UNIVERSITY OF SOUTHAMPTON

A STUDY OF THE RELATIONSHIP BETWEEN ABCA I GENE POLYMORPHISMS, PLASMA LIPID LEVELS AND RISK OF ATHEROSCLEROSIS.

BY

NEDA MUSTAFA BOGARI

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Abstract

Accumulation of lipid-laden macrophages is a critical pathological process in atherosclerosis; a vascular anomaly which underlies coronary artery disease (CAD). Evidence suggests that this process is counteracted by efflux of cholesterol from macrophages, mediated by ATP-Binding Cassette Transporter A1 (ABCA1) which is located on the surface of macrophages. Mutations in the ABCA1 gene have been shown to cause Tangier disease, a rare Mendelian disorder manifesting extremely low plasma levels of high density lipoprotein and increased risk of coronary heart disease. To test the hypothesis that ABCA1 gene common variants are genetic factors that influence lipid levels and coronary heart disease in the general population, DNA from 1164 patients with angiographically confirmed coronary heart disease were genotyped for a number of polymorphisms in the ABCA1 gene. Genotypes were assessed by PCR to amplify DNA sequences containing the polymorphic site and restriction enzyme digestion to cleave one of the two alleles, followed by gel electrophoresis. Subsequently, genetic statistical analyses were carried out to determine whether ABCA1 genotypes and haplotypes were associated with CAD lipid levels and/or severity.

In patients not on statin treatment, HDL-cholesterol levels were associated with the I883M polymorphism, with carriers of the M allele having higher HDL-cholesterol levels than non-carriers ($p=0.001$), and there was a trend towards higher HDL-cholesterol levels in carriers of the I allele of the V825I polymorphism ($p=0.05$). In the sample as a whole and in patients on statin treatment, there was an association between HDL-cholesterol levels and the R1587K polymorphism, such that homozygotes of the K allele of the R1587K had the lowest mean level, compared with individuals of other genotype for this polymorphism ($p=0.028$ in the sample as a whole and $p=0.030$ in patients on statin treatment).

There was an association between the -407 G>C polymorphism and age of onset of symptomatic CAD, age of onset being lowest patients homozygous for the -407G allele, intermediate in heterozygotes, highest in those who were homozygous for the -407C allele ($p=0.002$). Age of onset of CAD was also found to be associated with the -565 C>T and -278 G>C polymorphisms ($p=0.01$ and $p=0.007$ respectively) which were in linkage disequilibrium with the -407 G>C polymorphism.

Functional studies showed that cells transfected with a plasmid expressing the 825I variant had a higher rate of apoAI-mediated cholesterol efflux with a lower rate of cholesterol laden foam cell formation, compared with cells transfected with a plasmid expressing the wild-type ABCA1 (825V). The studies also showed that cells expressing the 219K variant had a lower rate of cholesterol laden foam cell formation than cells expressing the ABCA1 wild-type (219R).

In conclusion, the results of this study support the underlying hypothesis that common polymorphisms in the ABCA1 gene could influence HDL-cholesterol levels and the development of atherosclerosis. Data also suggests that the ABCA1 gene polymorphisms have only moderate effects on these traits which are thought to have a multifactorial aetiology.

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List of Abbreviations

A	Adenosine
ABCA1	ATP Binding Cassette Subfamily-A Member1
ACE	Angiotensin-converting enzyme
AcLDL	Acetylated low density lipoprotein
AGEs	Advanced glycosylation end-products
AP-1	Activator protein 1
Apo A-I	Apolipoprotein A-1
Apo B	Apolipoprotein-B
Apo C	Apolipoprotein -C
Apo E	Apolipoprotein -E
APS	Ammonium Persulfate
BMI	Body mass index
C	Cytosine
C_aCl_2	Calcium chloride
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CE	Chylomicrons
CETP	Cholesteryl Esters Transfer Proteins
CHD	Coronary heart disease
CO_2	Carbon dioxide
COS-7	African Green Monkey Kidney fibroblast Cells
cSNP	Coding single nucleotide polymorphisms
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxribonucleotides (dATP, dCTP, dGTP and dTTP)
DR4	Direct repeat of nuclear hormone responsive element separated by four nucleotides
E.coli	*Escherichia coli*
e.g.	For example
EDTA	Ethylenediaminetetraacetic
FC	Free cholesterol
FCS	Fetal calf Serum
FH	Familial hypercholesterolaemia
FHA	Familial hypoalphalipoproteinemia
G	Guanine
GGPP	Geranylgeranyl-pyrophosphate
HCL	Hydrochloride
HDL-C	High-density-lipoprotein-cholesterol
HL	Hepatic lipases
HNF-3	Hepatocytes nuclear factor 3
I	Isoleucine
i.e.	That is
IDDM	Insulin-dependent diabetes mellitus
IDL	Intermediate-density-lipoprotein
IL-6	Interleukin-6
IVM	*In vitro* mutagenesis

K	Lysine
LB	Luria-bertani
LCAT	Lecithin-cholesterol acyl transferase
LD	Linkage disequilibrium
LDL-C	Low-density-lipoprotein-cholesterol
LDLr	Low-density-lipoprotein receptor
LRH-RE	Liver receptor homolog response element
LRP	LDL like receptor protein
LXR	Liver X receptor
M	Methionine
MADGE	Microplate array diagonal gel electrophoresis
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage colony-stimulating factor
$MgCl_2$	Magnesium chloride
MI	Myocardial infarction
MOD	Minimum obstruction diameter
MOL	Minimally oxidized LDL
mRNA	Messenger ribonucleic acid
MSD	Mean segment diameter
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
NBF	Nucleotide binding folds
NCEP	National Cholesterol Education Programme
NIDDM	Non-insulin dependent diabetes mellitus
NIH 3T3	Mouse embryonic fibroblast cell
NO	Nitric oxide
OD	Optical density
OxLDL	Oxidised low-density lipoprotein
PCR	Polymerase chain reaction
PG	Proteoglycans
PON	Paradoxonases
PPAR	Peroxisome proliferators activator receptor
PS	phosphatidylserine
R	Arginine
RCT	Reverse cholesterol transport
RE	Restriction enzyme
REGRESS	Regression Growth Evaluation Statin Study
RNA	Ribonucleic acid
RT	Room temperature
RXR	Retinoid X receptor
SAS	Southampton atherosclerosis study
SDS	Sodium dodecylsulfate
SGH	Southampton general hospital
SNPs	Single nucleotide polymorphisms
SR-B1	Scavenger receptor B1
SREBP	Sterol regulatory element 1- binding protein
T	Thymine
TAE	Tris-acetate
TBE	Tris-borate
TD	Tangier disease

TEMED	Tetramethylethylenediamine
TG	Triglyceride
TM	Transmembrane
TNF-alpha	Tumour necrosis factor-alpha
U.V	Ultra-violet light
USF	Upstream stimulatory factors
V	Valine
VLDL-C	Very-low-density-lipoprotein-cholesterol
VNTRs	Variable number of tandem repeats
ZNF202	Zinc finger protein 202

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Dedication

With special thanks to my mother Sumera Bogari and
my father Mustafa Bogari
and the ALL-knower God, for teaching man that which he knew not.

1 Introduction

Coronary artery disease (CAD) is the most common cause of death in industrialised countries, and its mortality and morbidity is increasing in developing countries[1]. The majority of CAD is caused by coronary atherosclerosis.

1.1 Atherosclerosis

1.1.1 Pathogenesis of atherosclerosis

Atherosclerosis is a progressive disease of large arteries and is characterised by the accumulation of lipid and fibrous materials in the vessel wall. The mechanisms underlying the development of atherosclerosis are complex and remain incompletely understood. A widely-recognised initial step of atherogenesis is infiltration of low-density-lipoprotein-cholesterol (LDL-C) from circulating blood into the sub-endothelium space of the vascular wall[2].

The intact endothelium is a selectively permeable barrier due to the presence of tight junctions between the endothelial cells. In the straight regions of the arteries where blood flow is uniform, fluid shear stress results in ellipsoidal endothelial cells which are aligned in the direction of flow. The tight junctions in these regions are particularly restrictive. In arterial bifurcations and bents however, where fluid flow is turbulent, the cells assume polygonal shape with no particular orientation. The tight junctions in these sites allow the passage of LDL and other macromolecules into the sub endothelial space[3,4,5]. Atherosclerotic lesions therefore, preferentially occur in areas of turbulent blood flow. The retention of LDL in the vascular sub endothelium is believed to be due to an interaction between apo B and the matrix proteoglycans in the vessel[6-8] . Not surprising therefore, other apo B-containing lipoproteins have the capacity of being retained in arterial vascular walls and of initiating atherosclerotic plaques. Another important physical force acting on endothelial cells is fluid shear stress, which has effects on EC morphology. This phenomenon alters the intercellular tight junctional complexes of the endothelium hence affecting its functions as a selectively permeable barrier between blood and tissues. This ultimately also contributes to the initial process of atherosclerosis.

Accumulation of LDL-C in the sub endothelial space is directly proportional to its serum level. Deposited LDL-C is first modified[9] in several ways (including oxidization, proteolysis, lipolysis and aggregation). Reactive oxygen species for LDL oxidation may come either from exposure to the oxidative wastes of vascular cells or lipoxygenase mediated oxidation. It has been shown that atherosclerosis is suppressed in mice deficient in 12/15 lipoxygenase[10]. Initial exposure of LDL to vascular cell wastes results in the so called minimally oxidized LDL (MOL) which, though lacking macrophage recognition signals, nevertheless possesses pro-inflammatory activities. When MOL is further modified by reactive oxygen species and a barrage of enzymes found in atherosclerotic lesions (myeloperoxidase, sphingomyelinase, phopholipase and other lipases), it becomes the so-called maximally (or highly) modified LDL.

The oxidized LDL-C exerts inflammatory effects by promoting recruitment of circulating monocytes to the vascular wall through stimulating endothelial cells to produce adhesion molecules (such as intercellular adhesion molecule 1, selectins, platelet endothelial cell adhesion molecule 1, and vascular adhesion molecule 1) and pro-inflammatory molecules (such as monocyte chemotactic protein 1 and macrophage colony stimulating factor) (Figure 1.1). Attracted monocytes in the lesion differentiate into macrophages and engulf a large quantity of transformed LDL through the macrophage scavenger receptors SR-A, CD36 and CD68. These LDL-loaded macrophages are termed *foam cells*, and the resulting lesion is called *fatty streak*.

Additional function of oxidized LDL includes inhibition of the production of nitric oxide (NO). This chemical mediator has multiple anti-atherogenic properties, including vasorelaxation. It has been demonstrated that mice devoid of endothelial NO synthase exhibited accelerated atherosclerosis mainly due to elevated blood pressure[11]. Apart from LDL-C other factors documented to modify inflammation, including already described shear forces, homocysteine levels, sex hormones, and infection. End products of glycation in diabetes are known to promote inflammation through interaction with endothelial receptors[12].

A fatty streak as earlier mentioned is the earliest evidence of atherosclerosis. Foam cells within the fatty streak can secrete factors (cytokines and growth factors) which promote smooth muscle cell proliferation and extra-cellular matrix deposition[13], which, together with a growing mass of extracellular lipid deposition form a more advanced lesion, the *atherosclerotic (or fibrous) plaque*. Cells, lipids, and matrix proteins are the main components of the atherosclerotic lesion, typically arranged in such a way that the central lipid core is enclosed by a "fibrous cap" made up of smooth muscle cells and extracellular matrix. Several atherosclerotic risk factors have been shown to operate at the level of fibrous plague, e.g. homocysteine and hypertension (through the action of angiotensin II) promote vascular smooth muscle cell proliferation.

Atherosclerotic plaques may lead to significant loss of vascular elasticity or might become complicated by calcification, ulceration or haemorrhage. They may grow extensively and compromise vascular lumen, or become ruptured and trigger the coagulation cascade, leading to thrombus formation and consequently an acute clinical event such as myocardial infarction. Plaque stability is an important determinant of the clinical outcome[14]. Plagues with a thin fibrous cap and high content of inflammatory cells tend to be very unstable. The finbrous cap is made up primarily by smooth muscle cells and extracellular matrix proteins such as collagens and proteoglycans. Both smooth muscle cells and extracellular matrix proteins provide the atherosclerotic plaque the strength to withstand mechanical stress. Reduced synthesis and increased degradation of extracellular matrix proteins contribute to the formation of unstable plaques. Studies have shown that interferon-gamma secreted by T-cells within the atherosclerotic plaque impairs the ability of smooth muscle cells to produce matrix proteins, while macrophage-foam cells secrete collagenases, gellatinases and other proteases that degrade extracellular matrix[13].

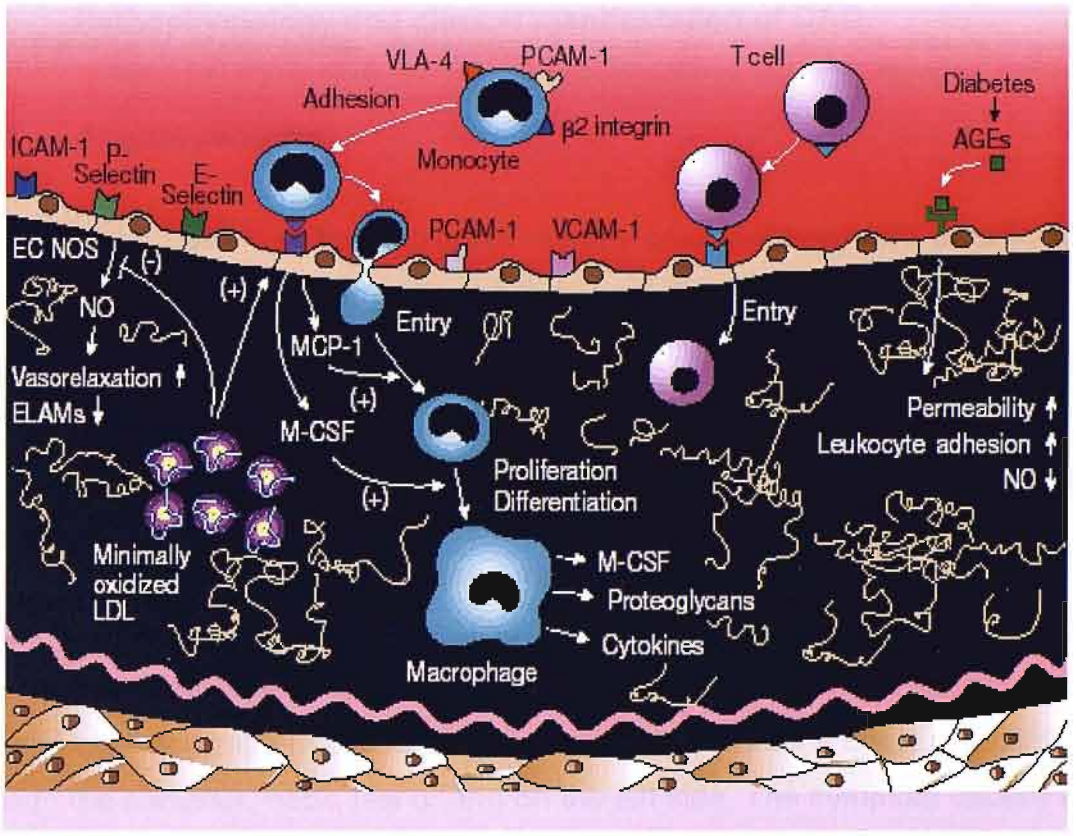

Figure 1.1 Diagrammatic representation of factors involved in atherogenesis

MOL reduces vasorelaxation by inhibiting the production of NO synthase, and promotes monocytes to adhere to, and migrate through, the endothelium by increasing the expression of adhesion molecules and chemoattractants, such as P-selectin, E-selectin and ICAM-1, by endothelial cells. The production of monocyte chemotactic protein-1 (MCP-1) and macrophage colony-stimulating factor (M-CSF) facilitates monocyte entry into the vascular wall, proliferation and differentiation into macrophages. Monocytes, as well as T-cells, carry on their surface, adhesion molecules (VLA-4, PCAM-1 and beta-2-integrin) which may interact with adhesion molecules on the endothelial cells (ICAM-1, P-selectin, E-selectin etc) and facilitate entry. Advanced glycosylation end-products (AGEs) produced in diabetes may also add to the pro-inflammatory effects of MOL reprinted from Lusis A 2000[14] with permission.

1.1.2 Pathophysiology and clinical manifestation of CAD

1.1.2.1 Angina pectoris

As reviewed by Lusis[14] in the setting of various environmental and genetic factors, atherosclerosis results in narrowing of the vascular lumen and reduced vascular elasticity, and can therefore reduce blood supply to the tissue. In the case of coronary atherosclerosis, this results in reduced blood supply to the distal myocardium. If the atherosclerotic lesion is relatively small, blood supply to the myocardium can be insufficient. An imbalance between oxygen demand and supply could occur under circumstances where there is increased myocardial oxygen demand (such as during physical exertion) or further decreased blood flow (e.g. resulting from coronary vasospasm or platelet aggregation at the site of an atherosclerotic lesion). This can cause the clinical symptom called angina which is typified by a chest pain which may radiate to the shoulder, neck, jaw or arm on the left side. The symptom usually occurs during physical exertion or emotional stress and is relieved within 5 to 10 minutes by rest or nitroglycerine. If the symptoms are relatively constant in duration, severity, frequency and precipitating factors for at least 6 weeks, the condition is called stable angina. If the symptoms occur at rest or their duration is prolonged or the symptoms can not be relieved by rest or nitroglycerine, the condition is called unstable angina.

1.1.2.2 Myocardial infarction

Ulceration or rupture of atherosclerotic plaques will expose blood to thrombogenic materials (such as tissue factor and collagen) in the atherosclerotic lesion, which can result in thrombus formation and a sudden dramatic reduction in blood supply. This can consequently lead to myocardial infarction. Atherosclerotic plaques that are prone to rupture have a thin fibrous cap and high content of inflammatory cells.

Myocardial infarction causes severe, persistent chest pain which is variously described as heavy, squeezing or crushing. It is often accompanied by anxiety, weakness, sweating, nausea and vomiting, and a sense of impending doom. Myocardial infarction may be associated with hypotension, loss of consciousness, and arrhythmias. The infarction results in electrocardiography changes and a raise of

plasma levels of enzymes released from the damaged myocardium. The condition is potentially lethal and the long-term outcome in survivors may be poor as a result of myocardial damage and associated heart failure.

1.1.3 Risk factors for CAD

Epidemiological studies have revealed a number of risk factors for CAD. These include:

1. Smoking
2. Dyslipidaemia
3. Hypertension
4. Diabetes mellitus
5. Obesity
6. Stress
7. Lack of exercise
8. Age
9. Male gender
10. Family history

1.1.3.1 Smoking

Tobacco smoke is a major risk factor for coronary artery disease. The risk of CAD is about 2-4 fold higher in smokers than in non-smokers[15]. It is estimated that smoking causes over 30,000 deaths a year from CVD in the UK[16].

It is postulated that smoking has a wide range of biological effects that increase the risk of developing atherosclerosis. These include altering lipid levels, increasing fibrinogen levels, and impairing endothelial and platelet functions. In addition, it has been proposed that smoking could augment the potential atherogenic effects of infection[17-19].

1.1.3.2 Dyslipidaemia

Numerous studies have shown that risk of CAD is positively associated with plasma levels of low-density-lipoprotein-cholesterol (LDL-C) and inversely associated with plasma levels of high-density-lipoprotein-cholesterol (HDL-C). Perhaps the most prominent study is the Framingham study which began in 1948, under the direction of the National Heart, Lung and Blood Institute, to identifying the common factors or characteristics that contribute to cardiovascular disease (CVD). The study followed CVD development over a long period of time in a large group of participants who had not yet developed overt symptoms of CVD or suffered a heart attack or stroke. It began by recruiting an Original Cohort (http://www.framinghamheartstudy.org/originalcohort.asp) of 5,209 men and women between the ages of 30 and 62 from the town of Framingham, Massachusetts. This study showed that the CAD risk decreased by half for each 20-mg/dL increase in HDL-C[20] and that reduced HDL-C levels (< 40 mg/dL) were associated with an increased risk of CAD even if the total cholesterol (Total-C) level is normal (< 200 mg/dL)[21].

The role of LDL-C in the development of atherosclerosis has been briefly discussed above. Apart from its ability to injure endothelial and smooth muscle cells,[22,23], modified LDL is chemotactic for other monocytes. It can also up-regulate the expression of genes for macrophage colony stimulating factor[24,25] and monocyte chemotactic protein-[26] by endothelial cells. Thus, it may help expand the inflammatory response by stimulating the replication of monocyte-derived macrophages and the entry of new monocytes into lesions. Studies have shown that removal and sequestration of modified LDL can have a protective effect through reducing in the inflammatory response[27,28].

The anti-atherogenic effect of HDL-C is probably mainly due to its role in reverse cholesterol transport which will be discussed in other sections later on in this thesis. In addition, HDL-C possesses several other anti-atherogenic properties including anti-inflammatory, anti-oxidant, anti-aggregatory, anti-coagulant and pro-fibrinolytic activities[29]. The antioxidant properties of HDL are due in part to serum paraoxonase,

an esterase carried on HDL that can degrade certain biologically active oxidized phospholipids[30,31].

1.1.3.3 Hypertension

The WHO defined hypertension (high blood pressure) as the presence of either systolic pressure of 140mmHg or a diastolic of above 90mmHg or both. There is an independent association between risk of CAD and blood pressure, especially diastolic blood pressure, and it has been shown that a reduction by 5 mmHg in diastolic blood pressure reduces by one fifth the risk of CAD and a reduction of 10 mmHg leads to nearly a one third reduction on CAD risk[32].

Hypertension promotes the development of atherosclerosis mainly through increased shear stress on the endothelium, leading to andothelial dysfunction. The endothelium-dependent vascular relaxation is impaired in hyperhension[33]. The abnormalities in endothelial function are responsible partly to the increase in the risk of CHD in hypertension because they promote vasoconstriction, thrombogenesis and the action of proliferative substances[34]. Angiotensin II is elevated in patients with hypertension[13] leading subsequently to the stimulation of smooth muscle cells through the activation of phospholipase C. Phospholipase C acts by increasing intracellular calcium concentrations, smooth-muscle contraction, increased protein synthesis, and smooth muscle cell hypertrophy[35]. Further, it increases smooth-muscle lipoxygenase activity, which enhances inflammation and the oxidation of LDL.

1.1.3.4 Diabetes mellitus

Diabetes mellitus is largely divided into Type I and Type II. Type I occurs in the young mainly due to immune mediated injury to the pancreas, leading to significantly reduced production of insulin by pancreatic beta-cells. Type I diabetic patients require regular injection of insulin, and therefore the condition is called insulin-dependent diabetes mellitus (IDDM). Type II diabetes is seen from the middle aged individuals as a result of relative deficiency of insulin or insulin resistance, and is called non-insulin dependent diabetes mellitus (NIDDM) as patients do not require regular insulin injection.

