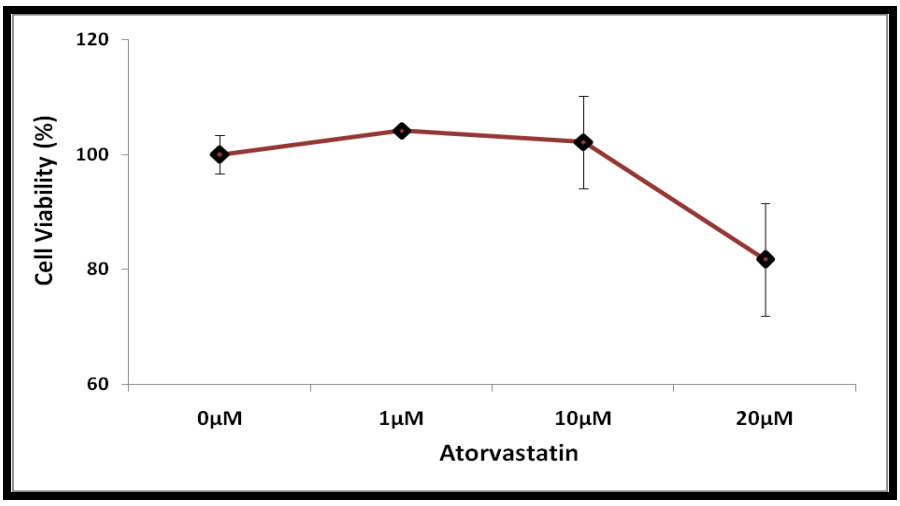


Figure 5.6 Cell viability of $HepG_2$ cells treated with atorvastatin for 24 hours. Data is represented as a percentage ± standard deviation relative to the untreated control (0µM).

The lowest measure of proliferation/metabolism was observed at 20μM, with 82±9.8% (Figure 5.6). No statistical differences between treatments were found. According to an *in vitro* study, 60μM atorvastatin was used to assess its effect on primary human-derived hepatocytes (Schroder et al., 2011). For all subsequent tests in our study, HepG2 cells were treated with 20μM atorvastatin for 24h.

Statin treatment increased the release of LDH (an intracellular enzyme), as measured in cell culture fluid after 24h. A simultaneous assessment of intracellular energy levels indicated a reduction in ATP levels (Figure 5.7).

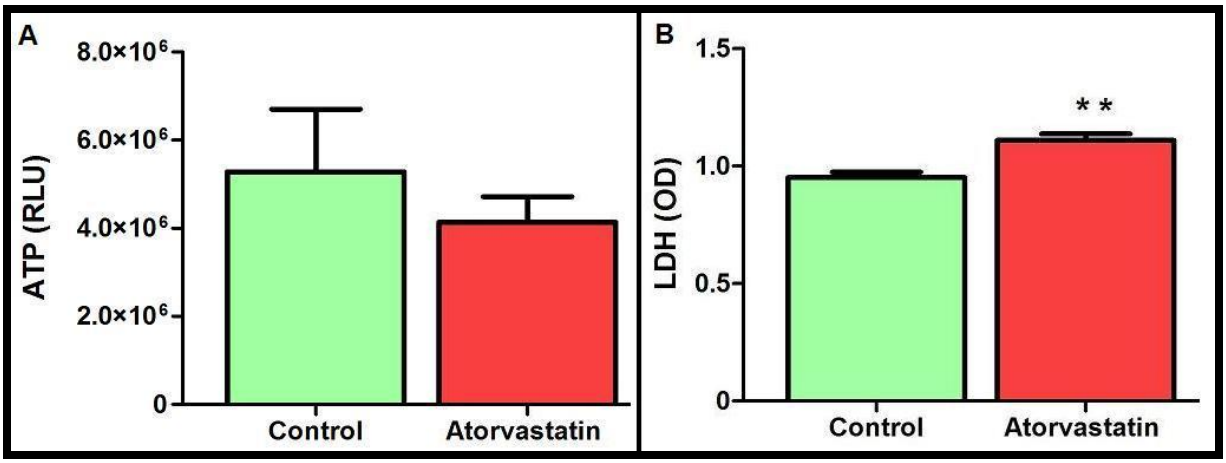


Figure 5.7 Levels of (A) ATP and (B) LDH activity in atorvastatin treated HepG2 cells. RLU: relative light units, OD: optical density. **p=0.001, Mann Whitney test, Mann Whitney U: 0.00 and Sum of ranks: 26, 63.

Basal levels of ATP in the control sample measured $5.28x10^6 \pm 0.82x10^6$ RLU and decreased to $4.13x10^6 \pm 0.34x10^6$ RLU in the presence of atorvastatin (Figure 5.7A); this finding however, was not statistically different. The decrease in ATP levels is in keeping with the decrease in cell proliferation/metabolic activity as measured by the MTT assay (Figure 5.6). Statin treated

cells had increased LDH levels (1.11±0.03 units) as compared to untreated cells (0.95±0.02 units) (p=0.001).

In order to evaluate the effect of atorvastatin on oxidative stress and NO synthesis, lipid peroxidation and nitrites levels were measured.

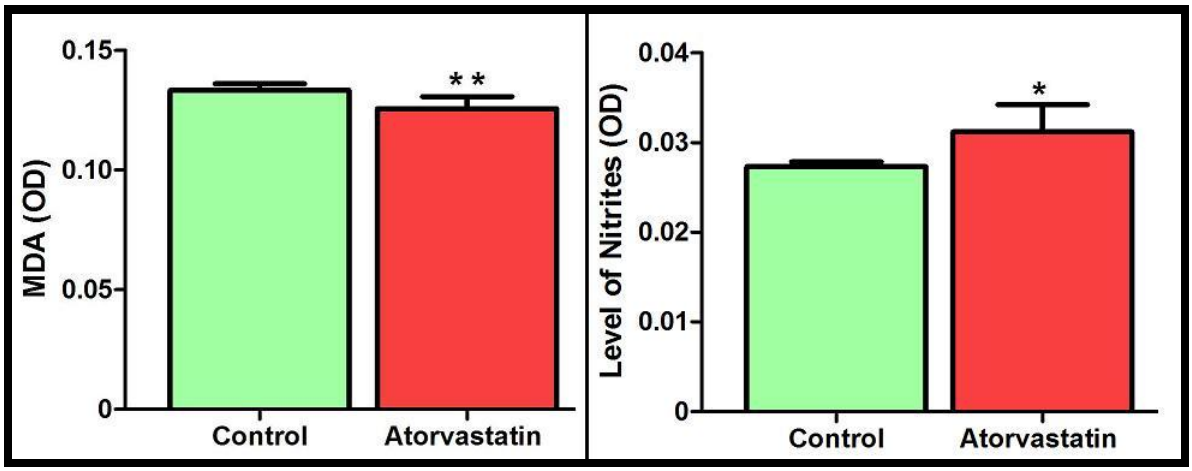


Figure 5.8 Levels of (A) MDA and (B) Nitrites from atorvastatin treated HepG2 cells. OD: optical density. **p=0.0092 (A), Mann Whitney test, Mann Whitney U: 8.00 and Sum of ranks: 142, 29; *p=0.044 (B), Mann Whitney test, Mann Whitney U: 4.00 and Sum of ranks: 25, 41.

The MDA levels in atorvastatin treated HepG2 cells was reduced from 0.133±0.003 units in control cells to 0.126±0.005 units (p=0.009). Also, atorvastatin increased the nitrite levels as compared to the controls (0.0312±0.003 units vs. control: 0.027±0.001 units, p=0.044). A significant finding, confirming the antioxidant potential of statins, was found (Figure 5.8A).

5.3.2 MicroRNA expression profile in statin treated HepG2 cells

No study, to date, assessed the role of miRNAs in HepG2 cells treated with atorvastatin. The miRNA pathway finder was used to profile the atorvastatin induced differential regulation of miRNAs in HepG2 cells (Figure 5.9, appendix 5).

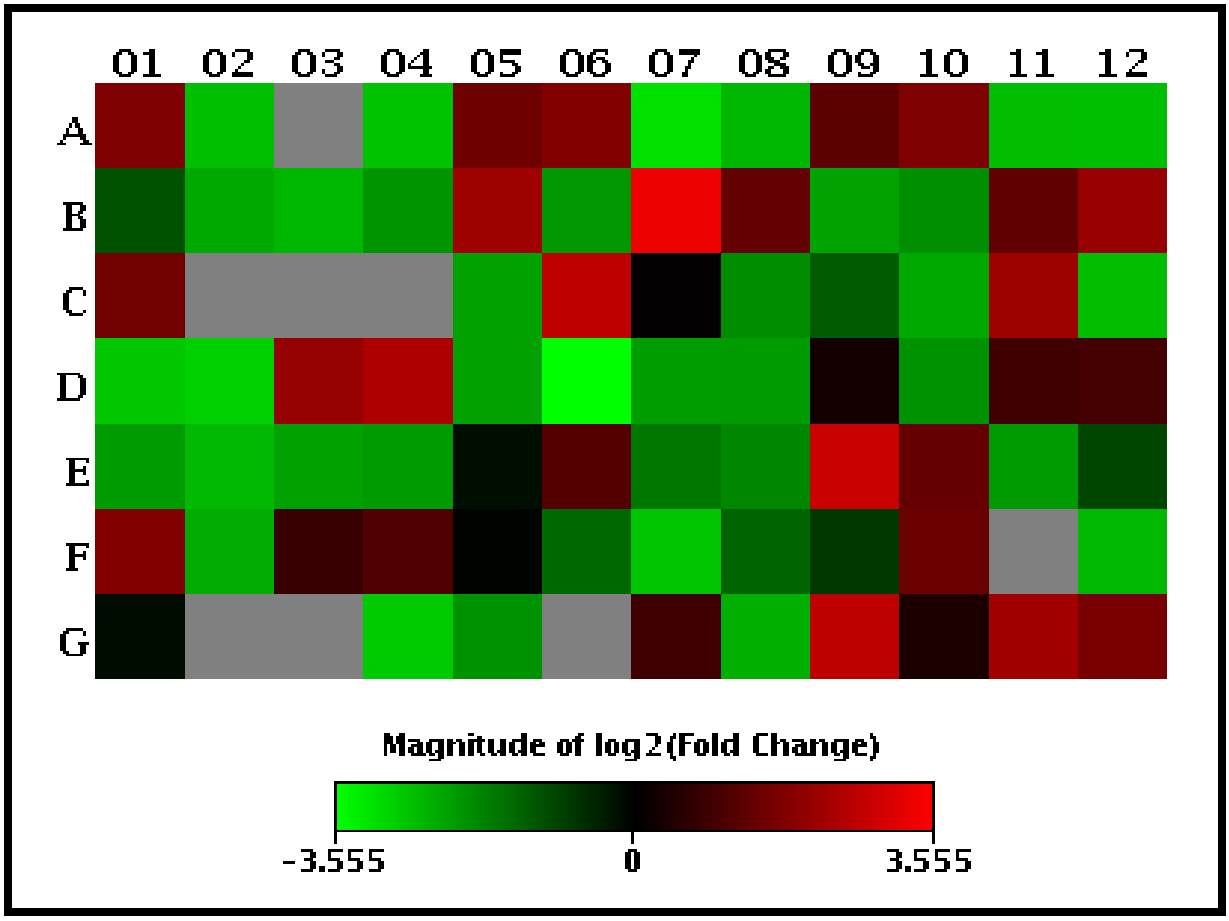


Figure 5.9 The differential expression of miR in atorvastatin treated HepG2 cells (represented as a heatmap).

MicroRNAs most significantly upregulated by atorvastatin included miR-302a-3p (3.05-fold), miR-302c-3p (3.61-fold), miR-124-3p (3.90-fold) and miR-222-3p (4.4-fold) (Figure 5.10); miR-

19a-3p, miR-101-3p and let-7g were downregulated (3.63-fold, 2.92-fold, 2.81-fold, respectively) (Figure 5.10).

The TargetScan tool was used to assess the mRNA targets of differentially regulated miRs. A list of the top 1000 gene targets was compiled for the 4 most up-regulated miRs (miR-222, miR-302a, miR-302c and miR-124a).

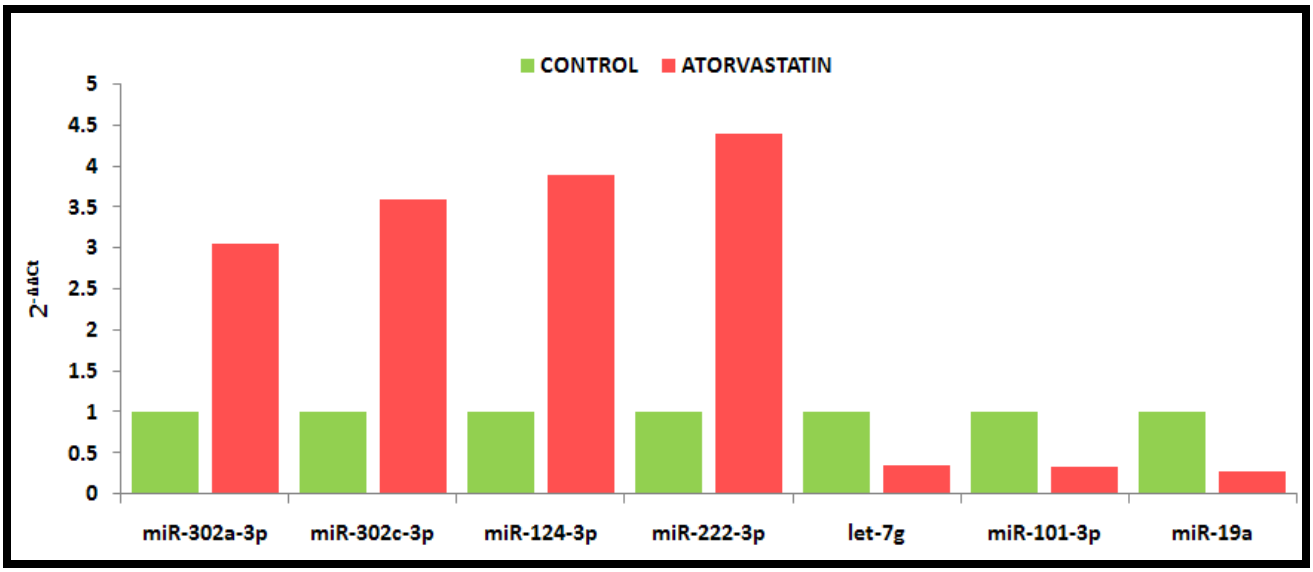


Figure 5.10 Fold change of miRs in HepG2 cells treated with 20μM atorvastatin for 24 hours. Data represented as a fold change relative to the untreated control.

Following a thorough screening of the potential targets, a list of targets was compiled. These were separated according to their role in specific biological processes such as metabolism (Table 5.1) and inflammation.

Table 5.1 Target genes of [a]miR-124a, [b]miR-222 and [c]miR-302a/c associated with carbohydrate and lipid metabolism and transport.

Target Gene	Representative transcript	Gene name
[a] ACAA2	NM_006111	acetyl-CoA acyltransferase 2
[c] ACADSB	NM_001609	acyl-CoA dehydrogenase, short/branched chain
[a,c] ACADVL	NM_000018	acyl-CoA dehydrogenase, very long chain
[c] ACOX1	NM_001185039	acyl-CoA oxidase 1, palmitoyl
[a] ACSL1	NM_001995	acyl-CoA synthetase long-chain family member 1
[c] AGPAT3	NM_001037553	1-acylglycerol-3-phosphate O-acyltransferase 3
[a] AGXT2L1	NM_001146590	alanine-glyoxylate aminotransferase 2-like 1
[a] ALDH1L2	NM_001034173	aldehyde dehydrogenase 1 family, member L2
[c] ALDH3A2	NM_000382	aldehyde dehydrogenase 3 family, member A2
[c] ALDH3B1	NM_000694	aldehyde dehydrogenase 3 family, member B1
[c] ALDH7A1	NM_001182	aldehyde dehydrogenase 7 family, member A1
[a] ATAD2B	NM_001242338	ATPase family, AAA domain containing 2B
[a] ATPAF1	NM_001042546	ATP synthase mitochondrial F1 complex assembly factor 1
[c] B3GALT5	NM_006057	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5
[c] B4GALT6	NM_004775	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 6
[a,c] C1GALT1	NM_020156	core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase, 1
[b] CAMKK1	NM_032294	calcium/calmodulin-dependent protein kinase kinase 1, alpha
[c] CAMTA1	NM_015215	calmodulin binding transcription activator 1
[c] CERK	NM_022766	ceramide kinase
[a] CERS2	NM_022075	ceramide synthase 2
[c] COA5	NM_001008215	cytochrome C oxidase assembly factor 5
[c] COX15	NM_004376	COX15 homolog, cytochrome c oxidase assembly protein (yeast)
[c] COX19	NM_001031617	COX19 cytochrome c oxidase assembly homolog (S. cerevisiae)
[a] CPT1A	NM_001876	carnitine palmitoyltransferase 1A (liver)
[a] CRAT	NM_000755	carnitine O-acetyltransferase
[a] CRTC3	NM_001042574	CREB regulated transcription coactivator 3
[a,c] CYCS	NM_018947	cytochrome c, somatic
[c] DAK	NM_015533	Dihydroxyacetone kinase 2 homolog (S. cerevisiae)