Diabetes substantially increases the risk of CAD. Men with Type 2 diabetes have a two to fourfold greater risk of CAD, and women with Type 2 diabetes have an even higher (three to fivefold) CAD risk[36]. The National Cholesterol Education Programme (NCEP) considers diabetes a CHD risk equivalent. It estimated that diabetes confers a 10-year CHD risk equal to that of persons with existing CHD, or >20% and recommends treating patients with type 2 diabetes the same as patients with established CHD[37]. Despite the lower level of CAD in Type 1 as compared with Type 2, adolescents with type 1 diabetes mellitus have increased levels of subclinical atherosclerosis as demonstrated by carotid intima-media thickness and by radial tonometry[38,39]. It has been shown in children that type 1 diabetes mellitus is independently linked with oxidative modification of LDL cholesterol[40].

Epidemilogical studies have shown a correlation of diabetics with hypertension, dyslipidaemia and hyperfibrinogenaemia, and it is thought that the increased cardiovascular risk in diabetic patients is, in part, a result of hypertension, dyslipidaemia, and hyperfibrinogenaemia, although the mechanisms leading to hypertension, dyslipidaemia and hyperfibrinogenaemia in diabetic patients are poorly understood. In addition, hyperglycaemia, and advanced glycation end products generated in hyperglycaemic conditions, may also have direct effects on the functions of cells in the vascular wall, e.g. by damaging the endothelium and promoting smooth muscle cell proliferation[41].

1.1.3.5 Obesity

Overweight and obesity, defined as a body mass index of 25-30 kg/m^2 and 30kg/m^2 respectively, are associated with hypertension, hyperlipidaemia and non-insulin dependent diabetes which are associated with increased risk of CAD as discussed above. A study from the World Health Organisation in 2002 suggested that raised body mass index (BMI) was responsible for over 7% of all disease burden in developed countries, and that approximately a third of CHD and ischaemic stroke and nearly 60% of hypertensive disease in developed countries was associated with BMI beyond the ideal lowest of 21 kg/m^2[16] . Recently the INTERHEART case-control study estimated that 63% of heart attacks in Western Europe and 28% of heart attacks in Central and Eastern Europe were associated with central obesity. The

study showed that the relative risk of heart attack in individuals with central obesity was twice that those without central obesity[42].

Obesity contributes to the formation of atherosclerosis mainly through the effects of adipose tissue. Adipose tissue elaborates a large number of bioactive mediators that affect the alterations in lipids, blood pressure, coagulation, fibrinolysis and inflammation, leading to endothelial dysfunction and atherosclerosis[43]. Associations between abdominal obesity, inflammation, and abnormal insulin sensitivity have recently become a subject of intense investigation[44]. Using in vivo techniques, it has been estimated that approximately 25 to 30% of total circulating interleukin-6 (IL-6) originates from subcutaneous adipose tissue in healthy adults. Tumour necrosis factor-alpha (TNF-alpha) is also expressed by adipocytes[45]. Both IL-6 and TNF-alpha are inflammatory mediates that promote atherosclerosis. In addition, there is an association between obesity and dyslipidaemia characterized by increased levels of triglycerides and total cholesterol, very low-density lipoprotein (VLDL) cholesterol, and small dense LDL particles, and reduced levels of lower high density lipoprotein (HDL) cholesterol[46]. This lipid profile is proatherogenic as discussed above.

1.1.3.6 Metabolic syndrome

Metabolic syndrome is defined as having any three or more of following traits:

- A body mass index of 30 kg/m² or greater (obesity);
- HDL cholesterol less than 1.04 mmol/L (40 mg/dL) if male or less than 1.29 mmol/L (50 mg/dL) if female;
- Triglycerides greater than or equal to 1.69 mmol/L (150 mg/dL) if fasting or greater than or equal to 4.52 mmol/L (400 mg/dL) if not fasting;
- Blood pressure greater than or equal to 130/85 mmHg or on anti-hypertension medication;
- Glucose greater than or equal to 6.1 mmol/L (110 mg/dL) if fasting or two-hour post-load glucose greater than or equal to 7.77 mmol/L (140 mg/dL).

Compared to people with no metabolic syndrome factors, the risk of CHD death is twice as high for people with one to two of the above factors and three-and-a-half

times higher for people with metabolic syndrome (three or more factors)[47]. Central to the risk of metabolic syndrome is the concept of insulin resistance. Promotion of dyslipidaemia and other metabolic abnormalities are responsible for proatherogenic environment found in the setting of insulin resistance[48]. In addition insulin resistance elevates serum glucose levels. Studies had shown progressive relationship between cardiovascular disease and glycaemia from as low a fasting glucose of 70mg/dl[49,50]. Insulin resistance is also associated with hypertension through urinary sodium retention, increased sympathetic nervous system activity,129 and stimulation of vascular smooth muscle growth.130 Insulin levels have been found to be significantly higher in adult patients with essential hypertension131– 133 and borderline hypertension134 than in normotensive control patients. In addition hyperinsulinemia is known to directly stimulate the formation of the atherogenic plaque by promoting smooth muscle proliferation, connective tissue formation, and LDL deposition in the plaque[51].

1.1.3.7 Male gender

CAD is more prevalent in males than females. Below the age of 60, men develop CAD at more than twice the rate of women[52]. However, women after menopause have comparable CAD rate to man of the same age. It is proposed that oestrogen might have an anti-atherogenic effect, and it has been shown that oestrogen can cause endothelial mediated coronary vasodilation[53]. This could be a reason that, in part, explain the lower prevalence of CAD in pre-menopausal women as compared with men.

1.1.3.8 Family history of CAD

CAD often aggregates in families[54] . A family history remains a significant risk factor of CAD after adjusting for other major risk factors such as smoking, dyslipidaemia, hypertension, diabetes and obesity[55]. Although familial clustering of CAD could be due to common environmental factors exposed by the family members, there is evidence suggesting that it is at least in part explained by genetics. The best evidence of a genetic basis has come from twins studies. For example, Marenberg *et al* found have shown significant high concordant rates of CAD death in monozygotic twins than in dizygotic twins[56]. .

1.1.4 Cholesterol metabolism

As discussed above, dyslipidaemia, particularly increased LDL-C and reduced HDL-C levels, is a major risk factor for CAD. This is particularly relevant to this thesis studying the ATP Binding Cassette Subfamily-A Member1 (ABCA1) gene. Therefore, this section will discuss the cholesterol metabolism pathways in detail.

1.1.4.1 Sources and functions of cholesterol

Understanding the details of cholesterol metabolism is necessary in order to elucidate the different factors that interact in the development of atherosclerosis and also to explore various treatment strategies. Cholesterol is a highly fat soluble multi-ring molecule containing 27 carbon atoms, available from many dietary sources and readily absorbed (together with triglycerides) in the gastrointestinal tract: this is called exogenous cholesterol. An alternative source of cholesterol in the body comes from hepatic synthesis, hence called hepatic or endogenous cholesterol. The third possible source is cholesterol secreted by the intestine[57].

Despite its untoward effects on the cardiovascular system, cholesterol remains an essential molecule in humans; virtually every cell depends on it for maintaining proper function of its membrane. In all cells, the plasma membrane is a dynamic structure that is made of two-molecule-thick lipid bilayer. In addition, cholesterol is a source for the generation of steroid hormones including oestrogen, progesterone, androgen and corticosteroids, that are enssential to normal physiology.

A number of drugs have been developed to decrease the levels of LDL-C with variable success[15,58]. Among these, the most commonly used today is the statins which function by reducing the cholesterol synthesis by the liver[59]. As reviewed by Grundy[57], it is estimated that the American diet provides, on average, 500-750mg of cholesterol per day, mainly from meat, eggs and diary products. Based on studies of primates, it is estimated that hepatic production of cholesterol in man is approximately 325 to 450 mg per day[57]. Initial steps in cholesterol synthesis include the sequential formation of acetoacetyl CoA, /3-hydroxyl-/3-methyl glutarate (HMG CoA), and mevalonic acid. The conversion of HMG CoA to mevalonic acid appears to be the rate determining reaction of cholesterol formation. Statins, which are HMG-

CoA reductase inhibitors, inhibits the conversion of HMG CoA to mevalonic acid, therefore reducing cholesterol synthesis in, and secreted by, the liver. The benefits of statin therapy has been consistently demonstrated in a number of studies, and it has been shown that statins can reduce the risk of CHD even in patients with low HDL-C who are usually at increased risk of cardiovascular complications[60].

Niacin is another drug for treating dyslipidemia. In particularly, it is often combined with other cholesterol lowering drug to treat triglycerdemia and also to raise HDL level[61,62]. Niacin is the only drug that has been demonstrated clinically to lower lipoprotein (a) level[63] . Fibrates are also used for treatment of hypertriglyceridaemia especially[64]. Several intervention studies with fibrates or nicotinic acid have demonstrated the clinical benefit of raising HDL-C levels and reducing CHD outcome[65].

1.1.4.2 Lipid transport pathways

Lipoproteins	Subclasses	Apolipoproteins contained
VLDL	6 subclasses different on sizes; from V1 for the smallest to V6 for the largest particles	apolipoprotein-B, -C and E
IDL	Only one species of IDL	apolipoprotein-B, -C and –E
LDL	3 subclasses designated L1, L2 and L3 in order of increasing size and decreasing density	apolipoprotein-B, -C and –E
HDL	HDL_3 or nascent HDL and HDL_2 (globular cholesterol-rich HDL particles)	Apolipoprotein-A-II, A-IV, and –C
Lipoprotein (a)	Unknown	Apolipoprotein-B and -(a)

Table 1.1 A summary of lipid particles

The three significant pathways that cholesterol follows in the body are represented by chylomicrons (exogenous pathway), LDL-C (endogenous pathway) and HDL-C (reverse cholesterol transport – RCT). Exogenous cholesterol can be effectively controlled by dietary means[66] and a number of drugs have been used to reduce LDL-C with some success,[58,67]Despite the fact that HDL-C level has been shown to be inversely associated with LDL-C level and susceptibility to atherosclerosis[29], HDL-C has largely remained un-targeted therapeutically, due mainly to incompleteness in our understanding of its metabolism and functions.

1.1.4.2.1 Exogenous lipid transport pathway

The exogenous lipid transport pathway is illustrated in the left hand part of Figure 1.2. After a meal, the lipid portion is emulsified by acids in the bile (mainly glycocholate) and is then acted upon by the appropriate enzymes and absorbed at the terminal ileum. The intestinal cells that absorb lipids incorporate triglyceride and cholesterol in the core of lipoprotein particles and secrete them into the blood stream as chylomicrons. Apolipoprotein B-48 (ApoB-48) is required as a co-factor in the

assembly and secretion of chylomicrons[68]. ApoB-48 is one of the two protein isoforms coded by the ApoB gene, the other isoform being ApoB-100. The ApoB-48 is synthesized exclusively by the small intestine, whereas ApoB-100 is synthesized by the liver. Both isoforms are coded by a single mRNA transcript of over 16 kb. APOB48 is generated when a stop codon (UAA) at residue 2153 is created by RNA editing[69-73]. As a result, the two isoforms share a common N-terminal sequence, but APOB48 lacks C-terminal LDL-receptor binding region.

Synthesised in muscle and fat cells and using apolipoprotein CII as co-factor, lipoprotein lipase catalyzes the hydrolysis of triglycerides in chylomicrons into fatty acids and glycerol which then serves as energy fuel or is stored as fat (ApoCIII may inhibit Lipoprotein lipase activity thereby affecting triglyceride hydrolysis)[74]. The resulting chylomicron remnant acquires apolipoprotein E which binds with LDL like receptor protein (LRP) on liver cells and mediates chylomicron remnant uptake by the liver. The cholesterol in the liver is then either re-secreted into the blood circulation in the form of very-low-density-lipoprotein-cholesterol (VLDL-C), or eliminated into the bile[74].

This concludes the fate of exogenous cholesterol which has been shown to be positively associated with atherosclerosis if the normally mild and transient postprandial hyperlipidemia becomes excessive and/or prolonged[66]. A number of factors could affect this pathway. For instance, the lipoprotein lipase gene is highly polymorphic, and mutations (e.g. Gly188Glu) can reduce the function of the enzyme[75]. Furthermore, since insulin stimulates lipoprotein lipase, the activity lipoprotein lipase may be depressed in diabetes[76,77]

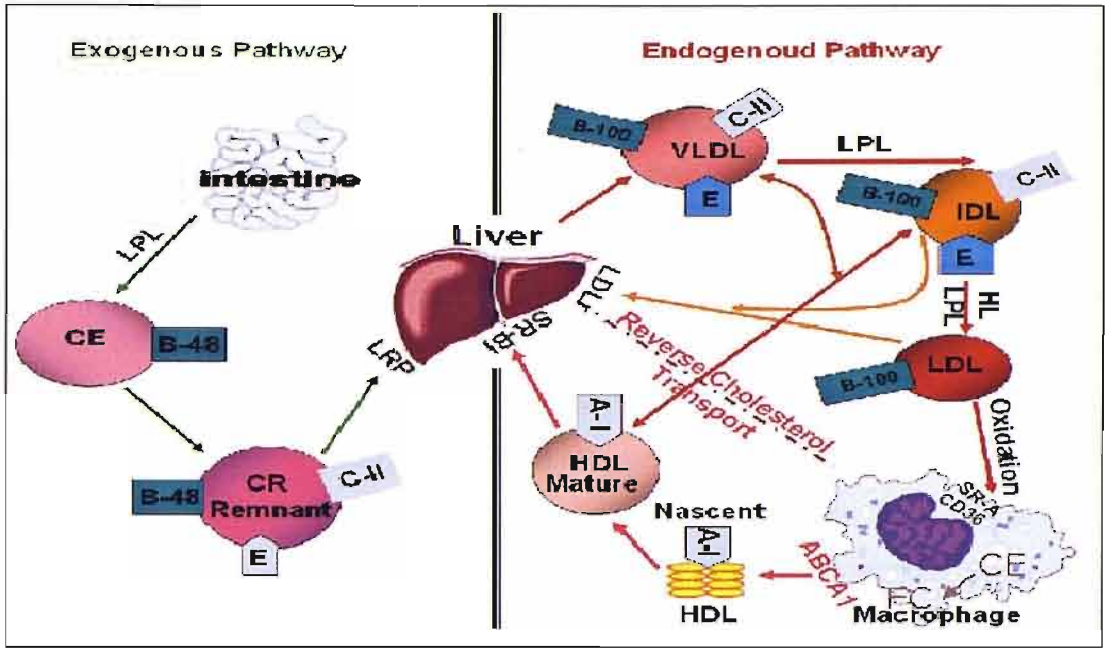

Figure 1.2 Overview of lipoprotein metabolism

The green coloured arrows represent exogenous pathway of lipid transport, and the red arrows represent transport of endogenously produced lipids, to cells. Reverse cholesterol transport is represented by pink arrows.

1.1.4.2.2 Endogenous lipid transport pathway

The endogenous lipid transport pathway is illustrated in the right hand part of figure 1.2 As well as salvaging lipids from other lipoproteins, the liver has the capacity to synthesise cholesterol and triglycerides *de novo*. Cholesterol is synthesised from acetate via an acetyl COA intermediate while triglycerides are made from fatty acids (acquired from diet or synthesised by the liver) and glycerol. Using apo B-100 as co-factor, the synthesised lipids together with phospholipids are packaged and secreted into the plasma as VLDL. It is interesting to note that apo B-48 (in the intestine) is encoded by the same gene that encodes apo B-100 (in the liver) with the important difference that in apo B-48 synthesis, the full length mRNA is modified to contain only 48% of the original transcript[74]. In the plasma, VLDL takes on additional apolipoproteins which include apo C (I, II and III) and apo E. In conditions of insulin resistance or high fat intake, there is excess triglyceride synthesis in the liver (due to the increased return of free fatty acids which cannot be incorporated into fat cells),

and this in turn drives more VLDL synthesis. Lipoprotein lipase acts on the secreted VLDL particles and hydrolyzes their triglycerides into fatty acids and glycerol, utilising apolipoprotein C-II as co-factor[74]. The resulting smaller and denser particles, intermediate-density-lipoprotein (IDL, also called VLDL remnants), either return to the liver via LDL receptor (LDLr)[74] or are acted upon by a combination of lipoprotein and hepatic lipases (HL) yielding an even denser LDL particles. Either way, Apo E plays a central role. Its main functions are in the assembly, processing, and removal of plasma lipoproteins. It has been shown that the presence of ApoE increases triglyceride content in newly formed VLDL, whereas the fast removal of chylomicrons requires the presence of a cellular pool of apoE ready to surface and bind to membrane proteoglycans and possibly to lipoprotein receptors such as the LDL receptor-related protein (LRP). Fazio *et al.* [78]recently discovered that VLDL-ApoE internalized by hepatocytes was partly protected from lysosomal degradation. It also recycled through the Golgi apparatus and clearly associated with physiologic role in lipoprotein assembly, remnant removal, and cholesterol efflux.

About 70% to 80% of available LDL is taken up by LDL receptors in the liver and other tissues while the remainder is phagocytosed by macrophages via the CD36 and SR-A receptors[68]. It is important to note that native LDL lipoproteins must first be transformed (by oxidization) before they can be taken up by macrophages[79].

Dyslipoproteinaemias involving defects in the endogenous lipid transport pathway are by far the commonest conditions associated with premature atherosclerosis. LDL has been shown by experimental and epidemiological studies to be highly associated with the risk of developing atherosclerosis[80]. In contrast, the role of VLDL still remains uncertain. However, when VLDL is enriched with cholesteryl esters, it is also atherogenic[81-83]. IDL is similarly atherogenic[84].

Elevated LDL might arise from monogenic disorders; either from defective LDL receptors (Familial Hypercholesterolaemia)[85] or its ligand (Familial Defective Apo B-100)[86], or from less severe multiple gene defects (Polygenic hypercholesterolaemia)[87].

Familial hypercholesterolaemia (FH) is an autosomal dominant disorder due to mutation in LDL receptor gene which results in a failure of LDL receptor to bind LDL

and consequently a failure of hepatic uptake and elimination of LDL. LDL particles therefore accumulate in the plasma, causing an increase in plasma cholesterol to up to 1000 mg/dl in homozygotes[88]. Although the homozygous form is very rare, the less severe heterozygous forms are relatively common with a frequency of 1/500 in the population[89].

Familial Defective Apo B-100 is also an autosomal, dominantly inherited disorder, which leads to increased serum cholesterol levels. The condition is due to substitution of glutamine for arginine at residue 3500 of apolipoprotein (apo) B-100, resulting in defective binding of apo-B containing lipoproteins with LDL receptors. The frequency to the Arg3500Gln mutation is about 3% in the population[90].

The elimination of the Apo-B containing lipoproteins requires binding with LDL receptors on the liver but a contribution is often needed from apo-E. Apo-E exists in three isoforms; Apo-E3 is the wild type while Apo-E2 (Cys158Arg) appears to be anti-atherogenic whereas Apo-E4 (Arg112Cys) is associated with accumulation of cholesterol and triglycerides and subsequent development of CAD. Carriers of Apo-E4 allele have been shown to have a significantly greater risk of CAD than subjects with Apo-E3 and Apo-E2 alleles[91]. Clinical trials have shown benefits of cholesterol reduction[92], with Apo-E4 allele conferring better response[93].

Mutations in the lipoprotein lipase gene may impair the enzyme's ability to breakdown VLDL and IDL resulting in their accumulation and consequent decrease in HDL due to CETP action and catabolism of Apo-A1 by triglycerides. At least one variant of the LPL gene Ser447→Stop (Ser447Ter) is associated with mild elevation of HDL and reduced risk of CAD[94].

1.1.4.2.3 Reverse cholesterol transport

Reverse cholesterol transport (RCT) is also illustrated in the right hand part of figure 1.2. RCT is so-called because, unlike the endogenous and exogenous pathways which provide cholesterol for body cells, it on the other hand transports cholesterol from the cells to the liver for recycling or elimination. RCT therefore unloads body cells of their cholesterol burden. This pathway is responsible for transporting to the liver, a fairly large amount of cholesterol synthesised by body cells, which has been estimated at about 10 mg per kilogram body weight daily[95].

Many cells in the body have the capacity to synthesise cholesterol for incorporation into their membranes, but apart from hepatocytes, only those cells capable of steroid genesis (e.g. gonads and adrenals) can metabolize cholesterol[57,96]. A cholesterol dependant feedback system has been described which balances a cell's cholesterol synthesis according to its cholesterol load, and for most cells, this appears efficient to prevent any overload[97]. In the liver, an additional mechanism exists; intracellular cholesterol regulates the *de novo* hepatic synthesis of cholesterol and the expression of LDL receptors on hepatocytes[98]. Macrophages which are able to mop up extra cholesterol from the body by endocytosis or phagocytosis, however, are not feedback regulated, and often ingest more than they can incorporate into their membranes. The excessively ingested cholesterol in macrophages is therefore stored as esters, and must be transported to the liver for elimination.

Generally, three mechanisms exist for cholesterol efflux from body cells to HDL particles and these serve as necessary feeders to the reverse cholesterol transport system. Firstly, cholesterol could passively diffuse between cell membranes and HDL particles in a bidirectional manner[99], a process which appears to be driven by the proportion of phospholipids in the HDL particle[100]. Secondly, although HDL utilises the scavenger receptor B1 (SR-B1) to deliver its load of cholesteryl esters to the liver and steroid-genic cells, it could also acquire unesterified cholesterol from the cells through direct interaction with this receptor[101]. This mechanism is independent of diffusion and seems to involve specific binding between the receptor and the HDL particles[102]. Because these processes are bidirectional, their effectiveness for net transfer of cholesterol to HDL is limited. Passive aqueous diffusion is of-course, concentration driven, hence could not preferentially concentrate cholesterol on HDL.

Furthermore, it has been shown that SR-B1 shows a disproportionately higher affinity to large globular cholesterol-rich-HDL than to the relatively cholesterol poor Apolipoprotein A1, emphasising the fact that *in vivo*, the preferred direction of cholesterol transfer via SR-B1 is from HDL to the cells[103].

The third and most important pathway is ABCA1 mediated cholesterol efflux (figure 1.3). Unlike passive diffusion and SR-B1 mediated efflux, it is an active, unidirectional process which requires an acceptor molecule (in the form of apo-A1, apo-AII or even apo-E) and causes a net transfer of cholesterol and phospholipids[3,104]. It has been demonstrated that apart from apoA-I, cholesterol efflux mediated by other apolipoproteins such as apoA-II, apoA-IV, apoC-I, and apoC-III are all decreased in Tangier cells[105]. It is suggested that powered by ATP hydrolysis, the transporter, lying within the plane of the membrane, spins along its long axis exposing molecules bound to the intra-cytoplasmic side to the outer leaflet[106,107]. By flipping phospholipid molecules from inner to outer leaflets of the plasma membrane, ABCA1 causes change in membrane morphology which is believed to facilitate acceptor molecule binding. Apo-A1 binds preferentially to a region of the membrane disturbed by ABCA1[108,109]. Lateral mobility helps to bring these components together, in fact a direct cross linking between apo-A1 and the extracellular loops of ABCA1 has been observed[104]. It has been recently shown that apo-A1 binds to ABCA1 using its terminal helix10, but while this binding is necessary and cholesterol efflux is directly proportional to the apo-A1 binding, it is not by itself sufficient to bring about efflux[110,111]. The end point is that the acceptor molecule ApoAI and other nasent HDL acquire cholesterol from ABCA1. The mechanism of this transfer is still unclear.