[a]	DGAT2	NM_032564	diacylglycerol O-acyltransferase 2
[c]	DGKB	NM_004080	diacylglycerol kinase, beta 90kDa
[b]	DGKE	NM_003647	diacylglycerol kinase, epsilon 64kDa
[c]	DGKQ	NM_001347	diacylglycerol kinase, theta 110kDa
[a]	DHCR24	NM_014762	24-dehydrocholesterol reductase
[c]	DHFRL1	NM_001195643	dihydrofolate reductase-like 1
[a]	DHRS1	NM_001136050	dehydrogenase/reductase (SDR family) member 1
[c]	EHHADH	NM_001166415	enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase
[a]	ELOVL5	NM_001242828	ELOVL fatty acid elongase 5
[a]	FAR1	NM_032228	fatty acyl CoA reductase 1
[c]	FOXRED2	NM_001102371	FAD-dependent oxidoreductase domain containing 2
[b]	FPGT	NM_001199328	fucose-1-phosphate guanylyltransferase
[c]	FUT4	NM_002033	fucosyltransferase 4 (alpha (1,3) fucosyltransferase, myeloid-specific)
[c]	GALK2	NM_001001556	galactokinase 2
[c]	GALNTL1	NM_001168368	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 1
[a]	GCDH	NM_000159	glutaryl-CoA dehydrogenase
[c]	GFPT1	NM_002056	glutamine--fructose-6-phosphate transaminase 1
[a]	GFPT2	NM_005110	glutamine-fructose-6-phosphate transaminase 2
[a,c]	GGPS1	NM_001037277	geranylgeranyl diphosphate synthase 1
[c]	GK	NM_000167	glycerol kinase
[a]	GK5	NM_001039547	glycerol kinase 5 (putative)
[c]	GLO1	NM_006708	glyoxalase I
[c]	GNPNAT1	NM_198066	glucosamine-phosphate N-acetyltransferase 1
[a]	GPAM	NM_020918	glycerol-3-phosphate acyltransferase, mitochondrial
[c]	GPD1L	NM_015141	glycerol-3-phosphate dehydrogenase 1-like
[a]	GPD2	NM_000408	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)
[c]	GPLD1	NM_001503	glycosylphosphatidylinositol specific phospholipase D1
[a]	GPT2	NM_001142466	glutamic pyruvate transaminase (alanine aminotransferase) 2
[a]	GSK3B	NM_001146156	glycogen synthase kinase 3 beta

[c]	GXYLT1	NM_001099650	glucoside xylosyltransferase 1
[c]	H6PD	NM_004285	hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)
[a]	HADH	NM_001184705	hydroxyacyl-CoA dehydrogenase
[a]	HADHA	NM_000182	hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit
[c]	HSD17B7	NM_016371	hydroxysteroid (17-beta) dehydrogenase 7
[c]	IDI1	NM_004508	isopentenyl-diphosphate delta isomerase 1
[a]	IMPAD1	NM_017813	inositol monophosphatase domain containing 1
[a]	INPP5B	NM_005540	inositol polyphosphate-5-phosphatase, 75kDa
[c]	INPP5F	NM_014937	inositol polyphosphate-5-phosphatase F
[c]	IVD	NM_001159508	isovaleryl-CoA dehydrogenase
[c]	KDSR	NM_002035	3-ketodihydrosphingosine reductase
[a]	L2HGDH	NM_024884	L-2-hydroxyglutarate dehydrogenase
[c]	LDHD	NM_153486	lactate dehydrogenase D
[c]	LDLR	NM_000527	low density lipoprotein receptor
[a]	LDLRAP1	NM_015627	low density lipoprotein receptor adaptor protein 1
[a]	LIPE	NM_005357	lipase, hormone-sensitive
[b]	LPPR1	NM_017753	lipid phosphate phosphatase-related protein type 1
[c]	LRP2	NM_004525	low density lipoprotein receptor-related protein 2
[a,c]	LRP6	NM_002336	low density lipoprotein receptor-related protein 6
[c]	LRPAP1	NM_002337	low density lipoprotein receptor-related protein associated protein 1
[a]	MTR	NM_000254	5-methyltetrahydrofolate-homocysteine methyltransferase
[c]	MVK	NM_000431	mevalonate kinase
[c]	NDUFA10	NM_004544	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10, 42kDa
[c]	NDUFC2	NM_001204054	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 2, 14.5kDa
[a]	OSBP	NM_002556	oxysterol binding protein
[a]	OSBP2	NM_030758	oxysterol binding protein 2
[a]	OSBPL10	NM_001174060	oxysterol binding protein-like 10
[a,b]	OSBPL3	NM_015550	oxysterol binding protein-like 3
[c]	OSBPL6	NM_001201480	oxysterol binding protein-like 6
[a,b]	OSBPL7	NM_145798	oxysterol binding protein-like 7
[a]	OSBPL8	NM_001003712	oxysterol binding protein-like 8

[b]	PAK1	NM_001128620	p21 protein (Cdc42/Rac)-activated kinase 1
[c]	PAK2	NM_002577	p21 protein (Cdc42/Rac)-activated kinase 2
[c]	PAK7	NM_020341	p21 protein (Cdc42/Rac)-activated kinase 7
[c]	PDP2	NM_020786	pyruvate dehyrogenase phosphatase catalytic subunit 2
[a]	PEX19	NM_001193644	peroxisomal biogenesis factor 19
[c]	PFKFB2	NM_006212	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2
[c]	PFKFB3	NM_001145443	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
[c]	PIK3AP1	NM_152309	phosphoinositide-3-kinase adaptor protein 1
[a]	PIP4K2C	NM_001146259	phosphatidylinositol-5-phosphate 4-kinase, type II, gamma
[a]	PLCB1	NM_015192	phospholipase C, beta 1 (phosphoinositide-specific)
[c]	PLCXD3	NM_001005473	phosphatidylinositol-specific phospholipase C, X domain containing 3
[c]	PNPO	NM_018129	pyridoxamine 5'-phosphate oxidase
[a,c]	PPARA	NM_001001928	peroxisome proliferator-activated receptor alpha
[a,c]	PPARGC1B	NM_001172698	peroxisome proliferator-activated receptor gamma, coactivator 1 beta
[a,c]	PXMP4	NM_007238	peroxisomal membrane protein 4, 24kDa
[c]	S1PR3	NM_005226	sphingosine-1-phosphate receptor 3
[c]	SC5DL	NM_001024956	sterol-C5-desaturase (ERG3 delta-5-desaturase homolog, S. cerevisiae)-like
[a]	SCD	NM_005063	stearoyl-CoA desaturase (delta-9-desaturase)
[b]	SCD5	NM_001037582	stearoyl-CoA desaturase 5
[a]	SGPP1	NM_030791	sphingosine-1-phosphate phosphatase 1
[c]	SIRT3	NM_001017524	sirtuin 3
[c]	SIRT5	NM_001193267	sirtuin 5
[c]	SLC16A12	NM_213606	solute carrier family 16, member 12 (monocarboxylic acid transporter 12)
[c]	SLC16A14	NM_152527	solute carrier family 16, member 14 (monocarboxylic acid transporter 14)
[c]	SLC2A1	NM_006516	solute carrier family 2 (facilitated glucose transporter), member 1
[c]	SLC2A3	NM_006931	solute carrier family 2 (facilitated glucose transporter), member 3
[a]	SORD	NM_003104	sorbitol dehydrogenase
[a]	SPHK1	NM_001142601	sphingosine kinase 1
[c]	SRD5A3	NM_024592	steroid 5 alpha-reductase 3
[a]	SUCLG2	NM_003848	succinate-CoA ligase, GDP-forming, beta subunit

[b]	TP53BP2	NM_001031685	tumour protein p53 binding protein, 2
[a,b,c]	TP53INP1	NM_001135733	tumour protein p53 inducible nuclear protein 1
[a]	TP53INP2	NM_021202	tumour protein p53 inducible nuclear protein 2
[c]	UGGT1	NM_020120	UDP-glucose glycoprotein glucosyltransferase 1

Several cytokines such as IL-8, IL-13 and IL-16 and cytokine receptor molecules of IL-2, IL-6, IL-17A, IL-17D and IL-21 were found to be targets of miR-124a-3p, miR-222 and miR-302a/c. Another significant target of interest involved in inflammation is the downstream signalling molecule, interleukin-1 receptor associated kinase-1 (IRAK1).

Epigenetic modification of DNA requires the expression and activity of several DNA-methyl-regulators. It is therefore interesting that a list of targets of miR-124a, miR-222 and miR-302a/c include the methyl-CpG binding protein (MECP2), methyl-CpG binding domain proteins 1 and 2 (MDB1 and MDB2) and DNA (cytosine-5-) methyltransferase 3 beta.

The liver is actively involved in metabolic processing of nutrients, drugs and waste products. Most of these processes are energy dependant and require the optimal functioning of the mitochondria, the energy hub of all cells. Statin induced overexpression of miRs that target crucial molecules for ATP generation: cytochrome c (CYCS) (miR-124a, miR-302a/c), cytochrome c assembly factor 5 (COA5) (miR-302a/c), coenzyme Q10 (COQ10B) (miR-302a/c), ATP synthase mitochondrial F1 complex (ATPAF1) (miR-124a) is therefore an important observation in this study.

Spermine oxidase, involved in polyamine synthesis, was also identified as a target of miR-124a. Polyamines are known to play a role in immunomodulation.

The liver and kidney are the two primary sources of creatine which is transported to peripheral tissue. The miR-124a specifically targets the mRNA of GAMT, a very important enzyme involved in creatine synthesis which catalyses the conversion of guanidinoacetoacetate (GAA) to form creatine.

5.3.3 Quantitative analysis of GAMT, AGAT and SMO mRNA expression and western blot analysis for GAMT and p53 protein expression

Two targets of miR-124a were chosen to assess the extent by which mRNA levels are affected. These include GAMT and SMO. A mechanism of statin induced creatine depletion was speculated and the mRNA levels of the enzyme, AGAT (involved upstream of GAMT in the creatine synthesis pathway), was assessed. The mRNA level of SMO was selected for assessment due to the role of polyamines in immunomodulation.

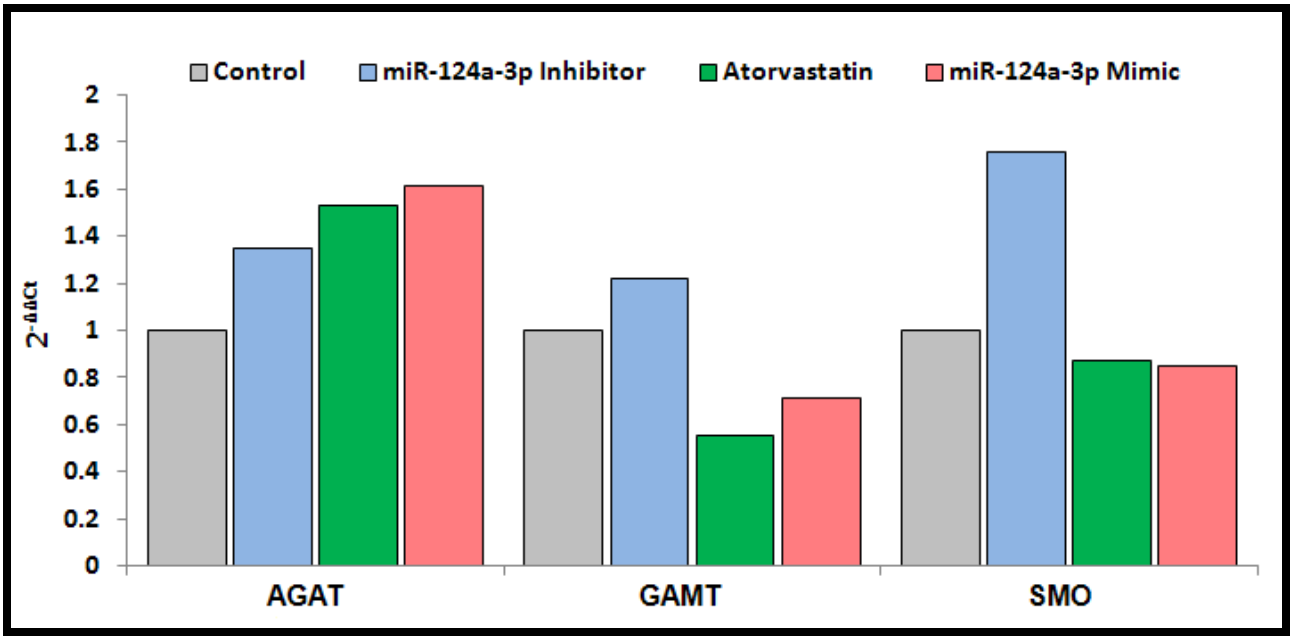


Figure 5.11 Relative fold changes in mRNA expression of GAMT, AGAT and SMO in HepG2 cells treated with atorvastatin, miRNA-124a-3p mimic and miRNA-124a-3p inhibitor.

Quantitative PCR was used to determine the effect of atorvastatin treatment on mRNA levels GAMT, AGAT and SMO in HepG2 cells. A 1.8-fold decrease in GAMT and 1.15-fold decrease in SMO mRNA levels was observed in atorvastatin treated HepG2 cells. An increase in mRNA level of AGAT was noted in HepG2 cells treated with atorvastatin by 1.53-fold (Figure 5.11).

In the HepG2 cells transfected with mimics of miR-124a-3p, a similar fold change in mRNA levels of GAMT, AGAT, SMO was observed. The reduction in mRNA levels, however, was not the same as that noted in the statin treatment with fold changes of 1.41-fold decrease in GAMT, 1.2-fold decrease in SMO and a 1.61-fold increase of AGAT. In the presence of the miRNA-124a-3p inhibitor, a much higher level of mRNA was detected compared to the control with a 1.76-fold change in GAMT, 1.22-fold in SMO and 1.35-fold in AGAT (Figure 5.11).

The binding of miRNAs to target mRNA sequence does not always translate to a reduction in protein expression. Therefore, the protein levels of GAMT in atorvastatin treated cells and cells transfected with miR-124a-3p mimics and inhibitors were assessed by western blotting. The level of phosphorylated p53, a transcription factor of GAMT, was assessed in atorvastatin treated cells and in cells transfected with miR-124a-3p mimics and inhibitors.

The level of GAMT was relatively lower than the untreated control (1.11-fold). HepG2 cells transfected with 50nM of miR-124a-3p mimic showed a 1.36-fold decrease in GAMT expression. No change in the protein expression of GAMT was noted in the miR-124a-3p inhibitor treatment (Figure 5.12).