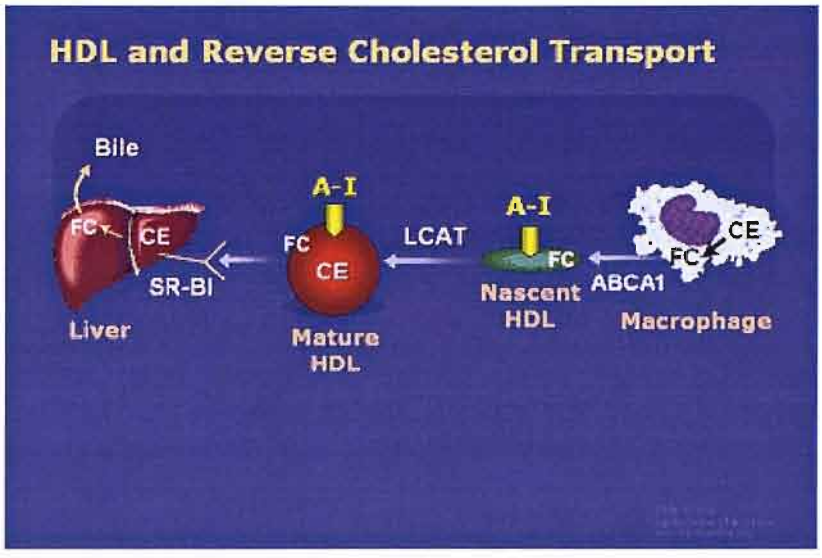

Figure 1.3 Demonstrating ABCA1 mediated cholesterol efflux

The figure demonstrates ABCA1 mediated cholesterol efflux from macrophages into lipid poor apolipoprotein A-1 to form mature HDL which is in turn taken up by hepatic SR-B1 receptors reprinted from lipid online, 2007[112], with permission.

Over the last three decades, the concept of reverse cholesterol transport gave emphasis to the central role of the transfer of HDL from periphery cells to the liver for ultimate excretion in the bile[113]. However, recent studies have shown that at least in mice, the ABCA1 pathway in peripheral macrophages contributes very little to plasma HDL level[114], and that the liver is the most important source of HDL production[115]. Timmins *et al.* showed that liver-specific ABCA1-KO mice (generated by crossing ABCA1 floxed mice in which ABCA1 exons 45-46 flanked by lox P sites and mice expressing Cre recombinase under the control of liver specific albumin promoter, resulting in exons 45-46 being deleted by liver-specific Cre recombinase and consequently an inactive ABCA1 protein) have HDL-C levels that are profoundly reduced by 80%[115], indicating that the liver is quantitatively the most important site for lipidation of lipid-poor apoA and ApoA-I carrying nascent HDL via ABCA1. In addition to the liver, the intestine appears to be also a major source of HDL production, as intestine-specific ABCA1-KO mice (generated by crossing ABCA1 floxed mice in which ABCA1 exons 44-45 are flanked by lox P sites and mice expressing Cre recombinase under the control of the intestinal epithelium-specific *Villin* promoter) show a decrease of 20% in HDL-C production[116]. Thus, the two organs that synthesise apoA-I, the liver and intestine, are also primarily responsible for lipidating

newly secreted lipid-poor apoA-I HDL via ABCA1-mediated lipid efflux. In contrast, it is estimated that macrophages only contribute 1% to the total HDL-C in the plasma. It should be emphasised, however, that although cholesterol efflux from macrophages accounts for only minimal part of the total plasma HDL, it is very important with regard to the development of atherosclerosis[117-119].

As discussed above, elevated LDL levels and reduced HDL levels are associated with increased risk of CAD. Like hypercholesterolaemia, HDL deficiency can be caused by genetic factors.

Tangier disease is a rare autosomal co-dominant disorder, with only 100 patients described worldwide. The disease is due to mutation of the ABCA1 gene. It is typified by low or absent HDL and a complex of conditions associated with the accumulation of cholesterol esters in the tissues and the macrophages. They have a six-fold increased risk of atherosclerosis after the age of 30. Unlike 'normals', Tangier disease sufferers can develop CAD without high levels of LDL[120-122].

Polymorphisms of apo-A1/CIII/AIV and hepatic lipase genes have been associated with low serum HDL concentration in the general population[123-125]. In addition, polymorphisms of the LCAT (Gly230Arg)[126] and the CETP genes (Taq1-B)[127] have been shown to be associated with increased atherogenic potential, probably through reduction of the HDL level and enhancement of CETP activity with consequent production of smaller dense LDL particles. CETP is a hydrophobic glycoprotein that is produced largely in the liver and subsequently secreted in to the plasma where it circulates bound mainly to HDL[128]. CETP enhances the transfer of cholesteryl esters from antiatherogenic HDLs to proatherogenic apolipoprotein B (apoB) – containing lipoproteins, including VLDLs, VLDL remnants, IDLs, and LDLs. A deficiency of CETP is associated with increased HDL levels and decreased LDL levels, a scenario typically antiatherogenic[129]. de Grooth *et al.* have highlighted the effect of CETP deficiency causing high plasma HDL-c levels in Asian populations[130].

1.1.4.3 Lipoproteins sub-species

Each of the major classes of lipoproteins can be further subdivided into subgroups. HDL, for instance, is a heterogeneous group of lipid transporting particles with differing size and density and differing, even opposing, functions. A study of

lipoprotein subclasses is therefore pivotal to understanding their relative contributions to atherosclerotic risk.

The protein components of lipoproteins, most of which function as membrane enzymes or receptor ligands, are called apolipoproteins. Plasma lipoproteins can be broadly classified on the basis of their apolipoprotein content. This classification has some significance in that a particular lipoprotein's association to the risk of atherosclerosis could be related to its apolipoprotein content. Apolipoprotein-B containing lipoproteins are believed to be atherogenic and these include VLDL, LDL, IDL and apolipoprotein (a)[131,132]. On the other hand, HDL, which contains Apolipoprotein-A and is devoid of apolipoprotein-B, has been epidemiologically shown to be anti-atherogenic[133]. This classification is however too simplistic to be of any practical use. For one, there are many more apolipoproteins than just A and B, and even these are further subdivided into different types. Furthermore, all lipoprotein particles contain more than a single apolipoprotein and these too may very well contribute to its characteristics. Lastly, the fact that some HDL subtypes may in fact predispose to atherosclerosis makes the picture all the more confusing. Because of all these drawbacks it becomes necessary to classify lipoproteins in a more detailed manner, and this could be achieved by several means such as nuclear magnetic resonance, which is the commonest method employed.

1.1.4.3.1 VLDL

VLDL contains apolipoprotein-B, -C and E. It is composed of 6 subclasses of different sizes; numbered from V1 for the smallest to V6 for the largest particles[134]. It consists mainly of triglycerides but also receives from HDL, cholesteryl esters transferred by Cholesteryl Ester Transfer Protein (CETP)[128,135].

1.1.4.3.2 IDL

There exists only one species of IDL. This arises as a VLDL remnant following the hydrolysis of the triglycerides contained in VLDL's core. IDL, as VLDL, is an apolipoprotein-B, -C and -E containing lipoprotein. Increased IDL level is associated with increased prevalence of CAD[57].

1.1.4.3.3 LDL

LDL is yet another apolipoprotein-B, -C and -E containing lipoprotein and is found to have 3 subclasses designated L1, L2 and L3 in order of increasing size and decreasing density[134]. Increased LDL level is particularly associated with the increased risk of atherosclerosis[67]. Among the LDL sub-species, small dense LDL particles are more atherogenic[68].

1.1.4.3.4 HDL

Unlike the other lipoproteins, HDL is an Apo-A containing lipoprotein; made mainly of ApoA-I (70 to 80% of its protein content), which appears to be crucial in HDL synthesis, but also contains Apo A-II and A-IV. All are synthesised in the liver and/or intestines and are released in the plasma as phospholipid-apolipoprotein complexes. These complexes acquire apolipoprotein C from other lipoproteins and supposedly coalesce to form discoid shaped cholesterol-poor nascent HDL particles called HDL_3. Other apolipoproteins on HDL_3 particles are apo E and apo J[29]. This form of HDL has a particularly high affinity for ABCA1 and the SR-B1 receptor in cell membranes, and is the major acceptor of cholesterol effluxed by ABCA1[136]. Lecithin-Cholesterol Acyl Transferase (LCAT), also synthesised by the liver, mediates the transfer of linoleic acid from lecithin to cholesterol on HDL particles using apo A-I as a co-factor. The cholesteryl ester so formed then migrates to the core making room for additional free cholesterol uptake. By increasing the capacity of HDL to receive more cholesterol, the action of LCAT converts HDL_3 to HDL_2 which are globular cholesterol-rich HDL particles.

The cholesterol in HDL_2 is eliminated from the plasma via one of three ways (figure 1.4). Firstly, the whole of the particle may be taken up by the hepatocytes, possibly with the help of proteoglycans, apo E and other factors[137]. The internalised HDL is then degraded and its cholesterol excreted in bile. The second pathway involves hepatic lipase and SR-B1 receptor. Here there is a selective uptake of cholesteryl esters from HDL and the cholesterol deficient particle is not degraded[137] but rather released for reuse. A third mechanism involves the regeneration of HDL_3 from HDL_2 by CETP and lipases[68]. CETP exchanges the cholesteryl esters in HDL_2 with triglycerides from the core of apo B containing lipoproteins while hepatic and lipoprotein lipases together and/or individually, hydrolyze triglycerides in the HDL

core regenerating HDL_3. The apo B containing lipoprotein remnants are then taken up by the liver via LDL receptor and LDL related protein.

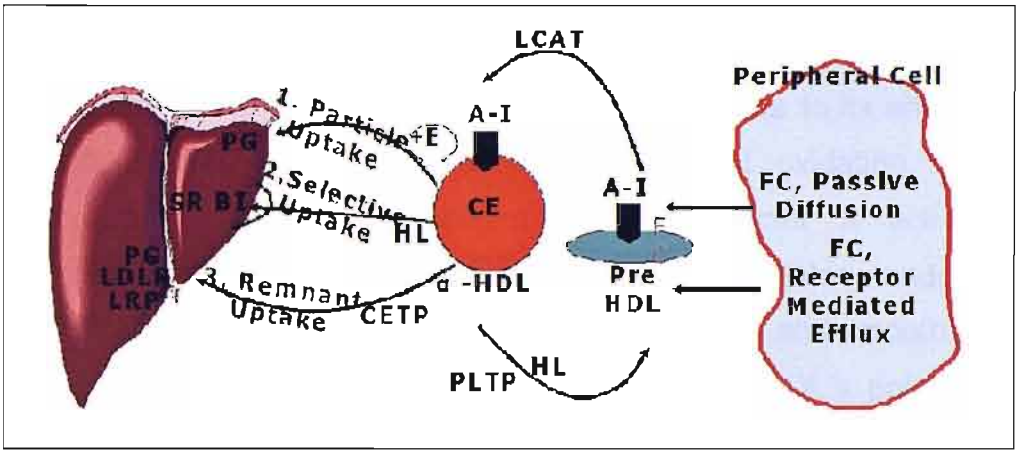

Figure 1.4 Pathways for the delivery of cholesterol to the liver by HDL particles

Free cholesterol (FC), CE (cholesteryl ester), Hepatic lipase (HL), Proteoglycans (PG), LDL receptor (LDLR), LDL receptor-related protein (LRP) adapted from Tall *at al.*2000[137].

Nuclear magnetic resonance subdivides HDL into 5 major subclasses from H1 to H5[134]. While H3, H4 and H5, which roughly correspond to HDL_2, are anti-atherogenic, H1 and H2 approximating to HDL_3 might in fact promote atherogenicity[68]. Epidemiological evidence suggests that low levels of HDL2 are more predictive of cardiovascular disease than low levels of HDL3[138,139]. Studies had shown that lipid-lowering drugs, exercise, and dietary interventions all appear to influence the levels of HDL2 than HDL3[139]. Colvin *et al.* have also shown that HDL3 was considered to accept cholesterol avidly which, on conversion to HDL2, can adequately execute the function of reverse cholesterol transport[140]. It is therefore, not strictly correct to regard HDL as being generally "good", but for the sake of simplicity, the term "HDL" in this report would always refer to the major anti-atherogenic portion of HDL unless otherwise specified. HDL_2 and HDL_3 can be separated by isopycnic ultracentrifugation but when whole HDL is subjected to gel electrophoresis, its different particles separate into three subgroups according to their mobility. Particles with mobility similar to albumin are said to have α-mobility, while those with less or more have pre-α and pre-β mobility, respectively. This separation has proven to be of

practical importance since HDL cholesterol and much of apo A-I are contained mainly in the alpha fraction and it is this fraction that is particularly anti-atherogenic[141].

As reviewed in Nofer *et al.*, apart from involvement in reverse cholesterol transport, HDL has several other anti-inflammatory, anti-aggregatory, anti-oxidant, anti-coagulant and pro-fibrinolytic activities which might contribute to its anti-atherogenic effect[29]. *In vitro* studies have shown that HDL inhibits LDL oxidation, LDL induced endothelial dysfunction/apoptosis, platelet activation, factor X activation, and monocyte chemotaxis and adhesion[29,142]. It also stimulates endothelial cell proliferation, prostacyclin and natriuretic peptide C synthesis, and smooth muscle cell proliferation. There however exists concern that some of HDL's potentially useful actions *in vitro* might not translate into a favourable outcome *in vivo*[29,142]. This might be either due to other compounding factors such as co-morbidity or the presence of additional risk factors or to the fact that the physiological actions of HDL are due to its various components (apolipoproteins, lipid, enzymes) which are distributed amongst the various HDL subclasses in a non-uniform manner. This matter is studied further in the next section.

1.1.4.3.5 Lipoprotein (a)

This is a poorly understood apo B containing lipoprotein. It also contains apolipoprotein (a) and is independently associated with increased risk of atherosclerosis[143]. This atherogenic activity may be partly explained by the fact that apolipoprotein (a), being homologous to plasminogen, inhibits the conversion of plasmin from plasminogen and also stimulates the synthesis of the plasminogen activator inhibitor-I[144], thereby decreasing fibrinolysis and promoting thrombosis on the surface of endothelial cells. Lipoprotein (a) also contains cholesteryl ester which contributes to its atherogenicity. Because lipoprotein (a) could not be measured by the conventional nuclear magnetic resonance, immunochemical techniques are used instead. Like LDL, lipoprotein (a) is also oxidized and HDL might inhibit this process, but Statins, which increase plasma HDL, have not been shown to decrease lipoprotein (a). Currently, niacin is the only drug that is used clinically to lower lipoprotein (a) level[63].

1.1.5 ATP binding cassette subfamily-A Member1 (ABCA1)

1.1.5.1 The ATP binding cassette superfamily

The ATP binding cassette superfamily is a group of about 50 genes including more than 100 ubiquitous transmembrane transport proteins[145,146] each having at least one highly conserved ATP binding cassette. Members of the family were first described in bacteria[147], but are also found in eukaryotic cells, and are involved in the transport, across intracellular and extracellular membranes, of a wide range of substrates which include peptides, amino acids, ions, lipids, metabolic products, sugars, drugs and complete proteins[148]. Members of the ABC transporter superfamily in mammals are grouped phylogenetically into 7 subfamilies (from ABCA to ABCG). The members in each subfamily are identified by numbers; thus the 12 members of the first subfamily are designated ABCA1 to ABCA10 then ABCA12 and ABCA13[147]; surprisingly no member is labelled ABCA11.

In general, special domains of the ABC transporters have been conserved through evolution; indeed the classification of a transmembrane transporter as an ABC transporter is based on its possessing the ATP binding cassette. All members bind ATP and energize their functions from ATP hydrolysis[149], but not all ATP binding pumps are classified as ABC transporters. Classification is based on the utilization of the so called nucleotide binding folds (NBF) as their ATP binding domains. Intra-cytoplasmic NBFs are typically made of two constant regions called walker A and walker B motifs which are separated by a short segment of an amino acids chain. Just upstream of the walker B motif a third element called signature or C motif is found (figure 1.5). The transmembrane region is made of an alpha-helix structure that traverses the membrane 6 to 11 times[150,151]. Being the portion that is in contact with both sides of the membrane, the transmembrane segment is responsible for a transporter's substrate specificity[151]. A functional unit of an ABC transporter consists of 2 identical halves each with a transmembrane and an NBF motif. Although some ABC transporters are produced as half transporters, they are assembled as dimers on the membrane.

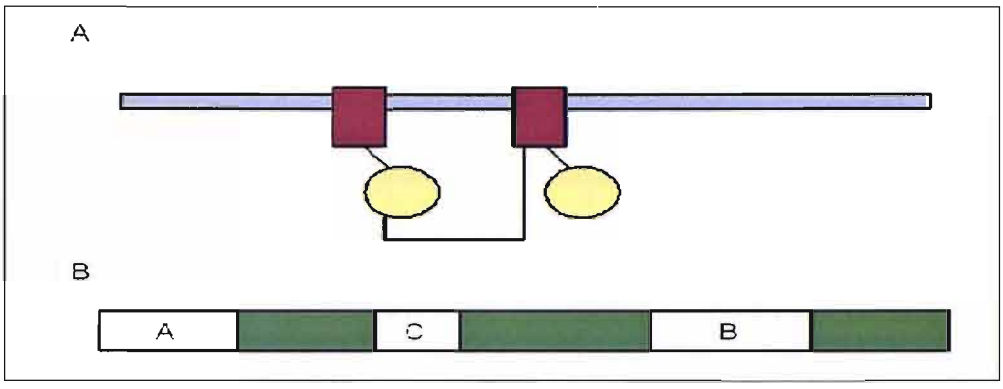

Figure 1.5 A model of representative ABC protein

The figure shows the general structure of ABC. A diagram of the structure of a representative ABC protein is shown with a lipid bilayer in blue, the transmembrane (TM) domains in plum, and the nucleotide binding folds (NBF) in yelow. Although the most common arrangement is a full transporter with motifs arranged N-TM-NBF-TM-NBF-C, as shown, NBF-TM-NBF-TM, TM-NBF, and NBF-TM arrangements are also found. B. The NBF of an ABC gene contains the Walker A and B motifs found in all ATP-binding proteins. In addition, a signature or C motif is also present. Above the diagram are the most common amino acids found in these motifs; subfamilies often contain characteristic residues in these and other regions adapted from Dean *at al.* 2001[147].

ATP binding cassette transporters have gained great clinical importance for their discovered roles in multiple cancer drug resistance (ABCC1 or MRP1), cystic fibrosis (ABCC7 or CFTR), Adrenoleuko-dystrophy (ABCD1), and progressive familial intrahepatic cholestases (ABCB11)[147] to mention but a few. More recently, and most relevant to cardiovascular research, is the characterisation of the defect in Tangier Disease and familial hypoalphalipoproteinemia (FHA) to ABCA1, a transporter exclusive to multicellular eukaryotic cells. As discussed above, ABCA1 plays a crucial role in the reverse cholesterol transport by pumping free cholesterol (and phospholipids) to lipid poor Apo-A1 particles which later develop into mature HDL particles and transport the cholesterol back to the liver for elimination.

1.1.5.2 Structure of ABCA1 gene and protein

The exact structure of ABCA1 protein is unknown but from the general structure of ABC transporters and the electron microscopy of closely related members, a model predicted for the structure of ABCA1 is shown below (figure 1.6).

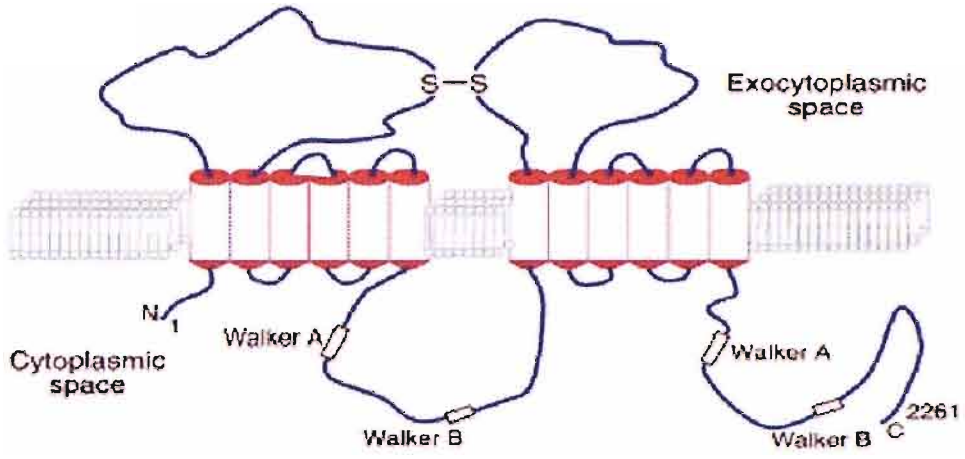

Figure 1.6 A schematic model of ABCA1 based on topologies of its closest relative ABCA4

Shown in the figure is the general structure of ABC consisting of two sets of six transmembrane domains in red, two large extracellular loops in blue joined by a disulfide bridge, and two paired cytoplasmic Walker and ABC signature motifs reprinted from Tall *at al.* 2002[136], with permission.

The human ABCA1 gene (GDB ID 305294, RefSeq genomic assemblies NC_000009.10 NT_008470.18) is a large gene spanning 149 kb of DNA on chromosome 9q31.1[152]. It contains 50 exons separated by 49 introns, which in turn contain 62 repetitive Alu sequences. It is located on GDB 6276759, coordinate 0.7534 and ordinals 112.5 Est MB on chromosome 9 integrated map. The mouse ortholog of the gene is located on chromosome 4 at 23.1 centimorgans from the centromere[146,153,154].

The ABCA1 protein has a molecular weight 250 KDa and has 2261 amino acids with a 45 amino acids long signal peptide at the N terminal which might be necessary in directing the protein into plasma membranes[154]. Although an earlier account of the ABCA1 gene sequence predicted a transcript of 2201 amino acids and absence of any leader sequence[148], this was most likely a truncated version and did not represent a full size ABCA1 gene.

1.1.5.3 Expression and regulation of ABCA1 gene

ABCA1 is expressed in almost all tissues of the body, and its expression in adults is most abundant in macrophages[148], the liver, lungs and adrenal glands. ABCA1 protein can be found in resident macrophages of various organs and tissues, in foam

cells in atherosclerotic lesions, in tubules and ducts of the kidneys and Leydig cells in testes and also in the epithelial cells of small intestine[148,155]. In these and other cells, the protein could be found associated with the cell membrane, Golgi network or prenuclear vesicles[156].

In intrauterine life, the gene is expressed abundantly in the placenta and fetal liver[148]. The gene's expression in fetal tissues is believed to support ABCA1's role. ABCA1 was reported to be involved in phosphatidylserine (PS) translocation, an event that dictates the rate of apoptosis[157-159]. Both PS translocation and apoptosis possessed determinant roles in placental development[160,161]. This regulation is necessary for a good outcome in pregnancy[162].

Just as the expression of ABCA1 gene is different in different tissues, so is its regulation and physiologic role. Although the transporter has the same primary function in each cell, an intra-membrane pump that actively transports cholesterol and phospholipids across the membrane, the physiologic aim may significantly differ. While it unloads cholesterol from macrophages for subsequent elimination, it may, in the liver serve to channel bile into the gall bladder and yet in the intestinal cells help to regulate cholesterol absorption[163].

While ABCA1 activity is sterol-dependent in fibroblasts and macrophages, hepatocytes seem to have different control signals[148,164]. ApoA-I however, promotes cholesterol efflux from all of these cells. Furthermore, evidence suggests that not only are the ABCA1 regulating signals different in hepatocytes, but even the gene's promoter in liver cells is unique. A study compared two transgenic mice; one containing the complete human ABCA1 transgene (BAC1, 255kb) and the other a shorter 171kb BAC2 transgene lacking the ABCA1 promoter and exon1. While all cells express human ABCA1 in the BAC1 transgene, mainly the liver and testis expressed the protein in BAC2 transgene suggesting that these cells possess an alternative ABCA1 promoter and exon1 (termed exon1A)[165]. Thus, the ABCA1 gene has two promoters one upstream of exon1 and the other upstream of exon1a (figure 1.7)[166]. It has been shown that in macrophages, ABCA1 expression is driven only by the promoter upstream of exon 1, whereas in liver cells, ABCA1 expression is controlled also by the internal promoter located in intron 1 upstream of exon1a[167].

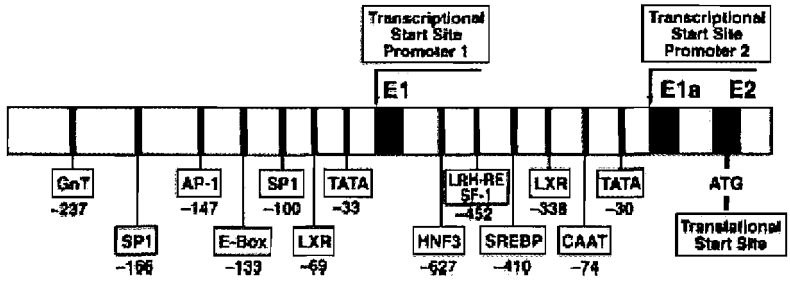

Figure 1.7 Schematic model of a segment of the ABCA1 gene

The figure shows the two promoters and their relation to the exons E1, E1a and E2. Sequences with potential regulatory roles have also been shown. AP-1,activator protein 1; HNF-3, hepatocyte nuclear factor 3; LRH-RE, liver receptor homolog response element; SREBP, sterol regulatory element 1-binding protein reprinted from Santamarina-Fojo *at al.* 2001[166], with permission.

The ABCA1 gene promoters contain a number of cis-elements important in the regulation of ABCA1 transcription (figure 1.7). The DR4 (direct repeat of nuclear hormone responsive element separated by four nucleotides) regulatory motif is responsible for LXR-mediated sterol activation of the gene (see below) while the SREBP site (sterol response element binding protein) may enable free cholesterol to directly regulate ABCA1 expression. It has been noted that SREBP in sterol depleted conditions, also induces the transcription of LDL receptor genes[168]. Several helix-loop-helix transcription factors (upstream stimulatory factors, USF1, USF2, and Fra2) are able to bind to the E-box motif and repress ABCA1 expression, and mutation or deletion of this motif results in augmentation of the gene's expression[166]. Another inhibitory motif for the ABCA1 gene is the GnT repeat which binds zinc finger protein 202 (ZNF202) and brings about repression[169].

1.1.5.4 Cholesterol-mediated regulation

In macrophages, the low basal ABCA1 expression (assessed by mRNA and cholesterol efflux) can be greatly augmented by treating them with AcLDL which induces cholesterol loading and reversed when the same cells are treated with HDL_3 which de-load them of cholesterol[148,164].

Two members of the nuclear hormone receptor superfamily called the liver X receptor (LXR) and the retinoid X receptor (RXR) have been known to affect many aspects of cholesterol metabolism[170,171]. Oxysterols, such as 22-OH cholesterol, 27-OH

cholesterol, or 24(S), 25-epoxycholesterol, are considered the natural ligands of LXR. For RXR the natural ligand is 9-cis-retinoic acid (CRA)[171,172]. LXR and RXR readily form heterodimers but while geranylgeranyl-pyrophosphate (GGPP), an inhibitor of LXR/RXR binding to DR4, blocks response of ABCA1 to cholesterol it fails to affect stimulation by 9-cis-retinoic acid. It therefore follows that GGPP inhibits the interaction by binding to the LXR portion only. Since cholesterol *per se* has failed to activate LXR, it stands to reason that, for cholesterol to affect the ABCA1 expression via LXR/RXR transcription factors, it necessarily needs to be converted into oxysterols such as hydroxycholesterols. Interestingly, mutation of the DR4 motif impairs the response of ABCA1 not only to oxysterols and CRA, but also to cholesterol[172]. The DR4 site is found on a number of other genes in agreement with the widespread role of LXR/RXR in regulating cholesterol metabolism.

The LXR signalling pathway appears to promote lipid metabolism and increase cholesterol efflux (figure 1.8 & 1.9) while, at the same time, repressing the expression of a number of inflammatory genes[173] (figure 1.10). It is in a sense anti-inflammatory in action and this becomes all the more important in view of the fact that atherosclerosis is not only a disorder of lipid metabolism, but is now considered as a chronic inflammatory disease as well[174].

The anti-inflammatory actions of LXR/RXR together with their multitude of gene targets with a significant role in cholesterol metabolism make these transcription factors particularly attractive as therapeutic possibilities against CAD.

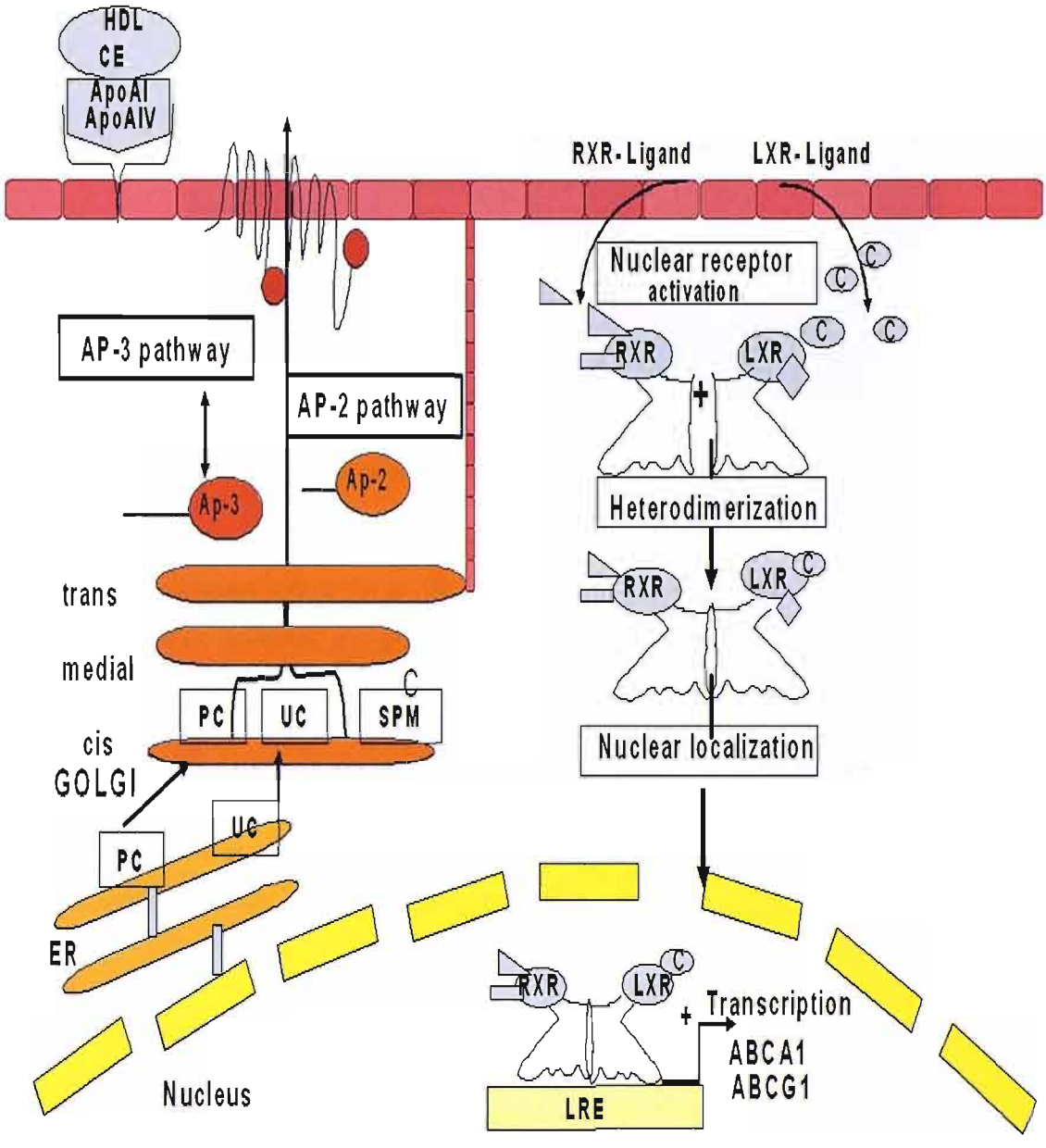

Figure 1.8 Graphical representation of intracellular lipid trafficking

Trafficking is from uptake of sterols leading to LXR/RXR dimerization which stimulates synthesis and secretion of ABCA1 pump followed by its subsequent localization to the plasma membrane. Role of ABCG1 in lipid trafficking is also shown adapted from Schmitz *at al.* 2001[175].

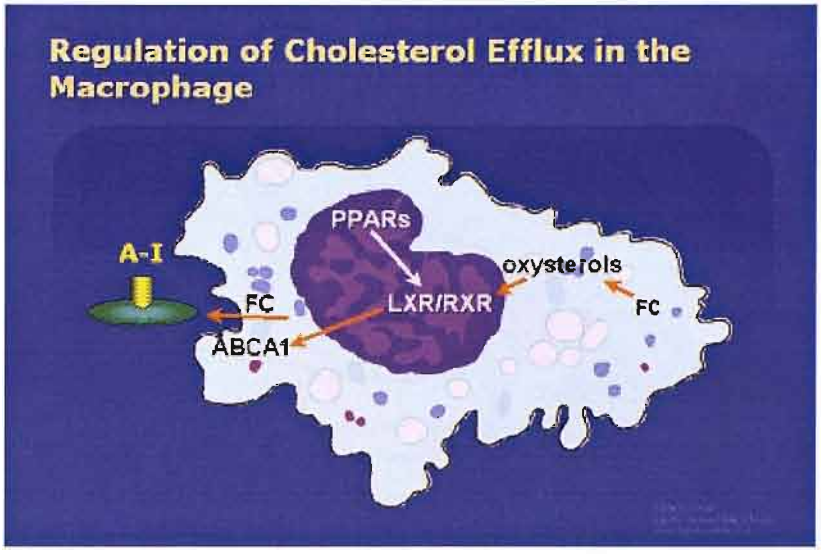

Figure 1.9 Illustrating the central role of LXR/RXR in the expression ABCA1 gene in macropages

The cellular transporter ABCA1 is an important protein that transports overload free cholesterol (FC) out of the cell to an acceptor, lipid-poor apo A-I. ABCA1 gene transcription is regulated by the nuclear receptor heterodimer liver X receptor/retinoid X receptor (LXR/RXR). Cellular oxysterols appear to be one of the endogenous ligands for LXR and promote the transcription of ABCA1. Oxysterols are generated from free cholesterol. Most likely, this characterizes a homeostatic mechanism by which macrophages clear themselves of overload free cholesterol. LXR gene transcription is also influenced by both peroxisome proliferator-activated receptor α (PPAR-α) and PPAR-γ reprinted from libid online, 2007[176], with permission.

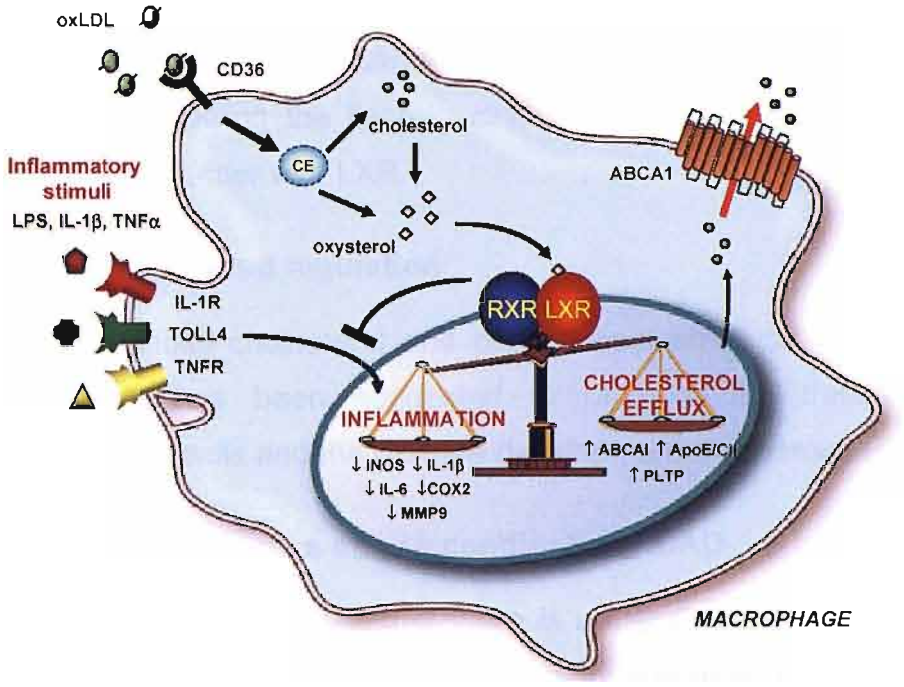

Figure 1.10 Effect of LXR/RXR on the expression of genes involved in cholesterol efflux and inflammation

When LXR/RXR heterodimer binds its oxysterol ligands, it stimulates the expression of ABCA1 and other target genes which play important roles in lipid metabolism. Concurrently, it also modulates the repression of inflammatory genes like the interleukin-1 & 6 and COX2 genes[170], with permission.

1.1.5.5 Cyclic AMP-mediated regulation

The precise motif through which cAMP might regulate ABCA1 transcription has not been identified as yet, nevertheless several studies have provided insight into the regulatory role of cAMP on ABCA1 transcription. Incubation of cells with 8-Br-cAMP resulted in a 10 fold increase in ABCA1 mRNA and protein levels, enhancement in cell surface expression of ABCA1, enhancement of Apo-A1 binding to surface ABCA1 and Apo-A1 mediated cholesterol efflux. These are all reversed within 6 hrs of discontinuing cAMP[177,178].

1.1.5.6 PPAR-mediated mediated regulation

It is evident that agonists of transcription factors peroxisome proliferators activator receptor (PPAR) alpha and gamma, can mediate ABCA1 regulation via the stimulation of LXR[179]. Chinneti *et al.* demonstrated that PPAR agonists induced increases in levels of mRNA of ABCA1 and LXRα in normal subjects but not in TD

patients[180]. Although no PPAR response element has yet been described on the ABCA1 promoter, PPARgamma knockout mice show significantly less enhancement of cholesterol efflux following the ligand activation of PPARgamma[181]. Like RXR, PPAR also forms heterodimer with LXR.

1.1.5.7 Cytokine-mediated regulation

Interferon gamma inhibits cholesterol and phospholipid efflux by reducing ABCA1 expression and this has been suggested to promote the transformation of macrophages into foam cells and the ultimate development of atherosclerosis[166,182].

1.1.5.8 The ABCA1 gene as a strong candidate for CAD

Epidemiological studies have shown that there is a significantly raised risk of CAD amongst twins and first degree relatives of a person who died of the disease. Twin studies have shown higher concordance of death from CAD among monozygotic twins than dizygotic twins[56,183,184]. These studies suggest a genetic component in the aetiology of CAD. Premature CAD is a clinical manifestation of several monogenic disorders such as familial hypercholesterolaemia, familial Defective Apo B-100, and Tangier disease. However, these Mendelian disorders are quite rare, accounting for a small portion of CAD cases in the general population. For most CAD cases, the disease is caused by the interaction of many environmental factors (such as smoking and high fat diet) and genetic factors.

A number of genes have been reported to be associated with CAD. Examples include angiotensin-converting enzyme (ACE), apolipoproteins, paraoxonases (PON) and factor V. However, these genes probably do not account for all the variance of CAD susceptibility in the population and therefore, it is likely that additional susceptibility gene may exist that have not yet been identified.

The discovery that Tangier disease, in which premature CAD is one of the major characteristics, is caused by mutation in the ABCA1 gene has indicated the ABCA1 gene to be a strong candidate for CAD susceptibility in the general population. Although mutations that have a dramatic effect on ABCA1 function and cause Tangier disease are rare (hence there have been only about 100 reported Tangier disease cases), it is possible that common, but milder, variants of this gene may

contribute to genetic susceptibility to CAD in the general population. There is emerging evidence to support this view, which is discussed in more detail in the following section.

1.1.5.9 Polymorphisms in ABCA1 gene

In any given population, there may be more than one allele for a particular gene. This characteristic is called mutation or polymorphism. The term "mutation" is usually dedicated to rare genetic changes that severely alter the function of the protein or the encoded enzyme. "Polymorphism" on the other hand, denotes common variations with the rarer allele having a frequency of greater than 1% in the population. Polymorphisms usually are either functionally neutral or have only a small functional effect on the function or expression of the encoded protein or enzyme. It has been estimated that polymorphisms occur on average once every 200-1000 base-pairs[185]. They are thought to be maintained within a given population by two main factors; mutation and natural selection[186].

There are several types of polymorphisms: single nucleotide polymorphisms (SNPs), variable number of tandem repeats (VNTRs), insertion/deletions of one nucleotide or more, and gene copy number variation. SNPs are the commonest type of polymorphism accounting for about 90% of sequence variations in humans[187] and arise due to difference in single base pair. VNTRs on the other hand, denote a variability in the number of repeats of repetitive DNA sequences between individuals[188]. There are two major classes of repetitive DNA namely the interspersed repeats and satellite DNA[189]. The term satellite DNA refers to sequences that comprise head-to-tail tandem repeats which are highly polymorphic and result probably from unequal DNA exchange during cell division. Although the majority of DNA polymorphisms are functionally inert, some of them could nevertheless affect gene regulation or the function of its product, and may contribute to genetic susceptibility of human disease.

There are a number of SNPs at the ABCA1 locus. Prior to the start of this project, several polymorphisms in the ABCA1 gene had been studied in relation to lipid levels and cardiovascular traits by several groups. These studies are summarised below.

1.1.5.9.1 A study by Clee *et al.*

Clee *et al.*[190] studied 10 coding region polymorphisms (R219K, V399A, V771M, T774P, K776N, V825I, I883M, E1172D, R1587K, S1731C) (figure 1.11) in 804 Dutch men with proven CAD who participated in the Regression Growth Evaluation Statin Study (REGRESS). The minor allele frequencies of these polymorphisms were 0.46 (R219K), 0.16 (V399A), 0.58 (V771M), 0.06 (T774P), 0.05 (K776N), 0.16 (V825I), 0.234 (I883M), 0.53 (E1172D), 0.44 (R1587K) and 0.0 (S1731C) in this sample.

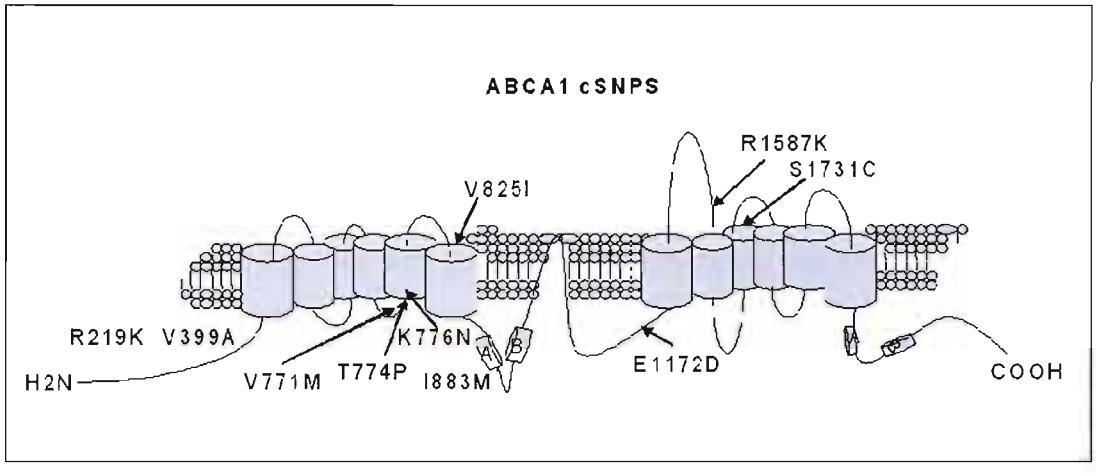

Figure 1.11 ABCA1 coding polymorphism adapted from Clee *et al.*2001 [190]

In this study, coronary atherosclerosis was measured by computer-assisted quantitative coronary angiography[191] to determine the mean segment diameter (MSD) along the vessel which reflects diffuse atherosclerotic differences, and the minimum obstruction diameter (MOD) at an obstructed site which reflects focal atherosclerotic changes. The study showed that the R219K polymorphism was associated with CAD. Carriers of the 219K allele were found to have a reduced severity of CAD and fewer coronary events. Carriers of the 219K allele had decreased triglyceride levels and a trend toward increased HDL-C levels. Carriers of the V825I cSNP (n=103 VI + 4 II) had no obvious differences in lipid levels or baseline MSD or MOD, but they did have a significantly increased number of events during the trial (44% versus 33% in noncarriers, P=0.0008; odds ratio, 2.31; 95% CI, 1.41 to 3.83).

The I883M polymorphism was found to be associated with increased progression of coronary progression and cardiovascular events. No association with lipid levels was found.

The R1587K polymorphism was found to be associated with HDL-C levels, carriers of the K allele having decreased HDL-C. No significant differences in CAD or events were found in carriers compared with noncarriers.

The V771M polymorphism was found to be associated with the degree of coronary atherosclerosis, carriers of the 771M allele had decreased focal atherosclerosis and a trend toward less diffuse atherosclerosis. Carriers and non-carriers had no difference in lipid levels.