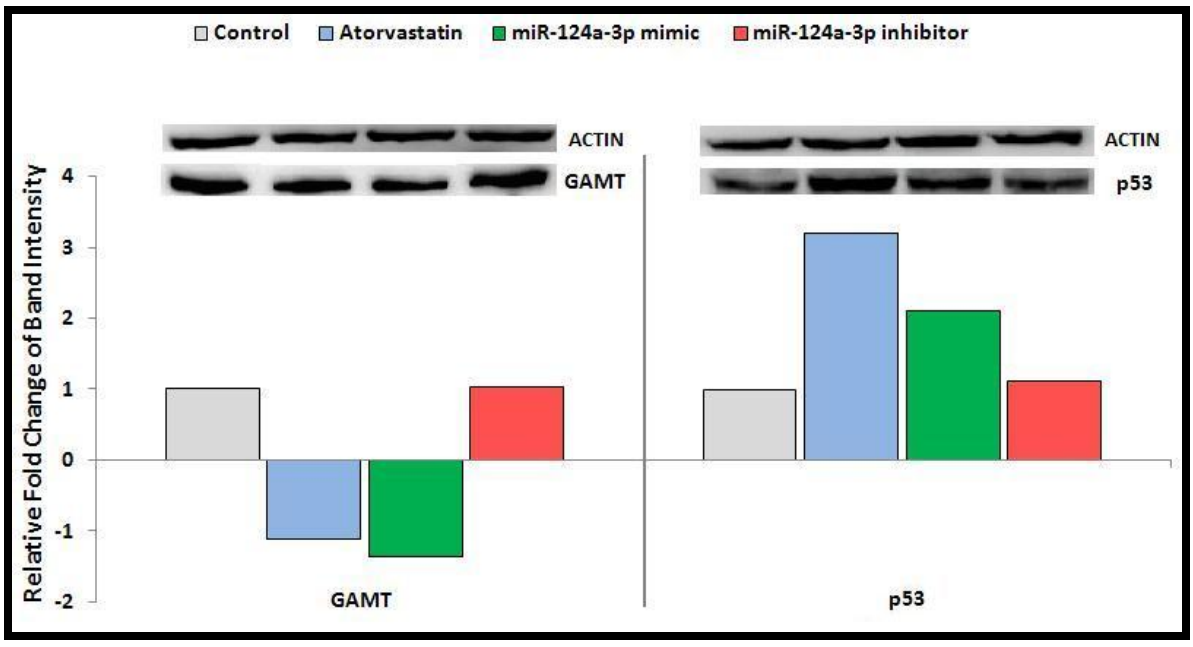


Figure 5.12 Relative fold changes in protein expression of GAMT and phosphorylation of p53 (where n=2 and normalised against actin) following treatment with atorvastatin, and a mimic and inhibitor of miR-124a-3p.

The highest level of p53 phosphorylation was observed in atorvastatin treated HepG2 cells (3.2-fold increase). In the presence of the miR-124a-3p mimics the level of p53 phosphorylation was also higher than the control by 2.1-fold. A similar level of p53 phosphorylation between the control and miR-124a-3p inhibitors was noted (Figure 5.12).

5.4 Discussion

Statins are unequivocally the most widely prescribed drug for the primary and secondary prevention of CVD worldwide (Eidelman et al., 2002). Atorvastatin is a more commonly prescribed drug from the statin family due to its lower therapeutic dose compared to simvastatin, lovastatin and cerivastatin (Strandberg et al., 2004). The well-tolerated and high benefit-risk ratio of atorvastatin has been demonstrated in clinical trials (Strandberg et al., 2004).

The liver, being the central hub of metabolism, plays a crucial role in glucose homeostasis, cholesterol and lipoprotein synthesis, and xenobiotic metabolism.

Statins have not only received the spotlight for their positive effects, but have also been implicated for their adverse effects in patients (Chalasani, 2005). It was reported that liver function enzymes rose asymptomatically after therapy. The guidelines for patients on atorvastatin therapy recommended a liver function test be performed before administration, followed by biannual monitoring of liver function (Chalasani, 2005).

In our study, we observed an increase in LDH activity after treatment of HepG2 cells with atorvastatin (Figure 5.7). This increased LDH activity was only observed at the higher concentration of 20µM atorvastatin. LDH activity in cell culture media indicates that atorvastatin may have induced membrane damage or leakage due to its lipophilic nature. The metabolic activity in hepG2 cells treated with atorvastatin as measured by the MTT (Figure 5.6) and ATP (Figure 5.7) assays, showed compromised metabolic function. The inhibition of HMGCR and subsequent accumulation of acetyl-coA in the mt, can be transported back into the cytosol following conversion to citrate. This may lead to a decrease in reducing equivalents, and hence the lower levels of MTT conversion and intracellular ATP production.

The positive effects of statins (LDL-C reduction, the protection against oxidative stress, vaso-relaxant properties of increased NO synthesis and anti-inflammatory potential) out-weigh the minor, asymptomatic adverse effects. Statins decreased production of superoxide by inhibiting geranylgeranylpyrophosphate (GGPP) activation of ras, preventing the stimulation of NADPH oxidase (Wagner et al., 2002; Wassmann et al., 2002; Wang et al., 2008). Also, cytosolic citrate undergoes a two-step reaction catalysed by ATP-citrate lyase and malate dehydrogenase to form pyruvate. The first reaction utilises NADH and the second produces NADPH. Both, the inhibition of NADPH oxidase and the previously mentioned reaction, provides NADPH which can be used for the regeneration of the intracellular antioxidant, GSH. A significant reduction in lipid peroxidation noted in atorvastatin treated cells (Figure 5.8) in our study may be due to the antioxidant properties of statins and increased GSH.

The inhibition of eNOS by GGPP occurs via GGPP stimulation of G protein Rho (Figure 5.2). Reduced oxidative stress and statin inhibition of HMGCR prevents GGPP formation and contributes to increased eNOS activity and an elevation in nitric oxide (Endres, 2005). A significant increase in nitrites was observed in atorvastatin treated HepG2 cells (Figure 5.8). This strongly suggests that atorvastatin increases eNOS activity. This result is in keeping with the antioxidant property of statins.

In the Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin, 17,802 apparently healthy men and women with LDL-C levels of less than 130 mg/dl (3.4 mmol/l) and high-sensitivity CRP levels of 2.0 mg/l or higher were assessed. It was concluded that rosuvastatin significantly reduced the incidence of major cardiovascular events in healthy persons without hyperlipidaemia. Of the 17,802 subjects involved in the study, 16% presented with muscle weakness, stiffness or pain (Rao and Milbrandt, 2010).

The duration of myopathy from statin therapy ranges from a few weeks to two years (Phillips et al., 2002). To date, little is known about the mechanism by which statin therapy leads to muscle weakness or fatigue. A study on statin-associated myopathy found pathology even in the presence of normal creatine kinase (CK) levels (Phillips et al., 2002). Creatine kinase is abundant in energy-demanding tissues such as skeletal muscle. The reaction catalysed by CK is the reversible conversion of creatine to phosphocreatine which serves as an energy reservoir for the instant regeneration of ATP *in situ*. Creatine, however, is produced primarily in the liver and kidney and is transported in circulation to peripheral tissues including muscle.

Our study assessed the effect of atorvastatin on 84 well characterised miRNAs in the HepG2 cells and showed a significant increase in several miRNAs species (miR-124a, miR-222 and miR-302a/c, Figure 5.10). At least two miRNA panels were used per treatment – hence the differentially regulated miRNA's were validated and repeated using the separate miRNA expression assays in which six replicates were analysed. Following an extensive analysis of predicted targets of up-regulated miRNAs, several molecules involved in atherosclerotic plaque development and progression were identified. The elevated miR-124a and miR-302a/c targets integral components of the electron transport chain (CYCS, COA5, COQ10B and ATPAF1) as well as a key enzyme in creatine synthesis (GAMT, target of miR-124a).

Figure 5.13 shows a plausible mechanism of statin induced energy depletion which may lead to myopathy. In order to validate the proposed mechanism, the mRNA levels of GAMT and AGAT – enzymes involved in creatine synthesis - were assessed and found to be decreased by 1.8-fold and increased by 1.53-fold, respectively (Figure 5.11). The lower expression of GAMT mRNA by atorvastatin was confirmed by western blotting (Figure 5.12). This decrease in protein expression will reduce creatine synthesis. A compensatory mechanism is observed as the rate-limiting enzyme (AGAT) of creatine synthesis up regulated by low creatine.