The other polymorphisms studied did not show any association with CAD or lipid levels.

As described in later chapters, the study of this project did not show an association of R219K with HDL-C or CAD, which is in contrast to the findings of Clee *et al.*, but is consistent with several other studies which also failed to show such an association[192-197].

1.1.5.9.2 A study by Wang *et al.*

Wang *et al.*[198] studied the R219K, V825I, I883M, and R1587K polymorphisms (which were referred to R/K159, V/I765, I/M823, and R/K1527 in their paper, different numbering from those in the Clee *et al.*[190] paper being due to a different translation start site being used as a reference).

They found in a group of 245 Inuit individuals that mean HDL cholesterol concentration was significantly higher in M823/M823 homozygotes than in subjects with the I823/I823 and M823/I823 genotypes.

The other polymorphisms studied were not found to be associated with HDL cholesterol concentration in this study.

As described in later chapters, in concordance to the Study of Wang *et al.*, our study also demonstrated that the carriers of the 883M allele had higher HDL-cholesterol levels than non-carriers.

1.1.5.9.3 A study by Lutucuta *et al.*

Lutucuta *et al.*[199] studied the C-477T polymorphism in the promoter of the ABCA1 gene in a group of 372 patients with angiographically defined CAD.

The researchers showed that the TT genotype was associated with severity of coronary atherosclerosis. The study also showed that heterozygotes had a moderate reduction in plasma HDL-C and apoA1 levels.

The C-477T polymorphism has more recently been referred to the C-565T or C-564T polymorphism following the finding that the transcription start site of the ABCA1 gene is different from what was thought previously.

As described in later chapters, our study found a correlation between age of onset of symptomatic and C-477T even after adjustment of confounding variables.

1.1.6 Hypotheses

Whilst rare, loss-of-function mutations in the ABCA1 gene severely affect HDL-cholesterol synthesis and cause the Tangier's disease, certain common polymorphisms in the ABCA1 gene can have a moderate effect on HDL-cholesterol levels and can have an influence on susceptibility to and/or severity of coronary heart disease.

1.1.7 Aims and Objectives

1.1.7.1 Aims

1. One of the aims of this study was to investigate whether the reported association of the R219K, V771M, V825I, I883M, R1587K and C-565T polymorphisms with CAD and/or lipid levels could be detected in a second sample, as replication is important in genetic association studies.

2. Another aim of this study was to test whether other polymorphisms in the proximal promoter were also associated with CAD and/or lipid levels, as these polymorphisms are located in the regulatory region and therefore might have a functional effect on ABCA1 expression but had not been studied previously. These polymorphisms were G-407C, C-302T, G-278C, G-99C, and C-14T (figure 1.12).

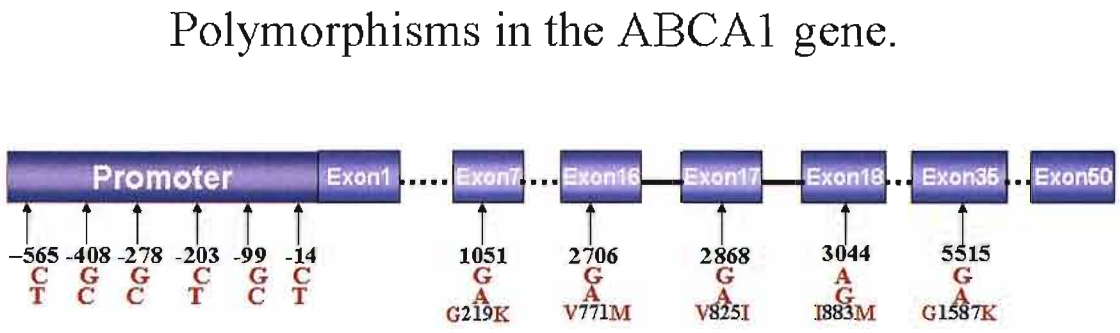

Figure 1.12 Diagram showing regulatory and coding SNPs to be studied in this project

3. It was unknown whether and which of the common polymorphisms mentioned above had any functional effect. Therefore, another aim of this study was to investigate whether any of the above polymorphisms had a functional effect.

1.1.7.2 Objectives

1. To genotype the Southampton Atherosclerosis Study (SAS) sample for 6 common polymorphisms in the proximal promoter and 5 common polymorphisms in the coding region of the ABCA1 gene, and carry out statistically analyses to investigate whether the polymorphisms are associated with HDL-cholesterol levels, total cholesterol levels, triglyceride levels, age of onset of symptomatic CAD.

2. To perform functional studies of the common polymorphisms in the proximal promoter of the ABCA1 gene, by the luciferase reporter assays.

3. To create ABCA1 expression plasmids for different alleles of the common polymorphisms in the coding region, transfect the expression plasmids into cultured cells, and perform cholesterol efflux assays and measure cholesterol accumulation.

2 Methods

2.1 Subjects

The subjects of this study were 1164 Caucasian patients with coronary atherosclerosis defined as >50% stenosis in at least one coronary artery, who were recruited from the Southampton General Hospital and participated in the Southampton Atherosclerosis Study (SAS). Demographic and clinical data for these subjects had been collected previously and stored anonymously in a computer database. These parameters included age, sex, occupation, smoking habit, weight, height, statin treatment for hyperlipidemia, plasma total cholesterol levels, plasma HDL-cholesterol levels, plasma triglyceride levels, hypertension, diabetes mellitus, and family history of coronary artery disease (CAD) in first-degree relatives. Data for total cholesterol, HDL-cholesterol, and triglyceride were available for 1100, 630, and 1017 patients, respectively. They were measured by the clinical chemistry department of the Southampton General Hospital. The demographic and clinical features of the subjects are summarised in table 2.1.

Genomic DNA was extracted from the blood samples of the study subjects by using a salt precipitation method, diluted to a concentration of 7 ng/μl and stored at 4°C. This was done by other members of the group, prior to this study.

	Mean (SD), or %
Age (years)	63.3 (10.0)
Male	70.5%
BMI (kg/m2)	27.5 (4.2)
Smoking	74.6%
Treatment of hyperlipidemia	56.5%
Cholesterol (mmol/l)	5.1 (1.0)
Triglyceride (mmol/l)	1.9 (1.2)
HDL (mmol/l)	1.3 (0.5)
Hypertension	45.0%
Diabetes	13.3%
Family history of CAD	48.5%

Table 2.1 Characteristics of subjects

2.2 PCR

2.2.1 Introduction

Since the mid 1980s, the discovery of the polymerase chain reaction (PCR) has injected an unprecedented impetus into molecular genetics with tremendous benefits to basic and clinical research. PCR is a relatively simple and versatile method for the *in vitro* amplification of defined target DNA sequences. The target DNA or cDNA template is amplified many thousand or million-fold in a cell free system[200].

The process requires the definition of a specified target sequence from a heterogeneous and homogeneous DNA source (which may be total genome DNA or complex cDNA population). Two primers, 15 – 25 nucleotides long, are designed whose sequences are complementary to nucleotide sequences in either strand of the target DNA.

The constituents of the reaction, which include a heat stable DNA polymerase (e.g. *Taq* DNA obtained from a hot-spring living micro-organism, *Thermus aquaticus*) together with the four DNA precursors (dATP, dCTP, dGTP and dTTP); primers;

reaction buffer; magnesium and optional additives, are mixed in to a PCR tube and placed in the theromcycler which will take the reaction through many PCR cycles. The series of temperature and time adjustments is termed as one cycle of amplification.

The first step in the cycle denatures the DNA by heating it to 93^0C – 95^0C for 15 seconds to 2 minutes. In this process, the two intertwined strands of DNA separate, producing the necessary single-stranded DNA templates. The next step reduces the temperature to between 50^0C and 70^0C, allowing the oligonucleotide to anneal with the separated target DNA strands serving as primers for DNA synthesis. This step lasts approximately 30-60 seconds. Finally, the synthesis of new DNA strands begins when the reaction temperature is elevated to the optimum for the DNA polymerase. For most thermostable DNA polymerases the temperature is about 72^0C, and this results in the extension of the primer by the polymerase. This step completes one cycle and the next step commences with the return to 93^0C – 95^0C for denaturation. The cycle repeats giving amplification of DNA increase in copies of the target DNA as a chain reaction is established; with newly synthesised strands acting as templates for the synthesis of newer ones. After 20-40 cycles, the amplified nucleic acid may then be analysed for size, quantity, sequence or utilized in further experimental procedures, e.g., cloning.

The following measures can be used to increase specificity and sensitivity, if necessary.: "Nested Primers" (where products of one PCR are used as the DNA source of the second while primers of the reactions are chosen in such a way that the primers of the second are nested within the sequence of the first), "Hot-Start PCR" (which involves physical separation of some of the PCR components until heat denaturation of the DNA occurs to minimise non-specific primer binding when all reactants were mixed ab-initio) and "Touch-Down PCR" (whereby the stringency of hybridization is kept initially high by raising the annealing temperature above *Tm* of the expected duplex to discourage non-specific products and then gradually reducing the temperature to below *Tm*).

The main advantages of PCR are its simplicity, rapidity, sensitivity and ability to amplify sequences of DNA from unusual sources (e.g. fossils and formalin fixed tissues). Major limitations of the PCR includes the need to design primers (which

require prior sequence information of part of the target DNA – normally obtained by cell based cloning), limitation of target DNA size (normally within the range of 0.1 to 5 kb compared with 2 Mb when using cell based cloning) and quantity of product DNA and infidelity of DNA replication (because *Taq* DNA polymerase lacks 3'-5' proofreading capability). Techniques of PCR have however, been refined and most of these shortcomings overcome. It is now possible to amplify DNA up to 42 kb or even more and, by using alternate DNA polymerase, the fidelity of DNA replication in PCR has been greatly improved.

One of the most significant constraints of conventional PCR is the limitation of the size of amplified segments. This arose due to the fact that *Taq I* polymerase enzyme lacks a proofreading ability and, as a result, has a high rate of incorporation of mismatched bases during strand synthesis. The longer the strand length, the higher the number of errors and this causes the enzyme to dissociate and strand synthesis to halt. By adding a proofreading enzyme (e.g. *Pwo* DNA polymerase) to the PCR reaction, the fidelity of DNA synthesis improves and so does the length of the synthesized strand.

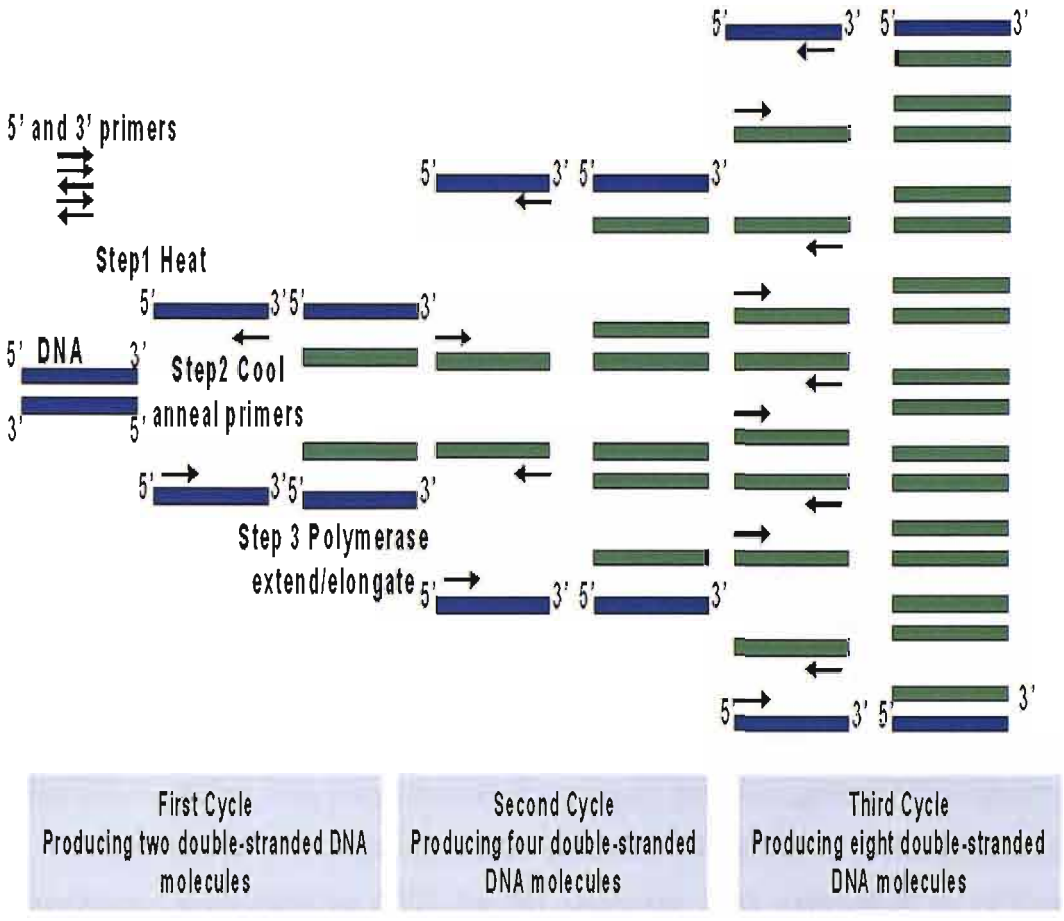

Figure 2.1 Polymerase chain reaction

2.2.2 Conditions in PCRs performed in this study for genotyping

In this study, to genotype the polymorphisms in the promoter region, a long range PCR was first carried out to amplify a 1451 bp fragment of ABCA1 promoter. The long range PCR products were then used as templates in secondary PCRs for genotyping the polymorphisms. For genotyping the coding region polymorphisms, genomic DNA was used as PCR templates. Long range PCR followed by secondary PCR was performed so as to conserve DNA samples and to increase the intensity of the PCR bands on the gel (described below).

For the majority of SNPs in this study, for following genotyping method was used which included 6 steps: (1) design PCR primers using a computer program primer

(http://cedar.genetics.soton.ac.uk/public_html/primer1.html) (2) perform PCR amplification of a DNA fragment containing the polymorphic site, (3) incubate the PCR products with a restriction enzyme that cleaves one of the two alleles, (4) carry

out gel electrophoresis to separate the cut and uncut PCR products, (5) stain and then scan of the gel to visualize the cut and uncut PCR products, and (6) determine the genotypes according to the cut and uncut patterns.

For the R219K SNP, the Tratra-primer ARMS-PCR was used, due to difficulty in optimizing the genotyping assay using the above method.

A total of 11 polymorphisms in the ABCA1 gene have been genotyped in the SAS cohort by our group. Of these, 6 (table 2.2) were genotyped by the author and the others were typed by other members of the research group.

The long range PCR to amplify the 1451 bp promoter sequence was achieved using the primers: 5'-GGG GTA CCG TGC AGC TGA ATG TCT GCA T -3' (Forward) and 5'-CCC TCG AGC TCA CTC TCG CTC GCA ATT A -3' (Reverse). The PCR contained 10 pmol of each PCR primer, PCR (Promega) buffer, 0.2 mM of dNTP, 1.5 mM of MgCl2, 1 unit of Taq polymerase (Promega), and 0.5 unit of PWO polymerase, and 20 ng of genomic DNA. The reaction included 34 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for two minutes.

The PCR primers used to genotype the 6 polymorphisms are described in table 2.2. Each PCR was carried out with 10 pmol of each primer, PCR buffer, 0.2mM of dNTP, appropriate concentration of MgCl2 as described in table 2.2, 0.5 unit of Taq polymerase(home made), and 1 µl of long range PCR products (or 20 ng of genomic DNA in the case of coding region polymorphism). The PCR conditions are described in table 2.2.

	Primers	Mg_2Cl	PCR condition
-407 G>C	5'-AAAAAAATTGCGGACTGCA-3' 5'-GCCCGCCAGCCTAAGAC-3'	2.91 mM	34 cycles of 95 °C, 30 seconds 62 °C, 30 seconds 72 °C, 30 seconds
-302 C>T	5'-GCTTCCCGCGCGTCTTCAGC-3' 5'-CCGCCCACGACACATCTAGGG-3'	1.5 mM	30 cycles of 95 °C, 30 seconds 60 °C, 30 seconds 72 °C, 30 seconds
-278 G>C	5'-TGCCGGGAACGTGGACTAGAGA-3' 5'-AGGGTCCGCGGTCTGGGT-3'	3 mM	34 cycles of 95 °C, 30 seconds 60 °C, 30 seconds 72 °C, 30 seconds
-99 G>C	5'-CTACATAAACAGAGGCCGGGTA-3' 5'-CTCACTCTCGCTCGCAATTAC-3'	1.2 mM	34 cycles of 95 °C, 30 seconds 62 °C, 30 seconds 72 °C, 30 seconds
-14 C>T	5'-CCACGTGCTTTCTGCTGAGTGA-3' 5'-TCGCAATTACGGGGTTTTTGTCG-3'	1.2 mM	34 cycles of 95 °C, 30 seconds 62 °C, 30 seconds 72 °C, 30 seconds
R219K	5'-CTGAGCTTTGTGGCCTACCCA-3' 5'-AGCCTCACATTCCGAAAGCATT-3'	2 mM	34 cycles of 95 °C, 30 seconds 60 °C, 30 seconds 72 °C, 30 seconds

Table 2.2 PCR primers, Mg2Cl concentrations and PCR conditions

2.3 Restriction enzyme digestion

2.3.1 Introduction

Hamilton Smith discovered the first restriction enzyme in 1970 and since then more than 3000 restriction enzymes have been reported[201]. The restriction enzyme is a bacterial enzyme used to recognise rather short sequences of double-stranded DNA as targets for cleavage. Different restriction enzymes have different target sequences. These sequences are about four to six nucleotides long. Following treatment with a suitable enzyme, the DNA molecule is cleaved into distinct fragments of varying lengths. These fragments can then be separated on the basis of their sizes by gel electrophoresis.

2.3.2 Restriction enzyme method

In this study, for the purpose of genotyping, 10 µl of the PCR product was digested with 3 units (0.3 µl) of an appropriate restriction enzyme (table 2.3) for 16 hours (overnight), followed by electrophoresis on a 12% acrylamide MADGE gel (described below). The cut and uncut PCR products were visualised by staining the gel with Vistra green (described below) and scanning the gel using a Fluoroimager (described below).

SNP	Restriction enzyme	Buffer	Temp.	Digests
-407 G>C	Pst I	NEBuffer 3	37°C	G allele: 136 bp, C allele: 19 bp, 117bp
-302 C>T	Pvu II	NEBuffer 2	37°C	C allele: 139 bp, T allele: 20 bp, 119bp
-278 G>C	RSA 1	NEBuffer 1	37°C	G allele: 110 bp, C allele: 18 bp, 92bp
-99 G>C	Taq^{α} I	NEBuffer Taq^{α} I	60°C	G allele: 142 bp, C allele: 22 bp, 120bp
-14 C>T	Pvu II	NEBuffer 2	37°C	C allele: 158 bp, T allele: 23 bp, 135bp
R219K	BstN1	NEBuffer 2	60°C	R allele: 144 bp, K allele: 20 bp, 124bp

NEBuffer 1: 10 mM Bis Tris Propane-HCL, 10 mM $MgCl_2$, 1 mM dithiothreitol

NEBuffer 2: 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol

NEBuffer 3: 100 mM NaCl, 50mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT

NEBuffer 4: 50 mM potassium acetate, 20mM Tris acetate, 10 mM magnesium acetate, 1 mM DTT

NEBuffer Taq^{α} I: 100 m M NaCl, 10 mM Tris-HCl, 10 mM MgCl,

BSA used with BstN1, PstI, Taq^{a} I, and Eco0109.

Table 2.3 Restriction enzymes used

2.4 Gel electrophoresis

The gel for electrophoresis is composed of either agarose or polyacrylamide, each of which has attributes suitable to particular tasks.

2.4.1 Agarose

Agarose is a linear polysaccharide extracted from seaweed. Typically, it is used in concentrations of 0.5 to 2%, and forms a gel matrix by stabilising hydrogen bonds when heated and allowed to cool. The higher the agarose concentration, the "stiffer" the gel. Agarose gel is extremely easy to prepare and its density or porosity depends on its concentration. Normally only a single-component agarose would be needed with no polymerisation catalysts required. The agarose powder is simply mixed with a buffer solution, which is then melted by heating and then allowed to cool. It is also non-toxic, but the resolving power is much less than that of polyacrylamide gels. Agarose gels however, have a large range of separation and by varying its concentration, fragments of DNA from about 200 to 50,000 bp can be separated using standard electrophoretic techniques. Figure 2.2 shows an example agarose gel.

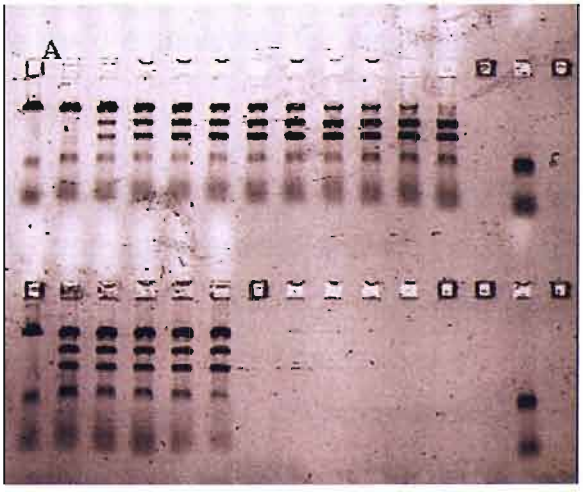

Figure 2.2 Agarose gel

2.4.2 Polyacrylamide

Polyacrylamide is a mixture of acrylamide and bisacrylamide. The ratio determines the site of the holes in the gel. To make the gel, Ammonium Persulfate (APS) and Tetramethylethylenediamine (TEMED) are added. APS provides source of superoxide ions to solidify the gel whilst TEMED acts as the catalyst for the APS reaction.

Polyacrylamide resulted from a cross-linked polymer of acrylamide formed by the vinyl polymerization crosslinked by a co-monomer simply called the cross-linker. The length of the polymer chains is dictated by the concentration of acrylamide used (usually between 3.5 and 20%) while the gel pore size is determined by concentration of both acrylamide and cross-linker. Polyacrylamide gels are comparatively more difficult to prepare than agarose gels; they must be poured between glass plates to expel oxygen which might inhibit the polymerisation process.

Polyacrylamide gels have a somewhat small range of separation, but very high resolving power. In the case of DNA, polyacrylamide is used for separating fragments of less than about 500 bp. However, under appropriate conditions, fragments of DNA differing in length by a single base pair are easily resolved. In contrast to agarose, polyacrylamide gels are used extensively for separating and characterizing mixtures of proteins.

There are generally two electrophoretic buffers in common usage both with almost identical resolving power and are prepared and stored as concentrate solutions. The most commonly used buffer for agarose gel electrophoresis contains ethylenediaminetetraacetic (EDTA) and Tris-acetate (TAE) which is cheaper and in which double stranded DNA normally migrates faster. The second buffer, which contains EDTA and Tris-borate (TBE) at a concentration of about 50 mM, although more expensive, has a higher buffering capacity than TAE.

2.4.3 Microplate-array diagonal gel electrophoresis (MADGE)

For high throughput researches in biomedical sciences and clinical lab, the conventional electrophoresis is not suitable. Electrophoresis in microplate format provides a convenient way for high throughput studies which allows for either liquid-phase reactions or solid-phase separations.

A robust system called microplate array diagonal gel electrophoresis, MADGE, has been designed to provide a means of multiple sample analysis (figure 2.3). It has the advantage of high resolution polyacrylamide gel electrophoresis and is compact and scalable[202].

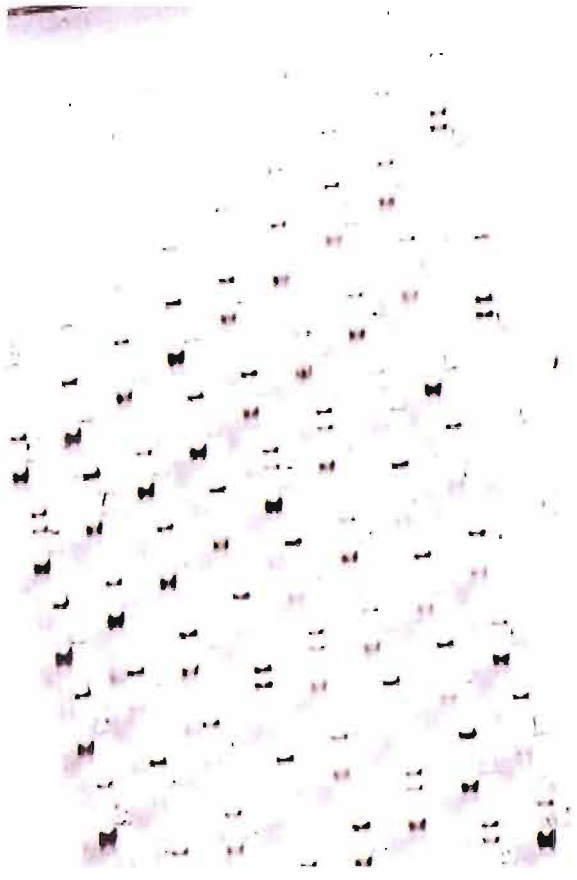

Figure 2.3 Polyacrylamide gel

2.4.4 Intercalating dyes

The DNA on a gel moves depending on its size and the percentage agarose or acrylamide of the gel. To visualise the DNA, it can be stained with a low concentration of fluorescent intercalating dye such as ethidium bromide or vistra green.

2.4.4.1 Ethidium bromide

Ethidium bromide (3,8 diamino-5-ethyl-6phenyl phenanthridinium bromide, dromalic) is the most commonly used nucleic acid stain for gel electrophoresis. It can stain single and double stranded nucleic acids (DNA and RNA). However, it has a greater affinity to double stranded nucleic acids than to single stranded nucleic acids. Typically, 10µl of ethidium bromide is added to 100ml of 10xTAE buffer.

2.4.4.2 Vistra green

Vistra green is a sensitive gel stain for double stranded DNA and is ten times more sensitive than ethidium bromide on a UV transilluminator. The high signal to noise ratio permits detection of less than 20 pg per band in both agarose and acrylamide gels. It works with denaturing and non-denaturing, agarose and polyacrylamide gels. Vistra green is non-fluorescent until it binds to DNA. The dye is thought to bind non-covalently to the DNA backbone.

2.5 Scanning

The Fluorimager 595 (Amersham Biosciences) system rapidly scans and analyses fluorescent labelled samples in both agarose and acrylamide gels. Scanned using a photomultiplier tube voltage of 800 V having filters for the absorption and emission spectra of the appropriate dye, the samples are scanned with a 25 nW 488 nm argon-ion laser beam. The flurorophores being excited to a higher energy state emit energy as light with longer wavelength 488 nm and 514 nm laser diode as they return to the ground state. This energy is captured by a fibre optic collector and amplified with a photomultiplier tube and then converted to a digital image. These images can be viewed and analysed for fragment size and band quantification with the help of ImageQuant and Fragment Analysis software. The program helps to enhance the

visualisation of very weak or very intense samples with a grey-scale adjustment feature. The Fluorimager system therefore, provides up to one hundred times the sensitivity of UV illuminated ethidium bromide visualisation in agarose and acrylamide gels.

2.6 Statistical analysis

The X^2 statistical method was used to test whether the genotype frequencies significantly deviated from Hardy-Weinberg equilibrium using 1 degree of freedom (df)[203].

To test association of ABCA1 polymorphism with lipid (HDL-cholesterol, total cholesterol and triglyceride) level, a linear regression analysis was carried out with lipid level entered as the dependent variable and the polymorphism (coded as 0 for major allele homozygotes, 1 for heterozygotes, and 2 for minor allele homozygotes) entered as the independent variable. The analysis was carried out first in the sample as a whole, and then in two subgroups stratified according to whether the patient was on statin treatment to lower cholesterol level.

To test association of ABCA1 polymorphism with age of onset of symptomatic CAD, a linear regression analysis was carried out, with age of onset entered as the dependent variable and the polymorphism (coded as 0 for major allele homozygotes, 1 for heterozygotes, and 2 for minor allele homozygotes) entered as the independent variable.

The above analyses were carried out using the SAS statistical software (SAS Institute Inc, NC).

The pair-wise linkage disequilibrium coefficient (D') and haplotype frequencies were determined using the program Thesias which employed an Expectation Maximization (SEM) algorithm. This program was also used to determine whether there was significant association of haplotypes with HDL-cholesterol, total cholesterol, triglyceride, and age of onset of symptomatic CAD.

2.6.1 *In vitro* site-specific mutagenesis

Site-directed mutagenesis was used to generate plasmids expressing the different coding variants, using a plasmid expressing the ABCA1 wildtype as a template (the ABCA1 wildtype expressing plasmid was provided by Dr. Christiane Albrecht). A PCR based site-directed mutagenesis method described by Scott *et al.*[204] was used as explained as follows. The PCR mixture contained 75 ng template plasmid DNA containing the ABCA1 cDNA (the plasmid was provided by Dr Christiane Albrecht and described in section 2.6.5.4), 125 ng of each oligonucleotide (table 2.4), 300 mM dNTPs, 3.75 U PfuTurbo® DNA polymerase (Stratagene, USA) and the buffer supplied with the polymerase in a total volume of 50 uL. PCR was performed under the following conditions; denaturation at 95°C for 2 min, followed by 16 cycles of denaturation at 95°C for 40 s, annealing at 58°C for 50 s and extension at 68°C for 1.5 min/kb. An aliquot of the PCR mixture was then examined by agarose gel electrophoresis to determine whether the correct sized product was obtained. Once the correct-sized PCR product was obtained, the reaction (50 mL) was incubated with 30 U of *Dpn*I for 3 h at 37°C. This step is critical because it preferentially digests the methylated, nonmutated parental DNA and thereby increases the frequency of clones with the desired mutation[205]. The digested PCR product was then ethanol precipitated and half of the resuspended pellet transformed into 50 uL of competent Top10â *E. coli* cells (Invitrogen, Carlsbad, CA, USA). Presence of the mutation and the integrity of the ATM DNA were confirmed by DNA sequencing of these constructs.

SNP	primers
219A FOR	GTGGCCTACCAAaGGAGAAACTGGCT
219A REV	AGCCAGTTTCTCCtTTGGTAGGCCAC
771M FOR	GCATGGCAGGACTACaTGGGCTTCACACTCA
771M REV	TGAGTGTGAAGCCCAtGTAGTCCTGCCATGC
825A FOR	CAATCTCACTACTTCGaTCTCCATGATGC
825A REV	GCATCATGGAGAtCGAAGTAGTGAGATTG
883G FOR	CAACCAGAAGAGAATgTCAGAAATCTGC
883G REV	GCAGATTTCTGAcATTCTCTTCTGGTTG
1587A FOR	GACAGGACTGGACACCAaAAATAATGTC
1587A REV	GACATTATTTtTGGTGTCCAGTCCTGTC

Mutated residues in primers are indicated in lower case.

Table 2.4 Primers used for site-directed mutagenesis

2.6.2 Competent cells

Since DNA is a very hydrophilic molecule, it won't normally pass through a bacterial cell's membrane. In order to make bacteria take in the plasmid, they must first be made "competent" to take up DNA. This is done by creating small holes in the bacterial cells by suspending them in a solution with a high concentration of calcium. Subsequently, competent bacteria can be transformed using the DNA as described in the protocol below (2.6)[206,207].

2.6.3 Preparation of competent *E.coli*

A fresh E.coli. (JM109) was inoculated in 25 ml LB medium and incubated overnight in a platform shaker at 225 rpm and at a constant temperature of 37°C. 1 ml of overnight culture was used to inoculate 100 ml LB medium (1:100 dilution) and allowed to grow at 37°C in a platform shaker at 225 rpm until an optical density reading (OD) of 0.4-0.6 was reached (approximately 2-3 hr). After this, the cells were centrifuged at 4,000 rpm at 4°C for 10 min. The supernatants were removed and discarded and the cell pellets were resuspended in 10 ml ice cold 0.1 C_aCl_2. The suspension was incubated on ice for 30 min. The cells were collected once more by centrifuging at 3,000rpm at 4°C for 5 min. Again the supernatants were discarded and the cell pellets suspended in 4 ml 0.1M C_aCl_2 before incubating on ice once more for a further 2 hr. The cells at this point were ready for transformation and could also be prepared for storage.

In order to freeze, 140 µl dimethylsulphoxide (DMSO) was added per 4 ml of cell and gently mixed. Cells were incubated on ice for 15 min, before another 140 µl of DMSO was added once more and gently mixed into the cells. Cell were then dispensed into aliquots in microcentrifuge tubes and liquid nitrogen was used to snap freeze samples before storing at -70°C.

2.6.4 Bacterial growth medium

2.6.4.1 Luria-Bertani (LB) medium

20g of LB broth base power was dissolved in 1 L of deionised water. The solution was autoclaved for 15 min at 121°C and stored at room temperature until required.

2.6.4.2 LB agar

32 g of LB agar powder was suspended in deionised water. The solution was autoclaved for 15 min at 121°C and stored at room temperature until required.

2.6.4.3 Ampicillin

A stock solution of 100 mg/ml ampicillin was prepared using sterilised, deionised water. The ampicillin was stored in aliquots at -20°C. The working concentration of ampicillin routinely used was 100 µg/ml.

2.6.5 Transformation

2.6.5.1 Introduction

The principle basically involves the introduction of a foreign plasmid into a bacteria, the plasmid then utilises the bacteria to multiply itself by allowing the production of substantial quantities of it. Plasmids are characteristically circular double-stranded DNA molecules distinct from the chromosomal DNA and capable of independent replication. They have variable sizes from as small as 1 to large as 400 kilobase pairs. They are found mainly in bacteria but are also isolated organisms. Every plasmid is made up of at least a single DNA sequence that serves as an origin of replication (a starting point for DNA replication), which enables the plasmid DNA to be duplicated independently from the chromosomal DNA.

The plasmid used to transform bacteria contains a gene that confers antibiotic resistance. The transformed bacteria are cultured in LB broth with an antibiotic (usually ampicillin). Using this approach, the antibiotics serve as a selection tool promoting the growth of the transformed bacteria. The plasmid is subsequently isolated from the bacteria.

2.6.5.2 Transformation conditions used in this study

Prior to the transformation, LB/agar plates containing the antibiotic ampicillin (100 µg/ml) were prepared for each sample and control. The transformation was achieved through the following process: ~20 ng of plasmid DNA was mixed gently with 100 µl of competent E.coli TOP10 cells (Invitrogen) and incubated on ice for 30 min. The samples were then heat-shocked in water bath at 42°C for a period of 2 min. They were then placed on ice for a further 2 min. 300 µl LB medium was added to each tube. The cells were allowed to recover by incubating at 37°C for 45 min in a platform shaker at ~150rpm. 200 µl of each transformation mix was plated onto LB/ampicillin plates and incubated overnight at 37°C. Transformants were selected by their resistance to ampicillin.

2.6.5.3 Analysis of transformants

In order to identify the clone of interest, several methods have been developed to screen transformants. The most popular of these methods is to isolate small-scale plasmid DNA preparations (minipreps) which can then either be sequenced or digested by restriction endonuclease enzyme of the desired clone, followed by fractionation of the products by gel electrophoresis. The method used in this study was sequencing. The purpose of sequencing the plasmids was to validate that the plasmids contained the designed allele following the *in vitro* mutagenesis and that there was no misincorporation during the PCR of the *in vitro* mutagenesis.

2.6.5.4 ABCA1 plasmid

A plasmid containing the ABCA1 cDNA was provided by Dr Christiane Albrecht. The cDNA contains the wild-type alleles for the R219K, V771M, V825I, I883M, and R1587K SNPs, and was used to generate the mutant alleles for these SNPs by *in vitro* mutagenesis as described above. The method used to generate the plasmid is described in detail in Albrecht *et al.*[208]. In brief, a full-length human ABCA 1 cDNA was produced by the method of RT-PCR using fibroblast RNA as template. The PCR product was cloned into the pCR^{R}4Blunt-TOPO vector (Introgen) and subcloned into the pGEM®-11Zf vector. The resulting construct contained an ABCA1-eGFP (green fluorescence protein) hybrid.

2.6.5.5 Plasmid DNA preparation

The importance of plasmid purification is to separate the plasmid DNA from the chromosomal DNA and the RNA of the bacteria cells. Many methods had been devised to remove part from the contaminating chromosomal DNA but also proteins and lipids so as to allow plasmid DNA free in solution. The methods include SDS-alkaline denaturation developed by Birnboim and Doly[209] in 1979 and modified by Birnboim[210] in 1983. This method has an advantage over others because of its versatility and efficiency. Other methods include salt-SDS precipitation developed by Hirt[211] in 1967 and rapid boiling first utilised by Holmes and Quigley[212] in 1981.

In this study Qiagen (Qiagen, UK) Hi-Speed plasmid midi kit was used. The kit based on a modified alkaline-SDS lysis method, in which the final plasmid DNA is extracted by adsorption to a silica membrane in the presence of high salts and the contaminants removed by washing.

The kit was used according to manufacturer's protocol. Bacteria were harvested from a 50 ml overnight culture by centrifugation at 6,000 rpm for 15 min. This step was performed in a 50 ml tube. The resulting bacterial pellet was re-suspended in 6 ml re-suspension buffer and lysed with 6 ml lysis buffer by 8 gentle inversions and incubation at RT for 5 minutes. The cell debris was then precipitated by addition of 6 ml Neutralisation/Binding buffer, and the solution was immediately transferred to a Qiafilter cartridge and incubated for 10 minutes at room temperature. The lysate that had passed through the cartridge was then transferred to a previously equilibrated HiSpeed Plasmid Midi tip (resin column) and allowed to enter the resin by gravity flow. The flow-through liquid was discarded and the binding column washed with 20 ml wash solution. The DNA was eluted with addition of 5 ml of Elution Solution, and precipitated with 3.5 ml isopropanol with incubation at room temperature for 5 minutes. The elute/isopropanol was filtered by using a Qiaprecipitator. The DNA bound to the Qiaprecipitator was washed twice with 2 ml 70% ethanol, and then eluted in 1 ml water.

2.6.6 Cell culture

Reagents: COS-7 cell line (kindly provided by Dr. C. Hodgkinson), 10X DMEM (Gibco UK), Penicillin/Streptomycin (Sigma, UK), Glutamine (Sigma, UK), Sodium Pyruvate (Sigma, UK), NaHCO3 (Sigma, UK), Fetal calf serum (Sigma, UK).

COS-7 cells were grown in 1X DMEM supplemented with 10% Fetal calf Serum (FCS), 2mM pyruvate, 2 mM glutamine, 100µg/ml penicillin/streptomycin, 5 mM NaHCO3 at 37°C in the presence of 5% CO2.

The morphology of the COS-7 cells was examined under a microscope and found to be similar to images from LGC Promochem which supplies COS-7 cells.

2.6.7 Trypsinisation

Cells were passaged every 3-4 days to ensure they did not reach confluence. Treatment with the protease trypsin detaches the cell from the plastic dish and allows sub-culturing. The culture medium was completely removed and for each 75 cm 2 flask, 5.0 ml of 1x HBSS solution was added to wash the cells, 2.0 ml of pre-warmed Trypsin-EDTA solution was added and the flask incubated at 37°C for 5 minutes and then lightly tapped in order to release the cells. Once in suspension, 8.0 ml of 1x DMEM with 10% FCS medium was added to inactivate the trypsin and the cells reseeded in fresh flasks as required.

2.6.8 Cryo-preservation of cells

To preserve stocks, cells were harvested by trypsinisation and pelleted by centrifugation at 1,000 rpm for 5 min. The supernatant was removed and the cell pellet re-suspended in 0.5 ml of DMEM medium. The cells were transferred to a cryotube and 0.5 ml of 10% DMSO (reduces cell lysing during freezing by interacting with plasma membranes) in FCS was added. The tube was frozen to -70°C in a container filled with isopropanol, which is specially designed to cool at a rate of less than 1°C per minute. Once frozen the cell were stored in liquid nitrogen (-190°C) until required.

2.6.9 Transient transfection of cells

2.6.9.1 Introduction

There are several transfection methods which are briefly discussed below.

2.6.9.1.1 DEAE

Transient transfection involves introducing foreign DNA into the nucleus of cell without integration into the chromosomes leading to high levels of expressed protein. DEAE-dextran is a cationic polymer that associates with negatively charged nucleic acids; the DNA-DEAE-dextran complexes have a positive charge, which induces association with the negatively charged cell plasma membrane. An osmotic shock to the cells induces endocytosis and the complexes pass into the cells.

2.6.9.1.2 Calcium phosphate co-precipitation

The method became popular following the publication of the work by Graham and van der Eb published in 1972[213]. Calcium phosphate co-precipitation is extensively utilised as the components are cheap and easily available. The procedure is straightforward with many different types of cultured cells being transfected. This method is routinely applied in both transient and stable transfection in different cells. Calcium chloride is mixed with DNA in a particular ratio to a buffered saline/phosphate solution leaving the mixture to incubate at room temperature. The precipitate produced is then dispersed on the cultured cells allowing it to be absorbed into the cells by either endocytosis or phagocytosis.

2.6.9.1.3 Electroporation

Electroporation was first documented for gene transfer by Wong and Neumann in 1982. The principle here involves disturbance of the cell membrane by an electrical pulse. This leads to the formation of pores that allow the passage of nucleic acids into the cell[214].

2.6.9.1.4 Lipofection

Cationic lipid-mediated DNA transfection into cultured cells was the original method of lipofection credited to Felgner *et al.* 1987[215]. The method was upgraded in 1993

through the replacement of the mono-cationic lipid reagent with a polycationic reagent called lipofectamine. The advantage of this method is its generally higher efficiency and ability to transfect diverse variety of cell lines. The method is also associated with comparatively low toxicity. Another merit of this procedure is efficient delivery of functional genes or viral genomes *in vivo.*

2.6.9.2 Transfection method used in this study

In this study, COS-7 cells were transfected with plasmids expressing ABCA1, using Fugene 6 transfection reagent which is a proprietary blend of lipid and other components. Advantages of FuGENE 6 Reagent include high transfection efficiency in many common cell types, including HeLa, NIH 3T3, COS-1, COS-7, and CHO-K1. More than 600 cell types have reported to have been successfully transfected with FuGENE 6 Reagent. In contrast to other transfection agents there is no need for extensive optimisation of DNA: Transfection Reagent ratios.

COS-7 cells were plated in 6 well plates in DMEM with 10% FCS and grown to 70% confluence for transfection. One the day of transfection, 1 µg plasmid DNA (a plasmid expressing ABCA1 wild-type, or the 219K, 771M, 825I, 883M or 1587K allele) was mixed with 3 µl FuGENE 6 Transfection Reagent, medium (serum-free, antibiotic-free) to a final volume of 100 ul, incubated at room temperature for 15 min, and then added to the cells. Figure 2.4 shows transfected COS7 cells. The fluorescence signal was produced the green fluorescent protein generated by the plasmid which contained the ABCA1-eGFP hybrid.

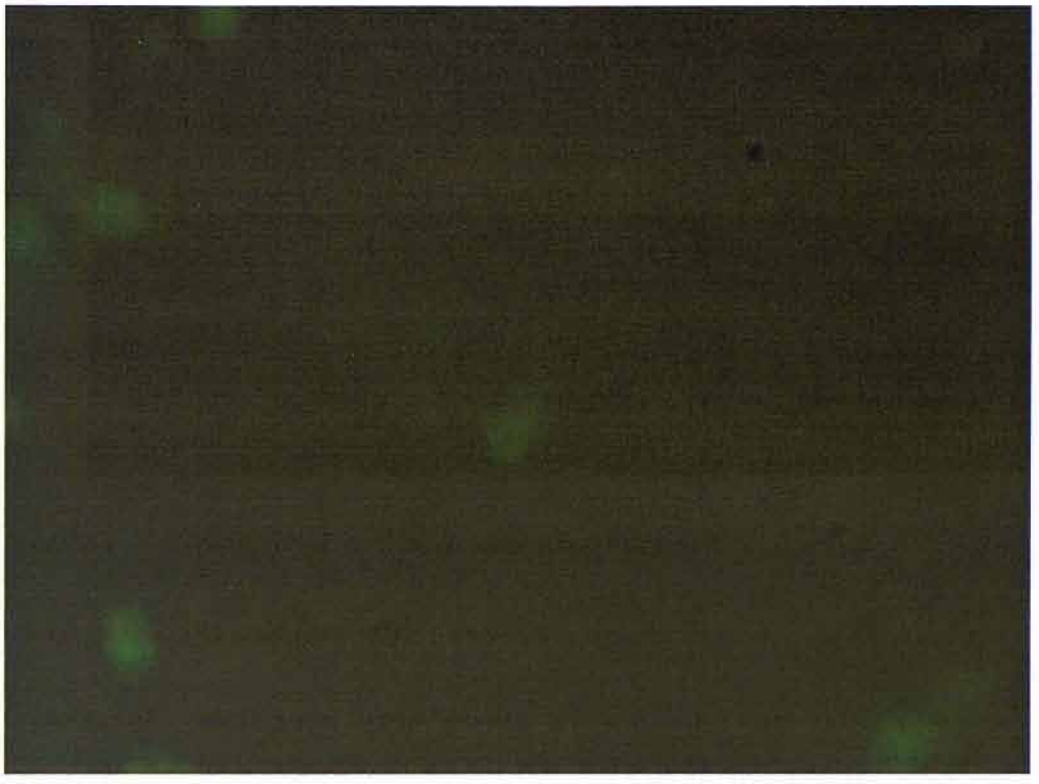

Figure 2.4 Transfection of COS-7 cells using Fugene 6

2.6.10 Foam cell formation assays

A series of optimization experiments were carried out to establish a method for producing cells that were transfected with the ABCA1 plasmids described above. The transfection proved to be difficult, probably due to the large size of the plasmids which were of approximately 15 kbp.