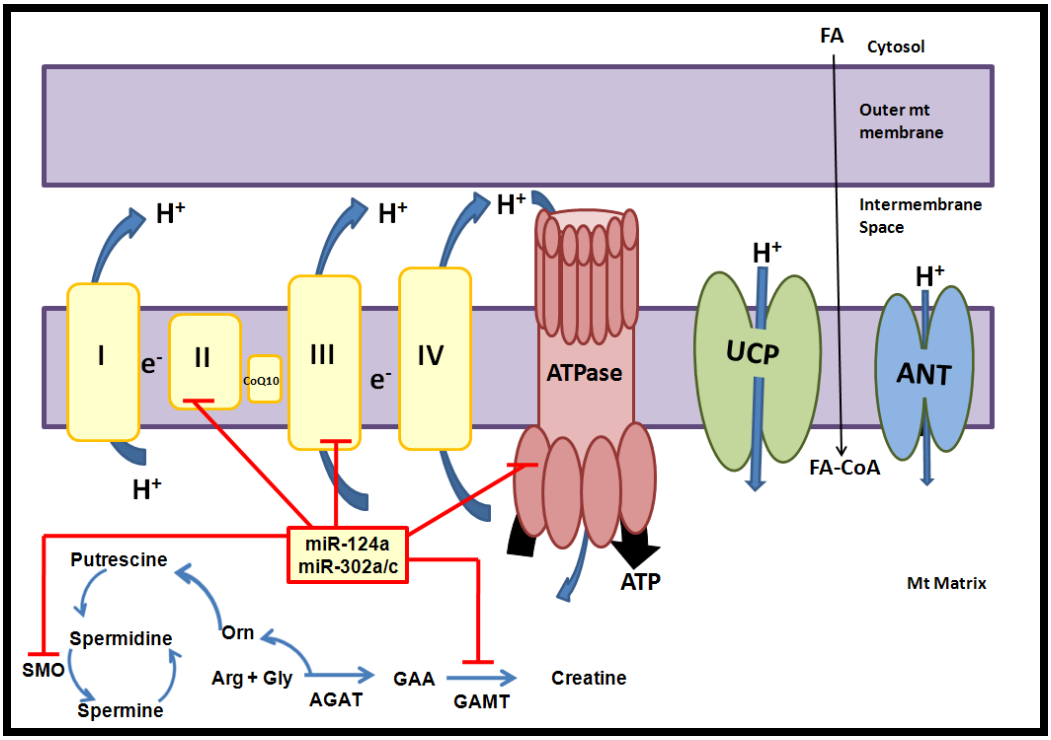


Figure 5.13 Targets of miR-124a and miR302a/c which are involved in the electron transport chain (Complexes I, II, III, IV, CoQ10: coenzyme Q10), creatine (arg: arginine, Gly: glycine, AGAT: arginine glycine aminotransferase, GAA: guanidinoacetoacetate, GAMT: guanidinoacetate aminotransferase) and polyamine (Orn: ornithine, SMO: spermine oxidase) synthesis pathways.

A study on left ventricular assist devices (LVADs) used to support a failing heart (found an unexpected elevation of AGAT mRNA levels in these patients compared to healthy donors. These patients were placed on pharmacological therapy of β_2-adrenergic agonists to improve cardiac function (Cullen et al., 2006). The authors however, did not use statin therapy as an exclusion criterion nor was the percentage of patients on statin therapy stated (Cullen et al., 2006). The compromised synthesis of creatine from the liver in the presence of statin therapy

may result in the increased expression of AGAT in the myocardium as a compensatory mechanism for the energy demands of the heart.

There is emerging evidence that p53 is involved in the regulation of energy metabolism and autophagy (Feng et al., 2005; Crighton et al., 2006; Bensaad and Vousden, 2007; Jones and Thompson, 2009). Metabolic activity of p53 is related to adenosine monophosphate kinase (AMPK) (Jones and Thompson, 2009), TP53-induced glycolysis and apoptosis regulator (TIGAR) (Bensaad and Vousden, 2007) and phosphoglycerate mutase (PGM) (Kondoh et al., 2005). Mutant cells lacking p53 showed enhanced glycolysis with decreased utilization of oxygen by mt respiration, commonly referred to as the Warburg effect (Bensaad and Vousden, 2007). A study on a link between p53, GAMT and FA oxidation provided new insight to the cells' response to nutrient availability (Ide et al., 2009). This study identified p53 activation (phosphorylation) under conditions of nutrient stress, which led to creatine biosynthesis and FA oxidation as an alternate source to regulate energy metabolism (Ide et al., 2009).

Our study atorvastatin elevated levels of miR-124a in the liver cells. The targets included the electron transport chain, and GAMT - both essential for creatine synthesis. The level of ATP in atorvastatin treated cells was lower than control cells, with no statistical significance. This change in AMP/ATP ratio will result in the phosphorylation of p53. The western blot results confirmed an increased phosphorylation of p53 in the presence of atorvastatin (Figure 5.12). The role of p53 in energy regulation leads to increased expression of GAMT and FA oxidation (Ide et al., 2009). In the presence of statin induced miR-124a-3p inhibition of GAMT, the alternate source of FAs would be utilized to compensate for energy requirements. This may

explain the decrease in free FA and triglycerides in circulation of patients on statin therapy. Furthermore, the upregulation of creatine kinase levels in patients on statins could possibly be a compensatory mechanism to preserve creatine stores.

A by-product of the AGAT catalysed reaction utilising L-arginine (Arg) and glycine (Gly) as substrates to form guanoacetoacetate, is ornithine (Orn) (Figure 5.6). Ornithine may then either enter the urea cycle or the polyamine synthesis pathway. Spermine oxidase, also a target of miR-124a, catalyses the conversion of spermine to spermidine, and when inhibited (1.15-fold in this study), results in an increase in spermine levels. The range of biological roles of spermine includes acting as a second messenger in cell signalling, regulation of DNA synthesis, cellular proliferation and inhibition of the immune response (Seiler and Atanassov, 1994). A study assessing the effects of spermine on stimulated human peripheral blood nuclear cells found an inhibition of pro-inflammatory cytokine synthesis: TNFα, IL-1 and IL-6 (Zhang et al., 1999).

The lipid-lowering properties of statins are well documented (Ebrahim et al., 1999; Heart Protection Study Collaborative Group, 2002; Law et al., 2003). In the CARE clinical trials (Ridker et al., 1998), statins were recognised for their anti-inflammatory effects. Since cytokines have been implicated as key mediators of atherosclerotic plaque development and progression, they have also become therapeutic targets of interest (von der Thusen et al., 2003). The inhibition of the synthesis of interleukins-8, -13 and -16, interleukin receptor 2 (IL-2R), 6 (IL-6R), 17A (IL-17AR), 17D (IL-17DR) and 21 (IL-21R) and IRAK1 are targets of statin-induced up-regulation of miR-124a, miR-222 and miR-302a/c. This may explain the anti-inflammatory effects of statin therapy. In hepatic cells IL-6R and IRAK1 are integral for

the binding of IL-6 and the downstream signalling of IL-1 ultimately resulting in the synthesis and release of CRP. A decrease in the levels of IRAK1 leads to reduced CRP synthesis in the presence of statins. In the clinical setting, this is a valuable finding as pro-inflammatory markers in circulation are reduced in patients on statin therapy.

The up-regulated miRNAs targeting the inflammasome appear to play role in regulating cytokine levels and their actions. This implies that and in conjunction with the presence of SNPs (IL-6 and CDRP) investigated in chapter 4, a more complex mechanism of control is present. A recent study found attenuated IL-6 production, decreased mRNA and protein levels of IL-6R and subsequent inhibition of STAT3 phosphorylation by added miR-124 in liver cell lines (Hatziapostolou et al., 2011). The mechanism of statin-reduced pro-inflammatory cytokine may be a result of up-regulation of miRNA-124. This may be necessary for CAD patients as inflammation is critical in the progression of atherosclerosis. Hence, the anti-inflammatory effects of statins may not only be a direct result of cytokine and cytokine receptor synthesis but also via indirect spermine regulation.

5.5 Conclusion

Atorvastatin limits metabolic activity and energy availability in hepG2 cells. Minimal cytotoxicity (LDH leakage) was noted with atorvastatin treatment. The antioxidant potential of atorvastatin was confirmed by a decrease in lipid peroxidation. The increase in nitric oxide production, also confirms the ability of statins to increase eNOS activity. Statin treated HepG2 cells exhibit a different miRNA profile compared to untreated controls. An assessment of

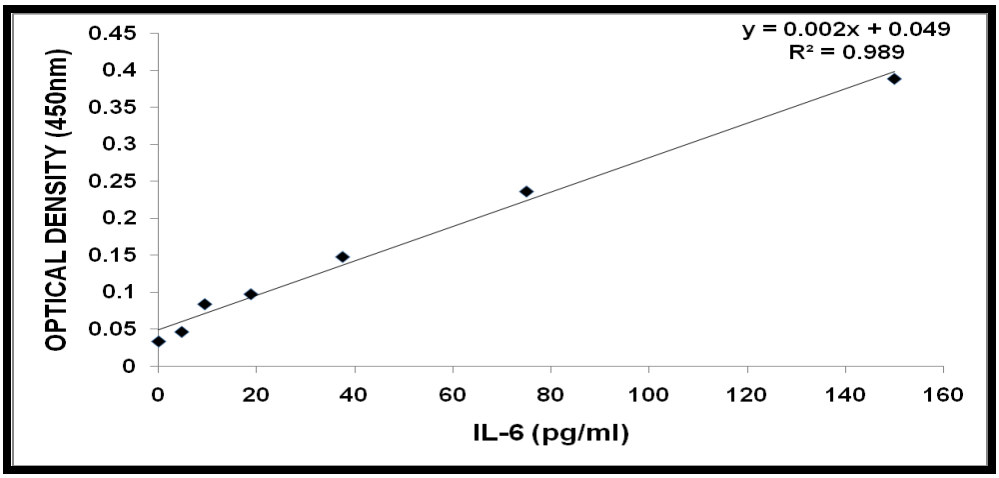
targets of up-regulated miR-124a, miR-222 and miR-302a/c show they play a crucial role in the regulation of inflammation (IL-6 and IRAK1, specifically) and metabolism (lipoprotein receptors and transporters, lipid and carbohydrate metabolism associated enzymes, specifically). The inhibition of GAMT by miR-124a may explain the creatine depletion and elevated creatine kinase levels observed in the clinical setting. The anti-inflammatory potential may also be attributed to the miR-124a inhibition of spermine oxidase. Taken together, several claims related to pleiotropic effects of statins have been confirmed in this study.