Initially, experiments were carried out in RAW264.7 cells which are murine macrophages. Transfection of RAW264.7 cells with the ABCA1 plasmids were first attempted using the FuGENE 6 as described above in section 2.6.9.2. However, few cells were transfected, as determined by examining the cells under a microscope to detect green fluorescence signal produced by the ABCA1-eGFP hybrid protein. Further experiments attempting to transfect the RAW267.4 cells with the ABCA1 plasmids were performed using another transfection reagent, GeneCarrier 2, which has been claimed to have high transfection efficiencies. The protocol for transfection using GeneCarrier 2 was the same as that using FuGENE 6 as described above. The experiments using GeneCarrier 2 also produce few transfected RAW264.7 cells.

Further optimization experiments were then carried out using a different cell line, HepG2, a hepatoma cell line. As for RAW264.7 cells, transfection were attempted using FuGENE 6 and GeneCarrier 2. However, as for RAW264.7 cells, few HepG2 cells were transfected.

Subsequently, a further cell line, the COS7 cell line, was tested. It proved that COS7 cell line could be transfected with high efficiency (between 20-25%) using FuGENE 6. Further, the transfection efficiency for the 6 different constructs representing the different ABCA1 variants was very similar with less than 4% differences. Therefore, further experiments were performed using COS7 cells and FuGENE 6 using the protocol described above in section 2.6.9.2.

For foam cell formation assays, COS7 cells were transfected with a plasmid expressing ABCA1 wild-type, or the 219K, 771M, 825I, 883M or 1587K allele. 48 Hours after transfection, 5µl acetylated LDL (50 µg/ml, Invitrogen, UK) was added and incubated at 37 °C for 5 h. After incubation, the medium was removed, and cells were fixed with 6% paraformaldehyde in PBS for 30 min and rinsed with propylene glycol for 2 min. Then 300 µl Oil Red O (5 mg/ml) was added to cover the cell for 30 min. Oil Red O stain was then removed, and the background stain was washed out with 6% paraformaldehyde. Numbers of cells with and without oil red O staining in five microscopic fields were counted. Photographs were taken for some of the fields. An example is shown in figure 2.5.

The rate of foam cell formation was calculated as follows: The percentages of Oil Red O stained cells were calculated for untransfected cells, and for cells transfected with the plasmid expressing the wild-type ABCA1, or the 219K, 771M, 825I, 883M or 1587K allele, respectively. The percentages of transfected cells display green fluorescence were calculated for cells transfected with the plasmid expressing the wild-type ABCA1 or the 219K, 771M, 825I, 883M or 1587K allele, respectively. Then the percentage of transfected cells was divided by the percentage of Oil Red O stained cells in untransfected cells minus the percentage of Oil Red O stained cells in the cells transfected with the plasmid expressing wild-type ABCA1, or the 219K, 771M, 825I, 883M or 1587K allele.

For example, if the percentage of Oil Red O stained cells in untransfected cells is 57 %, the percentage of Oil Red O stained cells in the cells transfected with the wild-type ABCA1 expressing plasmid is 46 %, and the percentage of cells transfected with the wild-type ABCA1 expressing plasmid is 19%, then the rate of foam cell formation is 0.19/(0.57-0.46)= 1.72. The t-test was then used to compare the foam cell formation rates from cells transfected with the wild-type ABCA1 construct and cells transfected with constructs expressing ABCA1 219K, 771M, 825I, 883M or 1587K.

Four independent experiments were carried out. In each experiment, transfection for each allele followed by foam cell formation assays was carried out in duplicate. Data presented are the average of the four experiments.

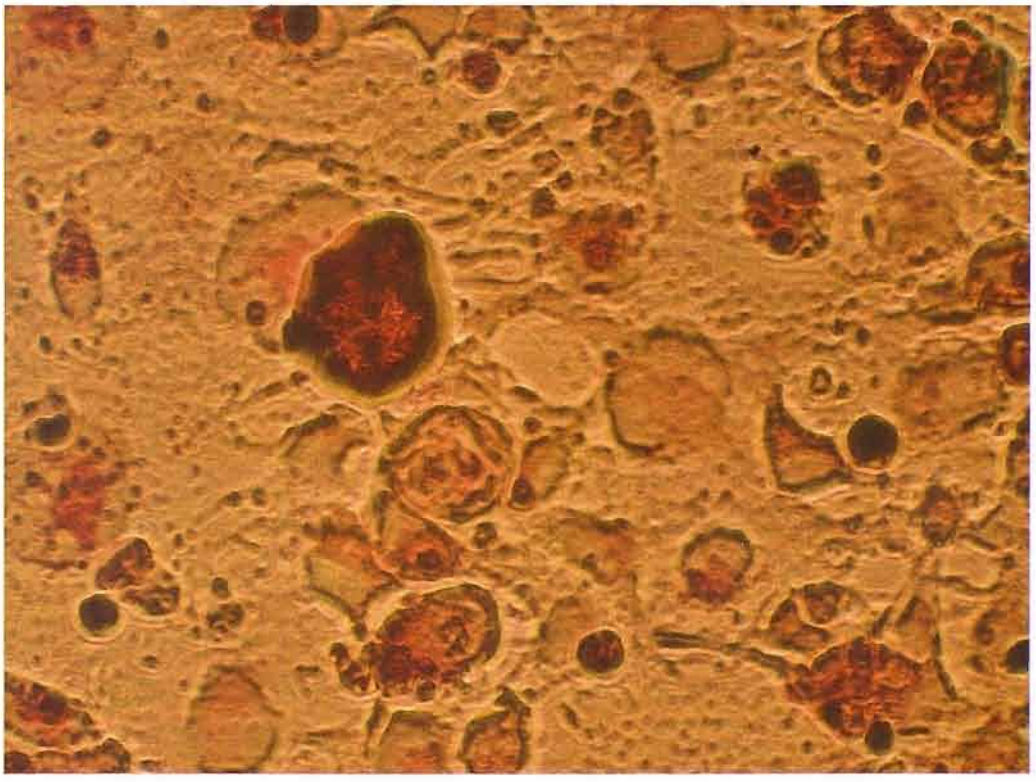

Figure 2.5 Foam cells are shown by the uptake of Oil-Red O stain

2.6.11 Promoter activity assays

These experiments were carried out by Dr. Theo Kyriakou, with Neda Bogari observing and providing minor assistance. In brief, transient transfection and reporter assays were performed to compare the strength of ABCA1 promoter of different haplotypes. For each haplotype, the corresponding ABCA1 promoter (from position -

588bp to +21bp relative to the transcriptional start site) was generated by PCR using genomic DNA as template and then inserted into a plasmid (pGL3-basic vector, Promega) containing a firefly luciferase reporter gene. All constructs were verified by DNA sequencing. RAW264.7 cells were transfected with each of the above constructs with the use of FuGENE 6 transfection reagent (Roche Diagnostics). A plasmid (pRL-TK, Promega) containing a renilla luciferase gene under the control of a thymidine kinase promoter, was co-transferred into the cells to serve as a reference for transfection efficiency. At 36 hour after transfection, the cells were lysed, and the activities of firefly luciferase and renilla luciferase in the lysates were measured with the use of a dual-luciferase assay kit (Promega). ABCA1 gene promoter activity was determined according to the ratio of firefly luciferase activity to renilla luciferase activity. Three independent experiments were performed. In each experiment, transfection and luciferase assay were carried out in duplicate for each construct.

2.6.12 Cholesterol efflux assays

Neda Bogari was involved in the generation of the expression plasmids, and the cholesterol efflux assays were performed by Dr. Enrique Viturro in Germany. In brief, COS7 cells were transfected with the different ABCA1 constructs respectively, using FuGENE 6 transfection reagent (Roche Diagnostics). At 24 hour post-transfection, transfection efficiency was determined by fluorescence microscopy. Cholesterol efflux assays were performed using a method described by Gelissen *et al.*[216] with minor modifications. Transfected cells were incubated with [^{3}H] cholesterol for 48 hours, washed and equilibrated for 18 hours in serum-free medium. The cells were then incubated in efflux medium with or without 20ug/ml apolipoprotein AI (apoAI, SIGMA) as an acceptor. Six hours later, efflux media were removed, and cells were washed and then dissolved in 0.1M NaOH solution. Cells and media were then assayed for radioactivity. Three independent experiments were performed, in each of which transfection and cholesterol efflux assay were carried out in triplicate for each construct. The rate of cholesterol efflux was calculated using the following formula: radioactivity in medium/(radioactivity in medium + radioactivity in cell extract). The values (apoA1-mediated efflux minus mean of unstimulated cells) for cells transfected with the plasmids expressing the ABCA1 219K, 771M, 825I, 883M or 1587K isoform

were compared with the mean of values (apoA1-mediated efflux minus mean of unstimulated cells) for cells transfected with the plasmid expressing ABCA1 wild-type.

3 Results

3.1 Allele and genotype frequencies of the ABCA1 gene polymorphisms

The SAS sample consisting of 1164 patients with coronary atherosclerosis (described in chapter 2) was genotyped for the -407 G>C, -302 C>T, -278 G>C, -99 G>C, -14 C>T, and R219K polymorphisms of the ABCA1 gene, using the methods described in Chapter 2. An example of the gel images obtained is shown in figure 2.3. Five other polymorphisms in this gene, i.e. -565 C>T, V771M, V825I, I883M, and R1587K, have been typed in this sample by other members of the group. The -565 C>T, -407 G>C, -302 C>T, -278 G>C, -99 G>C, -14 C>T, R219K, V825I, I883M, and R1587K polymorphisms represent all common polymorphisms in the proximal promoter and coding region of the gene, with a minor allele frequency > 0.05 according to previous studies reported in the literature. The V771M polymorphism, although it has a minor allele frequency <0.05, was found to associated with be the degree of atherosclerosis in the study by Clee *et al.*[191] as discussed above, and therefore was also examined in this project.

Genotype and allele frequencies of the 11 polymorphisms are shown in Table 3.1. Genotype frequencies of the 11 polymorphisms were consistent with expectation from Hardy-Weinberg equilibrium (Table 3.1).

Table 3. 1: Genotype and allele frequencies

		Number	Freq.
-565 C>T	CC genotype	351	0.305
	CT genotype	562	0.488
	TT genotype	239	0.207
	Total	1152	1.000
	T allele		0.451
-407 G>C	GG genotype	324	0.308
	CG genotype	509	0.484
	CC genotype	219	0.208
	Total	1052	1.000
	C allele		0.450
-302 C>T	CC genotype	683	0.661
	CT genotype	312	0.302
	TT genotype	39	0.037
	Total	1034	1.000
	T allele		0.189
-278 G>C	GG genotype	347	0.316
	GC genotype	522	0.476
	CC genotype	228	0.208
	Total	1058	1.000
	C allele		0.446
-99 G>C	GG genotype	570	0.539
	CG genotype	405	0.383
	CC genotype	83	0.078
	Total	1058	1.000
	C allele		0.270
-14 C>T	CC genotype	511	0.470
	CT genotype	448	0.412
	TT genotype	128	0.118
	Total	1087	1.000
	T allele		0.324
		Number	Freq
R219K	RR genotype	559	0.513
	RK genotype	439	0.403
	KK genotype	91	0.084
	Total	1089	1.000
	K allele		0.285
V771M	VV genotype	1012	0.954
	VM genotype	47	0.044
	MM genotype	2	0.002
	Total	1061	1.000
	M allele		0.024
V825I	VV genotype	966	0.890
	VI genotype	114	0.105
	II genotype	5	0.005
	Total	1085	1.000
	I allele		0.057
I883M	II genotype	724	0.734
	IM genotype	241	0.244
	MM genotype	21	0.022
	Total	986	1.000
	M allele		0.144
R1587K	RR genotype	540	0.579
	RK genotype	343	0.368
	KK genotype	49	0.053
	Total	932	1.000
	R allele		0.237

For the majority of the SNPs in this study, the genotyping success rates were approximately 90%. The genotyping success rates for I883M and R1587K, however, were only 85% and 80% respectively due to higher PCR failurer rates.

3.2 Association of ABCA1 gene polymorphisms with HDL-cholesterol levels

Since ABCA1 plays a pivotal role in the HDL-cholesterol synthesis, the ABCA1 gene polymorphisms described above were analysed in relation to HDL-cholesterol levels. The analysis was carried out first in the sample as a whole, and then in two subgroups stratified according to whether the patient was on statin treatment to lower cholesterol level.

Mean HDL-cholesterol levels in patients of different genotypes for the ABCA1 polymorphisms are shown in table 3.2. As presented in the table, there was an association between HDL-cholesterol levels and the R1587K polymorphism, such that homozygotes of the K allele of the R1587K had the lowest mean level, compared with individuals of other genotype for this polymorphism. This association was seen in the sample as a whole, in patients who were not on statin treatment, and in patients who were on statin treatment. However, the differences were statistically significant only in the sample as a whole ($p=0.02$) and in patients on statin treatment ($p=0.018$). The association was still observed after adjusting for age, gender, smoking, body mass index, hypertension, type 1 diabetes, type 2 diabetes, and family history of CAD ($p=0.047$ in the sample as a whole and $p=0.007$ in patients on statin treatment).

In the untreated group, HDL-cholesterol levels were associated with the I883M polymorphism. Carriers of the M allele had higher HDL-cholesterol levels than non-carriers ($p=0.0004$). The relationship remained significant after adjusting for age, gender, smoking, body mass index, hypertension, type 1 diabetes, type 2 diabetes, and family history of CAD ($p=0.001$).

In addition, there was a trend towards higher HDL-cholesterol levels in carriers of the I allele of the V825I polymorphism ($p=0.01$), which remained after adjusting for age, gender, smoking, body mass index, hypertension, type 1 diabetes, type 2 diabetes, and family history of CAD ($p=0.048$).

There was no association between HDL-cholesterol level and the other polymorphisms studied.

		All		Untreated		Statin treated	
-565 C>T	C/C	1.25 (0.34), 198	n.s.	1.25 (0.32), 81	n.s.	1.25 (0.35), 117	n.s.
	C/T	1.28 (0.33), 299		1.24 (0.30), 137		1.31 (0.34), 162	
	T/T	1.26 (0.35), 126		1.23 (0.28), 54		1.28 (0.40), 72	
-407 G>C	G/G	1.25 (0.35), 182	n.s.	1.24 (0.32), 76	n.s.	1.26 (0.36), 106	n.s.
	C/G	1.29 (0.34), 276		1.23 (0.30), 123		1.33 (0.36), 153	
	C/C	1.27 (0.34), 117		1.24 (0.27), 52		1.28 (0.38), 65	
-302 C>T	C/C	1.26 (0.33), 370	n.s.	1.26 (0.32), 165	n.s.	1.26 (0.33), 205	n.s.
	C/T	1.28 (0.36), 174		1.21 (0.27), 68		1.32 (0.40), 106	
	T/T	1.30 (0.38), 21		1.27 (0.26), 13		1.35 (0.53), 8	
-278 G>C	G/G	1.26 (0.34), 198	n.s.	1.25 (0.32), 80	n.s.	1.26 (0.35), 118	n.s.
	G/C	1.28 (0.33), 282		1.24 (0.30), 126		1.30 (0.35), 156	
	C/C	1.27 (0.36), 115		1.23 (0.28), 51		1.30 (0.42), 64	
-99 G>C	G/G	1.27 (0.35), 305	n.s.	1.25 (0.29), 139	n.s.	1.29 (0.38), 166	n.s.
	G/C	1.27 (0.35), 220		1.23 (0.31), 93		1.30 (0.36), 127	
	C/C	1.27 (0.27), 45		1.30 (0.28), 21		1.25 (0.27), 24	
-14 C>T	C/C	1.27 (0.33), 279	n.s.	1.25 (0.31), 126	n.s.	1.29 (0.35), 153	n.s.
	C/T	1.26 (0.33), 246		1.25 (0.32), 99		1.27 (0.34), 147	
	T/T	1.30 (0.38), 63		1.20 (0.23), 30		1.40 (0.47), 33	
R219K	R/R	1.29 (0.37), 286	n.s.	1.26 (0.30), 129	n.s.	1.32 (0.41), 157	n.s.
	R/K	1.26 (0.32), 241		1.23 (0.30), 104		1.28 (0.32), 137	
	K/K	1.23 (0.27), 47		1.25 (0.34), 18		1.21 (0.22), 29	
V771M	V/V	1.28 (0.35), 535	n.s.	1.25 (0.31), 244	n.s.	1.31 (0.37), 291	n.s.
	V/M	1.20 (0.23), 27		1.10 (0.22), 10		1.26 (0.22), 17	
	M/M	0.97 (-), 1		0.97 (-), 1		-	
V825I	V/V	1.27 (0.34), 532	n.s.	1.23 (0.30), 232	**P=0.01**	1.29 (0.36), 300	n.s.
	V/I	1.28 (0.35), 57		1.34 (0.31), 26		1.23 (0.38), 31	
	I/I	1.54 (0.07), 3		1.54 (0.07), 3		-	
I883M	I/I	1.26 (0.34), 396	n.s.	1.20 (0.27), 172	**P=0.0004**	1.30 (0.37), 224	n.s.
	I/M	1.31 (0.31), 134		1.39 (0.36), 56		1.25 (0.25), 78	
	M/M	1.32 (0.35), 13		1.34 (0.34), 9		1.25 (0.42), 4	
R1587K	R/R	1.29 (0.36), 283	**P=0.02**	1.27 (0.31), 142	n.s.	1.32 (0.39), 141	**P=0.018**
	R/K	1.25 (0.31), 194		1.24 (0.30), 84		1.26 (0.32), 110	
	K/K	1.12 (0.20), 23		1.18 (0.20), 9		1.08 (0.20), 14	

Table 3.1 **HDL levels [mean (SD), N] according to ABCA1 genotypes**

3.3 ABCA1 polymorphisms and total cholesterol levels

The total cholesterol levels according to ABCA1 genotypes in the sample as a whole, in patients not on statin treatment, and in patients who were no statin treatment are shown in table 3.3. As shown in the table, in patients not on statin treatment, those who where homozygous for the 771V allele had higher total cholesterol levels than those of the V/M of M/M genotype of the V771M polymorphism (p=0.006). The association remained significant after adjustment for age, gender, smoking, body mass index, hypertension, diabetes and family history of CAD (p=0.009). There was no association between total cholesterol level and the other polymorphisms studied.

		All		Untreated		Statin treated	
-565 C>T	C/C	5.14 (1.07), 327	n.s.	5.57 (1.17), 134	n.s.	4.84 (0.88), 193	n.s.
	C/T	5.15 (1.02), 520		5.57 (1.07), 233		4.81 (0.83), 287	
	T/T	4.99 (0.98), 228		5.33 (1.03), 94		4.75 (0.87), 134	
-407 G>C	G/G	5.14 (1.06), 303	n.s.	5.60 (1.12), 126	n.s.	4.82 (0.88), 177	n.s.
	C/G	5.19 (1.00), 472		5.63 (1.05), 204		4.87 (0.82), 268	
	C/C	4.95 (0.94), 207		5.21 (0.97), 89		4.75 (0.86), 118	
-302 C>T	C/C	5.16 (1.02), 640	n.s.	5.58 (1.05), 284	n.s.	4.83 (0.86), 356	n.s.
	C/T	5.02 (1.02), 289		5.43 (1.13), 117		4.74 (0.83), 172	
	T/T	4.99 (0.89), 34		5.12 (1.04), 15		4.89 (0.76), 19	
-278 G>C	G/G	5.13 (1.07), 325	n.s.	5.58 (1.16), 133	n.s.	4.82 (0.88), 192	n.s.
	G/C	5.15 (0.97), 483		5.59 (0.97), 209		4.83 (0.83), 274	
	C/C	5.00 (0.97), 216		5.32 (1.02), 90		4.78 (0.87), 126	
-99 G>C	G/G	5.10 (1.00), 528	n.s.	5.52 (1.08), 237	n.s.	4.77 (0.80), 301	n.s.
	G/C	5.14 (1.02), 373		5.52 (1.09), 158		4.86 (0.88), 215	
	C/C	5.27 (1.15), 76		5.67 (1.20), 34		4.95 (1.02), 42	
-14 C>T	C/C	5.16 (1.05), 475	n.s.	5.59 (1.09), 206	n.s.	4.83 (0.89), 269	n.s.
	C/T	5.10 (0.98), 416		5.48 (1.05), 178		4.80 (0.81), 238	
	T/T	5.04 (1.05), 121		5.45 (1.14), 47		4.78 (0.90), 74	
R219K	R/R	5.17 (1.04), 522	n.s.	5.60 (1.11), 233	n.s.	4.81 (0.83), 289	n.s.
	R/K	5.07 (1.01), 408		5.44 (1.06), 173		4.79 (0.89), 235	
	K/K	5.01 (1.01), 83		5.36 (1.15), 30		4.82 (0.87), 53	
V771M	V/V	5.13 (1.02), 942	n.s.	5.55 (1.08), 410	**P=0.006**	4.81 (0.85), 532	n.s.
	V/M	5.01 (0.92), 44		4.93 (0.77), 19		5.07 (1.03), 25	
	M/M	4.30 (-), 1		4.30 (-), 1		-	
V825I	V/V	5.13 (1.00), 900	n.s.	5.52 (1.05), 390	n.s.	4.83 (0.84), 510	n.s.
	V/I	5.09 (1.16), 108		5.72 (1.24), 42		4.69 (0.90), 66	
	I/I	4.74 (1.19), 5		4.52 (1.25), 4		5.60 (-), 1	
I883M	I/I	5.14 (1.02), 676	n.s.	5.55 (1.07), 295	n.s.	4.82 (0.84), 381	n.s.
	I/M	5.14 (1.06), 227		5.61 (1.11), 89		4.83 (0.90), 138	
	M/M	5.13 (1.18), 21		5.24 (1.46), 12		5.00 (0.71), 9	
R1587K	R/R	5.15 (1.06), 506	n.s.	5.57 (1.12), 241	n.s.	4.77 (0.83), 265	n.s.
	R/K	5.14 (1.01), 321		5.52 (1.09), 130		4.88 (0.86), 191	
	K/K	5.03 (0.89), 46		5.40 (0.83), 23		4.66 (0.79), 23	

Table 3.2 **Total cholesterol levels [mean (SD), N] according to ABCA1 genotypes**

3.4 ABCA1 polymorphisms and triglyceride levels

The triglyceride levels according to ABCA1 genotypes in the sample as a whole, in patients not on statin treatment, and in patients who were no statin treatment are shown in table 3.4. A moderate association between triglyceride level and the -278 G>C polymorphism in patients not on statin treatment (p=0.035) was found. However, this relationship was no longer statistically significant after adjustment for age, gender, smoking, body mass index, hypertension, diabetes and family history of CAD (p=0.098). No association was found between triglyceride levels and the other ABCA1 gene polymorphisms (table 3.4).