APPENDIX 1

ELISA STANDARDS

Preparation of samples of known concentration for the IL-6 ELISA

Standard Concentration	Volume IL-6	Volume Distilled water
150 pmol	50.0 µl	50.00 µl
75 pmol	25.0 µl	75.00 µl
37.5 pmol	12.5 µl	87.50 µl
18.8 pmol	6.25 µl	93.75 µl
9.4 pmol	3.13 µl	96.87 µl
4.7 pmol	1.56 µl	98.44 µl
0.00 pmol	0.00 µl	100.0 µl



Standard curve derived from the IL-6 standards.

Standard IL-6 concentrations were prepared and used to create the standard curve above. The curve was extrapolated and the equation (y=0.002x + 0.049) used to determine the serum IL-6 concentrations for each control and CAD patient.

APPENDIX 2

TABLE OF COMPONENTS PRESENT IN EAGLES MINIMUM ESSENTAILS MEDIA

Component	Concentration (mg/l)
Inorganic salts	
$CaCl_2.2H_2O$	186.0
KCl	400.0
KH_2PO_4	60.0
$MgSO_4.7H_2O$	200.0
NaCl	8 000.0
$NaHCO_3$	350.0
$Na_2HPO_4.7H_2O$	90.0
Other components	
Glucose	1 000.0
Phenol red	20.0
Amino acids	
L-Arginine-HCl	126.4
L-Cysteine	24.0
L-Histidine-HCl.H_2O	42.0
L-Isoleucine	52.4
L-Leucine	52.4
L-Lysine-HCl	73.0
L-Methionine	15.0
L-Phenylalanine	33.0
L-Threonine	47.6
L-Tryptophan	10.2
L-Tyrosine	36.2
L-Valine	46.8
Vitamins	
D-Capantothenate	1.0
Choline chloride	1.0
Folic acid	1.0
i-Inositol	2.0
Nicotinamide	1.0
Pyridoxine	1.0
Riboflavin	0.1
Thiamine-HCl	1.0

APPENDIX 3

THE $2^{-\Delta\Delta Ct}$ METHOD FOR ANALYSIS OF QUANTITATIVE PCR

The $2^{-\Delta\Delta Ct}$ method refers to the method of analysis used to obtain a relative fold change of a treated sample compared to an appropriate control group as described by Livak and Schmittgen (2001). The table below extracted directly from the original article highlights an example of relative quantification.

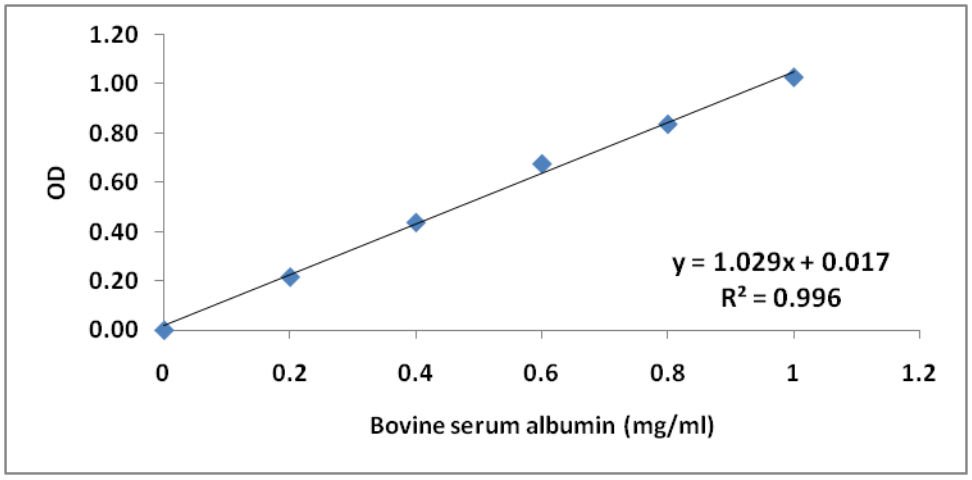
Treatment of Replicate Data Where Target and Reference Are Amplified in Separate Wells[a]

Tissue	c-*myc* C_T	GAPDH C_T	ΔC_T (Avg. c-*myc* C_T − Avg. GAPDH C_T)	$\Delta\Delta C_T$ (Avg. ΔC_T − Avg. $\Delta C_{T,Brain}$)	Normalized c-*myc* amount relative to brain $2^{-\Delta\Delta C_T}$
Brain	30.72	23.70			
	30.34	23.56			
	30.58	23.47			
	30.34	23.65			
	30.50	23.69			
	30.43	23.68			
Average	30.49 ± 0.15	23.63 ± 0.09	6.86 ± 0.17	0.00 ± 0.17	1.0 (0.9–1.1)
Kidney	27.06	22.76			
	27.03	22.61			
	27.03	22.62			
	27.10	22.60			
	26.99	22.61			
	26.94	22.76			
Average	27.03 ± 0.06	22.66 ± 0.08	4.37 ± 0.10	−2.50 ± 0.10	5.6 (5.3–6.0)

[a] Total RNA from human brain and kidney were purchased from Clontech. Using reverse transcriptase, cDNA was synthesized from 1 μg total RNA. Aliquots of cDNA were used as template for real-time PCR reactions containing either primers and probe for c-*myc* or primers and probe for GAPDH. Each reaction contained cDNA derived from 10 ng total RNA. Six replicates of each reaction were performed.

APPENDIX 4

Protein Quantification and Standardisation							
		Bovine serum albumin (mg/ml)					
		0	0.2	0.4	0.6	0.8	1
	OD1	0.001	0.212	0.432	0.674	0.821	1.021
	OD2	0.003	0.221	0.453	0.684	0.842	1.033
	OD3	0.003	0.219	0.429	0.668	0.844	1.023
	Average	0.00	0.22	0.44	0.68	0.84	1.03



		Control	Statin	mimic	inhibitor	
OD1		0.986	0.976	0.968	0.991	
OD2		0.978	0.976	0.97	0.982	
OD3		0.988	0.987	0.978	0.984	
Average		0.984	0.979667	0.972	0.985667	
Initial concentration		(mg/ml)	0.940	0.936	0.928	0.941
Final concentration		(mg/ml)	0.9	0.9	0.9	0.9
Final volume		(ul)	150	150	150	150
Initial volume		(ul)	144	144	145	143

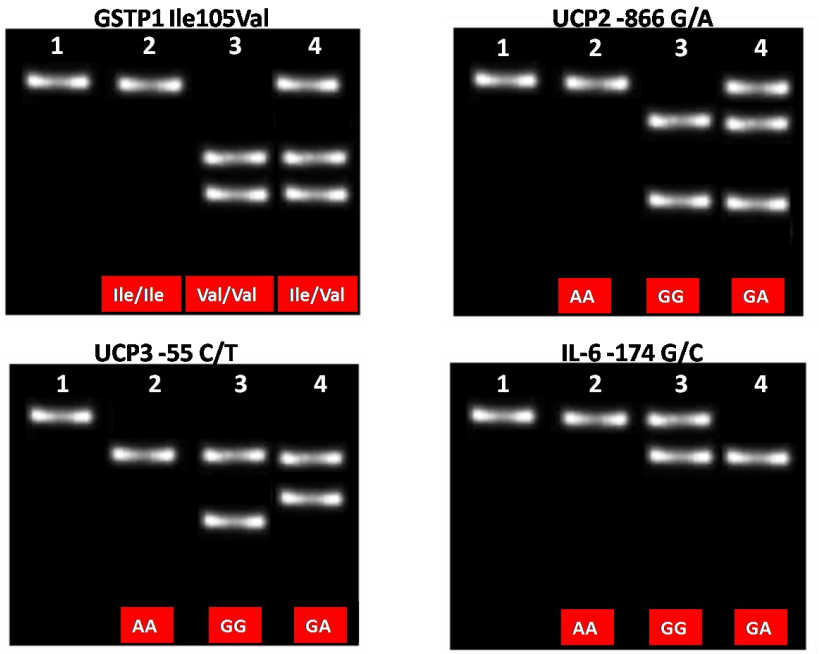
APPENDIX 5

miR Pathway Finder Array - Control vs. Atorvastatin

Observation	*Residual*	Rank	Rank prop	r based z score	Residual
miR-19a	-3.63	1	0.01	-2.51	-3.63
miR-101	-2.92	2	0.02	-2.09	-2.92
let-7g	-2.81	3	0.03	-1.87	-2.81
let-7f	-2.8	4	0.04	-1.72	-2.80
miR-140-5p	-2.78	5	0.05	-1.60	-2.78
miR-26b	-2.78	6	0.07	-1.50	-2.78
miR-126	-2.75	7	0.08	-1.41	-2.75
miR-425	-2.71	8	0.09	-1.33	-2.71
let-7e	-2.44	9	0.10	-1.26	-2.44
let-7a	-2.41	10	0.12	-1.20	-2.41
miR-29a	-2.35	11	0.13	-1.14	-2.35
miR-24	-2.26	12	0.14	-1.08	-2.26
miR-29c	-2.22	13	0.15	-1.03	-2.22
miR-194	-2.18	14	0.16	-0.98	-2.18
miR-27a	-2.1	15	0.18	-0.93	-2.10
miR-20a	-2.01	16	0.19	-0.88	-2.01
miR-30e	-2.	17	0.20	-0.84	-2.00
miR-30d	-1.93	18	0.21	-0.79	-1.93
miR-26a	-1.92	19	0.23	-0.75	-1.92
miR-27b	-1.88	20	0.24	-0.71	-1.88
miR-100	-1.87	21	0.25	-0.67	-1.87
miR-15b	-1.87	22	0.26	-0.64	-1.87
miR-19b	-1.84	23	0.27	-0.60	-1.84
miR-99a	-1.81	24	0.29	-0.56	-1.81
miR-424	-1.75	25	0.30	-0.53	-1.75
miR-9	-1.72	26	0.31	-0.49	-1.72
miR-30a	-1.72	27	0.32	-0.46	-1.72
miR-17	-1.49	28	0.34	-0.43	-1.49
miR-30c	-1.42	29	0.35	-0.39	-1.42
miR-93	-1.3	30	0.36	-0.36	-1.30
miR-195	-1.22	31	0.37	-0.33	-1.22
miR-16	-1.18	32	0.38	-0.29	-1.18
miR-125a-5p	-1.15	33	0.40	-0.26	-1.15
miR-125b	-1.09	34	0.41	-0.23	-1.09
miR-30b	-0.99	35	0.42	-0.20	-0.99
miR-25	-0.8	36	0.43	-0.17	-0.80
miR-151-5p	-0.78	37	0.45	-0.14	-0.78
miR-7	-0.72	38	0.46	-0.11	-0.72
miR-23a	-0.67	39	0.47	-0.08	-0.67

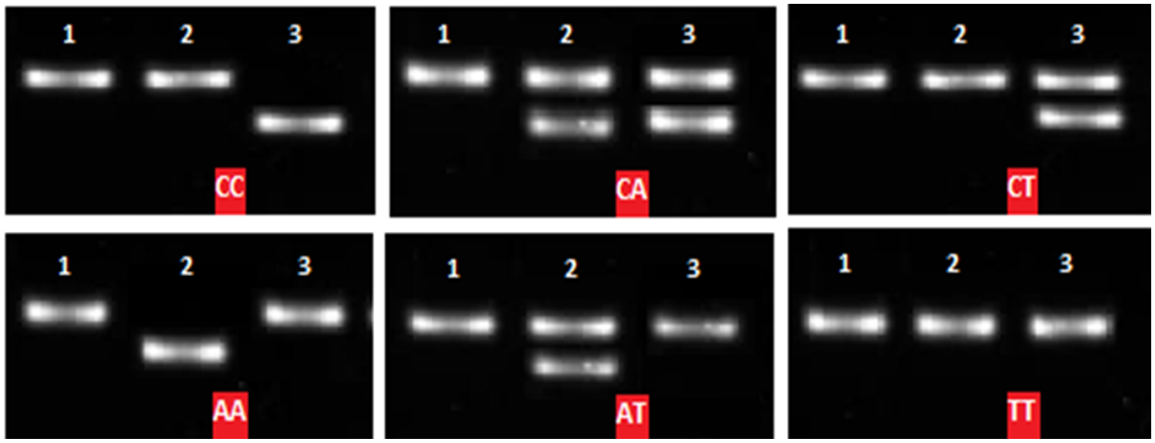
miR-21	-0.59	40	0.48	-0.05	-0.59
miR-374a	-0.56	41	0.49	-0.02	-0.56
miR-28-5p	-0.51	42	0.51	0.02	-0.51
miR-374b	-0.46	43	0.52	0.05	-0.46
miR-23b	-0.45	44	0.53	0.08	-0.45
miR-191	-0.17	45	0.54	0.11	-0.17
miR-103	0.41	46	0.55	0.14	0.41
miR-28-3p	0.47	47	0.57	0.17	0.47
let-7i	0.78	48	0.58	0.20	0.78
miR-320a	0.86	49	0.59	0.23	0.86
miR-376c	0.88	50	0.60	0.26	0.88
let-7c	0.97	51	0.62	0.29	0.97
miR-22	1.	52	0.63	0.33	1.00
miR-92a	1.12	53	0.64	0.36	1.12
miR-143	1.29	54	0.65	0.39	1.29
miR-15a	1.63	55	0.66	0.43	1.63
miR-32	1.7	56	0.68	0.46	1.70
miR-106b	1.75	57	0.69	0.49	1.75
miR-181b	1.77	58	0.70	0.53	1.77
miR-140-3p	1.79	59	0.71	0.56	1.79
miR-29b	1.84	60	0.73	0.60	1.84
miR-186	2.03	61	0.74	0.64	2.03
miR-181a	2.07	62	0.75	0.67	2.07
miR-196b	2.07	63	0.76	0.71	2.07
miR-142-5p	2.16	64	0.77	0.75	2.16
miR-128	2.18	65	0.79	0.79	2.18
miR-146a	2.19	66	0.80	0.84	2.19
miR-223	2.24	67	0.81	0.88	2.24
miR-122	2.25	68	0.82	0.93	2.25
miR-144	2.25	69	0.84	0.98	2.25
miR-185	2.36	70	0.85	1.03	2.36
let-7b	2.58	71	0.86	1.08	2.58
miR-150	2.62	72	0.87	1.14	2.62
miR-302b	2.62	73	0.88	1.20	2.62
miR-210	2.69	74	0.90	1.26	2.69
miR-141	2.74	75	0.91	1.33	2.74
let-7d	2.77	76	0.92	1.41	2.77
miR-130a	3.01	77	0.93	1.50	3.01
miR-200c	3.02	78	0.95	1.60	3.02
miR-302a	3.05	79	0.96	1.72	3.05
miR-302c	3.61	80	0.97	1.87	3.61
miR-124	3.9	81	0.98	2.09	3.90
miR-222	4.4	82	0.99	2.51	4.40

APPENDIX 6



Diagrammatic representation of GSTP1, UCP2, UCP3 and IL-6 PCR-RFLP restrictions. Lane 1 represents the PCR product. Genotypes are highlighted in red.

CRP -390 C/A/T Polymorphism



Diagrammatic representation of CRP -390 PCR-RFLP results. Each sample was digested with *Bfa I* (lane 2) and *Taq I* (lane3) for analysis of genotypes as illustrated. Lane 1 represents the PCR product. Genotypes are highlighted in red.

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