		All		Untreated		Statin treated	
-565 C>T	C/C	1.84 (1.09), 301	n.s.	1.86 (1.23), 122	n.s.	1.82 (0.98), 179	n.s.
	C/T	1.89 (1.13), 487		1.89 (1.10), 220		1.89 (1.16), 267	
	T/T	1.89 (1.12), 204		1.62 (0.79), 82		1.92 (1.29), 122	
-407 G>C	G/G	1.80 (1.06), 285	n.s.	1.83 (1.22), 118	n.s.	1.77 (0.93), 167	n.s.
	C/G	1.91 (1.09), 438		1.92 (1.04), 191		1.90 (1.13), 247	
	C/C	1.74 (1.06), 185		1.57 (0.79), 77		1.87 (1.21), 108	
-302 C>T	C/C	1.89 (1.15), 599	n.s.	1.85 (1.10), 265	n.s.	1.93 (1.18), 334	n.s.
	C/T	1.83 (1.08), 252		1.86 (1.10), 102		1.82 (1.06), 150	
	T/T	1.54 (0.69), 34		1.37 (0.61), 15		1.66 (0.74), 19	
-278 G>C	G/G	1.81 (1.08), 299	n.s.	1.87 (1.23), 121	**P=0.035**	1.77 (0.94), 178	n.s.
	G/C	1.89 (1.12), 454		1.88 (1.09), 199		1.89 (1.15), 255	
	C/C	1.79 (1.16), 191		1.56 (0.83), 77		1.94 (1.32), 114	
-99 G>C	G/G	1.89 (1.16), 497	n.s.	1.89 (1.21), 216	n.s.	1.89 (1.12), 281	n.s.
	G/C	1.83 (1.08), 345		1.81 (0.97), 147		1.84 (1.16), 198	
	C/C	1.68 (0.86), 69		1.57 (0.97), 32		1.78 (0.74), 37	
-14 C>T	C/C	1.85 (1.07), 444	n.s.	1.85 (1.00), 191	n.s.	1.86 (1.13), 253	n.s.
	C/T	1.88 (1.15), 379		1.80 (1.11), 161		1.94 (1.17), 218	
	T/T	1.67 (0.85), 113		1.67 (0.92), 44		1.63 (0.82), 69	
R219K	R/R	1.92 (1.17), 484	n.s.	1.88 (1.08), 216	n.s.	1.95 (1.23), 268	n.s.
	R/K	1.76 (1.08), 372		1.74 (1.17), 158		1.78 (1.02), 214	
	K/K	1.83 (1.00), 80		1.82 (0.87), 27		1.84 (1.07), 53	
V771M	V/V	1.84 (1.14), 866	n.s.	1.83 (1.12), 379	n.s.	1.85 (1.15), 487	n.s.
	V/M	2.04 (1.13), 39		1.87 (0.85), 14		2.13 (1.27), 25	
	M/M	1.10 (-), 1		1.10 (-), 1		-	
V825I	V/V	1.87 (1.12), 832	n.s.	1.86 (1.11), 361	n.s.	1.87 (1.13), 471	n.s.
	V/I	1.74 (0.92), 97		1.61 (0.85), 37		1.81 (0.96), 60	
	I/I	2.16 (1.72), 5		2.23 (1.98), 4		1.90 (-), 1	
I883M	I/I	1.83 (1.04), 623	n.s.	1.86 (1.05), 270	n.s.	1.80 (1.03), 353	n.s.
	I/M	1.86 (1.19), 210		1.73 (1.08), 80		1.93 (1.25), 130	
	M/M	1.77 (1.04), 20		1.86 (1.28), 11		1.64 (0.69), 9	
R1587K	R/R	1.83 (1.10), 470	n.s.	1.77 (1.12), 224	n.s.	1.87 (1.09), 226	n.s.
	R/K	1.90 (1.12), 291		2.00 (1.14), 117		1.84 (1.09), 174	
	K/K	1.87 (0.86), 44		1.74 (0.91), 21		1.97 (0.80), 23	

Table 3.3 **Triglyceride levels [mean (SD), N] according to ABCA1 genotypes**

3.5 ABCA1 gene polymorphisms and coronary stenosis

The ABCA1 gene polymorphisms were analysed in relation to the number of coronary arteries with significant (>50%) stenosis, and the results are shown in table 3.5. There was an association with the V771M polymorphism, with patients of the V/V genotype having greater mean number of coronary arteries with >50% stenosis (p=0.004). The association remained significant after adjustment for age, gender, body mass index, smoking, cholesterol, triglycerides, lipid-lowering medication, hypertension, diabetes and family history of CAD (p=0.031). The other ABCA1 polymorphisms studied were not found to be significantly associated with significant coronary stenosis.

Polymorphism	Genotype	Mean (SD), n	p-value
-565 C>T	C/C	1.83 (0.79), 351	n.s.
	C/T	1.83 (0.79), 562	
	T/T	1.91 (0.82), 239	
-407 G>C	G/G	1.84 (0.80), 324	n.s.
	G/C	1.80 (0.79), 509	
	C/C	1.93 (0.80), 219	
-302 C>T	C/C	1.83 (0.80), 683	n.s.
	C/T	1.90 (0.79), 312	
	T/T	1.82 (0.85), 39	
-278 G>C	G/G	1.84 (0.80), 347	n.s.
	G/C	1.81 (0.79), 522	
	C/C	1.92 (0.82), 228	
-99 G>C	G/G	1.84 (0.79), 570	n.s.
	G/C	1.88 (0.81), 405	
	C/C	1.81 (0.80), 83	
-14 C>T	C/C	1.83 (0.80), 511	n.s.
	C/T	1.84 (0.79), 448	
	T/T	1.88 (0.84), 128	
R219K	R/R	1.85 (0.82), 559	n.s.
	R/K	1.85 (0.80), 439	
	K/K	1.88 (0.77), 91	
V771M	VV	1.87 (0.80), 1012	**P=0.004**
	VM	1.57 (0.77), 47	
	MM	1.00 (-), 2	
V825I	V/V	1.85 (0.80), 966	n.s.
	V/I	1.81 (0.80), 114	
	I/I	1.60 (0.89), 5	
I883M	I/I	1.81 (0.80), 724	n.s.
	I/M	1.87 (0.81), 241	
	M/M	1.76 (0.89), 21	
R1587K	R/R	1.82 (0.78), 540	n.s.
	R/K	1.83 (0.80), 343	
	K/K	2.02 (0.77), 49	

Table 3.4 **Number of coronary arteries with >50% stenosis according to ABCA1 genotypes**

3.6 ABCA1 gene polymorphisms and age of onset of symptomatic CAD

When comparing different genotypes for the -407 G>C polymorphism, patients that were homozygous for the -407G allele had lowest, those who were heterozygous had intermediate, and those who were homozygous for the -407C allele had highest mean age of onset of symptomatic CAD (p=0.002, table 3.6).

Similarly, in, comparing different genotypes for the -278 G>C polymorphism, in -273G allele homozygote, the mean age of onset of symptomatic CAD was lowest, while in heterozygotes it was intermediate, and in -278C allele homozygotes was the highest (p=0.007, table 3.6).

Likewise, an additive allelic effect was also observed when comparing different genotypes for the -565 C>T polymorphism (p=0.01, table 3.6).

The association between age of onset of symptomatic CAD and these polymorphisms remained significant after adjusting for gender, body mass index, smoking, cholesterol, triglycerides, lipid-lowering medication, hypertension and diabetes and family history of CAD (p=0.03 for -565 C>T, p=0.004 for -407 G>C and p=0.01 for -278 G>C).

No statistically significant association was found between age of onset of symptomatic CAD and the coding region polymorphisms, although as shown in table 3.6, age of onset was over 3 years higher in homozygotes for the 825I allele and about 1 year higher in heterozygotes, than in homozygotes of the 825V allele.

Polymorphism	Genotype	Mean (SD), n	p-value
-565 C>T	C/C	58.95 (9.92), 318	p=0.01
	C/T	59.84 (9.59), 496	
	T/T	61.13 (10.1), 214	
-407 G>C	G/G	58.72 (9.99), 296	p=0.002
	G/C	59.79 (9.59), 458	
	C/C	61.54 (9.72), 191	
-302 C>T	C/C	59.45 (9.82), 619	n.s.
	C/T	60.38 (9.86), 276	
	T/T	60.90 (10.5), 33	
-278 G>C	G/G	58.94 (9.82), 313	p=0.007
	G/C	59.70 (9.67), 463	
	C/C	61.39 (10.3), 204	
-99 G>C	G/G	60.41 (9.90), 505	n.s.
	G/C	59.41 (9.68), 363	
	C/C	59.18 (9.35), 78	
-14 C>T	C/C	59.80 (9.69), 460	n.s.
	C/T	59.74 (9.80), 401	
	T/T	60.68 (10.5), 113	
R219K	R/R	60.07 (9.94), 503	n.s.
	R/K	59.82 (9.84), 392	
	K/K	59.33 (8.81), 78	
V771M	V/V	59.98 (9.85), 908	n.s.
	V/M	57.48 (11.45), 44	
	M/M	73.28 (-), 1	
V825I	V/V	59.70 (9.86), 866	n.s.
	V/I	60.75 (9.52), 100	
	I/I	63.46 (9.07), 4	
I883M	I/I	60.38 (9.66), 644	n.s.
	I/M	59.10 (9.65), 217	
	M/M	62.12 (6.36), 19	
R1587K	R/R	60.23 (9.95), 496	n.s.
	R/K	58.90 (9.50), 301	
	K/K	60.93 (9.12), 41	

Table 3.5 Age of onset of symptomatic CAD according to ABCA1 genotypes

3.7 Linkage disequilibrium between ABCA1 gene polymorphisms

The study demonstrated strong pair-wise linkage disequilibrium among the 6 polymorphisms in the promoter region (table 3.7). Linkage disequilibrium among the polymorphisms in the coding region was also observed. There was little linkage disequilibrium between these two blocks.

	-407G>C	-302C>T	-278G>C	-99G>C	-17C>T	R219K	V771M	V825I	I883M	R1587K
-565C>T	0.92**	0.94**	0.99**	-0.85**	0.98**	-0.11*	-0.51	0.12	0.04	0.07
-407G>C		0.98**	0.91**	-0.75**	0.90**	-0.10	-0.51	0.15	0.04	0.08
-302C>T			0.92**	-0.88**	0.84**	-0.17	-1.00*	0.03	0.04	0.08*
-278G>C				-0.85**	0.98**	-0.11	-0.51	0.12	0.02	0.06
-99G>C					-0.86**	0.04	0.19	-0.09	-0.12	-0.01
-17C>T						-0.05	-0.32	0.04	0.01	0.03
R219K							1.00**	-0.88**	0.25**	0.14**
V771M								-1.00	-1.00	-0.09
V825I									0.81**	-0.27
I883M										0.01

** $p<0.001$; *$p<0.05$

Table 3.6 **Coefficient (D') of pair-wise linkage disequilibrium between ABCA1 gene polymorphisms**

3.8 Haplotype frequencies

Because there was little linkage disequilibrium between the promoter polymorphisms and the coding region polymorphisms, haplotype analyses were carried out separately in these two blocks. Frequencies of haplotypes for the promoter polymorphisms and haplotypes for the coding region polymorphisms are shown in table 3.8.

Promoter halpotype	Frequency		Coding region haplotype	Frequency
CGCGGC	0.276181		RVVIR	0.515502
CGCGCC	0.243497		KVVIR	0.125573
TCTCGT	0.170695		RVVIK	0.116584
TCCCGT	0.130065		KVVIK	0.068971
TCCCGC	0.105794		KVVMR	0.047684
TCTCGC	0.015041		RVIMR	0.036479
TGCCGT	0.009603		KVVMK	0.021044
CCCGCC	0.005431		KMVIR	0.015694
CCCGGC	0.004845		RVVMR	0.013964
TCCCCC	0.004731		RVIMK	0.012637
TGCCGC	0.004445		RVVMK	0.010200
CGCGCT	0.004158		KMVIK	0.006310
TCCCCT	0.004095		RVIIR	0.003645
CCTGGC	0.003616		KVIIR	0.002198
CGCGGT	0.003369		RVIIK	0.002194
TCCGGC	0.003004		RMVIK	0.001321
CGCCGC	0.002613			
TCTCCT	0.002317			
TCTGGT	0.001904			
TCCGGT	0.001353			
CCTGCC	0.001096			
TGCGGC	0.001081			
CCCCGT	0.001066			

Table 3.7 ABCA1 gene haplotype frequencies

3.9 Haplotype analyses in relation to age of onset of symptomatic CAD

In haplotype analyses in relation to lipid levels and CAD traits, only those haplotypes which had a frequency of >0.01 were included in the analyses, as analysis of very rare haplotypes would not be reliable. The haplotype analyses showed that age of onset of symptomatic CAD was associated with ABCA1 promoter haplotypes, such that compared with the most common haplotype (C-G-C-G-G-C), the T-C-T-C-G-T and T-C-C-C-G-C were associated with higher mean age of onset of symptomatic CAD (table 3.9). This association remained significant after adjusting for gender, body mass index, smoking, lipid-lowering treatment, hypertension, diabetes and family history of CAD.

There was no association between the coding region haplotypes and age of onset of symptomatic CAD.

Neither the promoter haplotypes nor coding region haplotypes showed any significant haplotypic effect on HDL-cholesterol, total cholesterol or triglyceride levels, or on the number of coronary arteries with severe stensosis (>50%).

Haplotype	Haplotype frequency	Mean (95% CI) age of onset per haplotype	Inferred mean (95% CI) age of onset in homozygotes	Wald test	Wald test*
C-G-C-G-G-C	0.277	29.14 (28.44-29.84)	58.28 (56.88-59.68)	reference	reference
C-G-C-G-C-C	0.251	29.56 (28.16-30.96)	59.12 (56.32-61.92)	n.s.	n.s.
T-C-C-C-G-T	0.131	30.02 (29.01-31.03)	60.04 (58.02-62.06)	n.s.	n.s.
T-C-T-C-G-T	0.167	30.89 (30.07-31.71)	61.78 (60.14-63.42)	p=0.009	p=0.006
T-C-C-C-G-C	0.105	31.69 (30.47-32.91)	63.38 (60.94-65.82)	p=0.002	p=0.004

*Wald test with adjustment for covariates including gender, body mass index, smoking, lipid-lowering treatment, hypertension, diabetes and family history of CAD.

Table 3.8 ABCA1 gene promoter haplotypes versus age of onset of symptomatic CAD

3.10 Cholesterol efflux assay of ABCA1 gene coding polymorphisms

To explore whether any of the coding SNPs studied (R219K, V771M, V825I, I883M, and R1587K) had an effect on the activity of ABCA1 in facilitating cholesterol efflux, cells were transfected with plasmids expressing the different ABCA1 alleles then the rates of cholesterol efflux from the cells were determined (Neda Bogari was involved in the generation of the expression plasmids, and the cholesterol efflux assays were performed by Dr Enrique Viturro in Germany). A literature search was carried out to search for suitable cell lines for these experiments, and the search showed that a number of studies by various laboratories used COS7 cells to examine the effects of ABCA1 on cholesterol efflux[150,217,218], presumably because COS7 cells have low background ABCA1 expression and give a high transfection efficiency. The details of the experimental procedure are described in the Methods chapter.

The assays demonstrated that cells expressing the 825I isoform have higher rate of apoAI-mediated cholesterol efflux than cells expressing the wild-type (825V) isoform ($p=0.003$, figure 3.1).

Additionally, compared to the cells expressing the wild-type (883I) isoform, there was also a tendency towards increased rate of apoAI-mediated cholesterol efflux in cells expressing the 883M isoform.

In contrast, cells expressing the 771M isoform had reduced cholesterol efflux compared with cells expressing the wild-type (771 I), but the difference was not statistically significant (figure 3.1).

Cells expressing the 219K or 1587K isoform had similar rates of apoAI-mediated cholesterol efflux to those expressing the wild-type (219R and 1587R) isoform (figure 3.1).

The rate of cholesterol efflux in untransfected cells was substantially lower than that in cells transfected with the ABCA1 wild-type, 219R, 825I, 883M, 771M or 1587K construct (figure 3.1). A plasmid expressing an ABCA1 (V1704D and L1379F) mutant (labelled as K.O.) was included in these experiments to serve as a "control", as it had the same plasmid vector as the other constructs (wild-type, 219R, 825I, 883M, 771M

and 1587K) but the mutations in the ABCA1 gene in this plasmid resulted in complete loss of ABCA1 function as shown in a previous study[18]. The rate of cholesterol efflux in cells transfected with the K.O. plasmid was similar to that in untransfected cells and substantially lower than that in cells transfected with the ABCA1 wild-type, 219R, 825I, 883M, 771M or 1587K construct (figure 3.1), suggesting that the increase in cholesterol efflux rate in cells transfected with the ABCA1 wild-type, 219R, 825I, 883M, 771M or 1587K construct compared with untransfected cells is due to the ABCA1 gene rather than any other sequence in the plasmids or other reagents used during transfection.

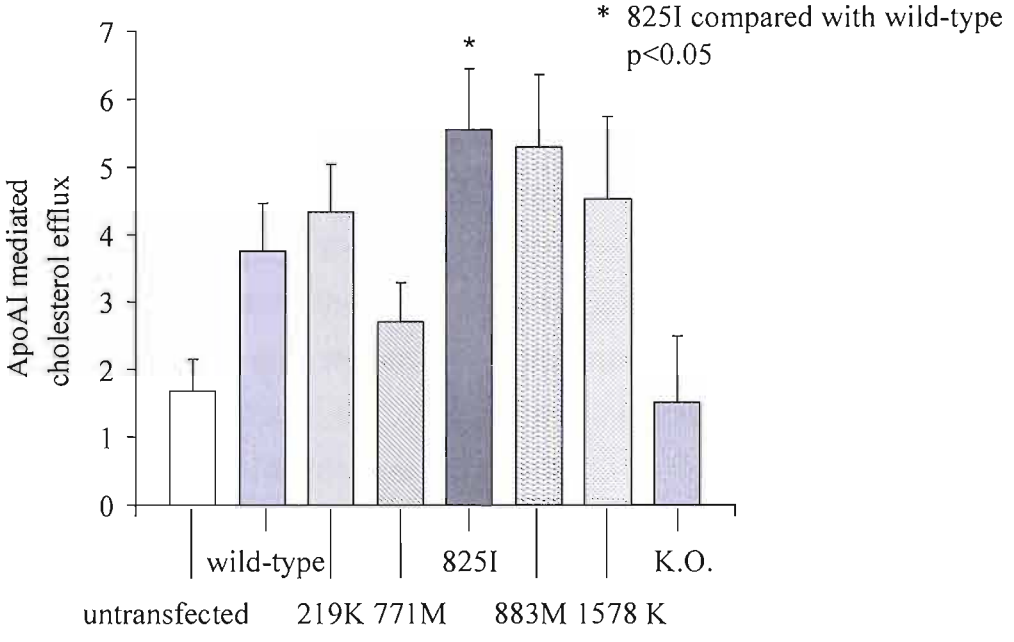

Figure 3.1 Results of apoAI-mediated cholesterol efflux assay of ABCA1 coding SNPs

Bars represent mean (standard error of mean) values from three independent experiments, in each of which transfection and cholesterol efflux assay were carried out in triplicate for each construct. Asterisk indicates $p<0.05$ comparing the rate of apoAI-mediated cholesterol efflux in cells transfected with the plasmid expressing the 825I isoform with that in cells transfected with the plasmid expressing the wild-type (825V) isoform.

3.11 Foam cell formation assay of ABCA1 gene coding polymorphisms

To investigate whether any of the coding polymorphisms had an effect on foam cell formation, cells were transfected with plasmids expressing the different ABCA1 alleles and incubated with acetylated LDL, followed by Oil-Red O staining for microscopic examination to determine the rate of cells that had become lipid-laden foam cells. The experimental procedure and method for calculated the rates of foam cell formation and statistical analysis are described in the Methods chapter, section 2.6.10.

The assays showed that the rate of cells that became lipid-laden foam cells were lower in those expressing the 219K isoform than those expressing the ABCA1 wildtype isoform ($p=0.012$, figure 3.2). The rates of cells that became lipid-laden foam cells were also slightly lower in those expressing the 771M, 825I, and 883M isoforms respectively, in comparison to the cells expressing the wild-type ABCA1, However, the differences did not reach statistical significance (figure 3.2). Comparing cells expressing the 1587K isoform and cells expressing the wild-type, there was little difference in the rate of lipid-laden foam (figure 3.2).

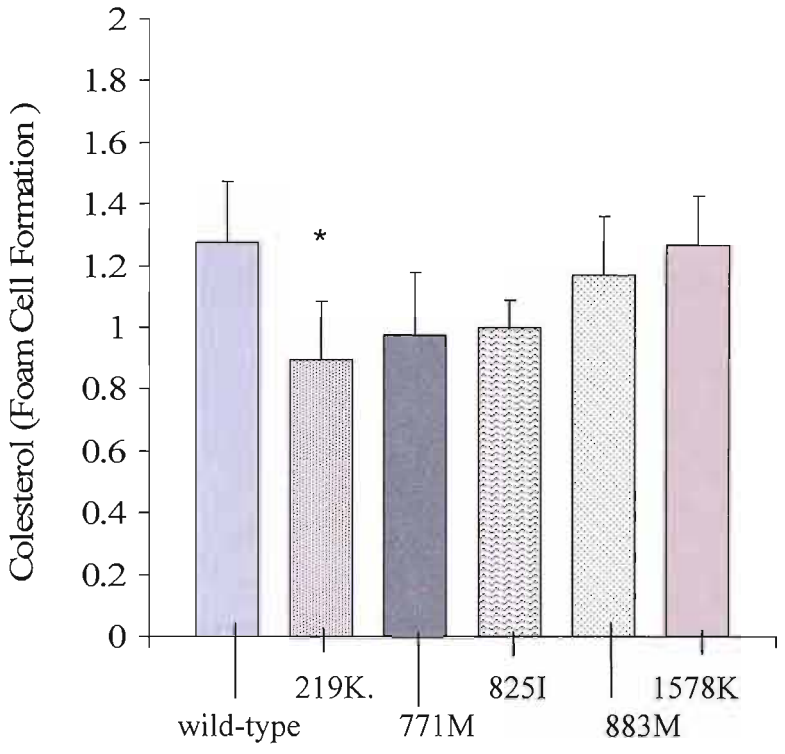

Figure 3.2 Results of foam cell formation of ABCA1 coding SNPs

Each bar shows an arbitrary unit of foam cell formation (the experimental procedure and method for calculated the rates of foam cell formation and statistical analysis are described in the Methods chapter, section 2.6.10.). Because the data shown are relative to the rate of foam cell formation in untransfected cells as described in section 2.6.10., the rate of foam cell formation in untransfected cells can not be drawn in the figure. Asterisk indicates p=0.012 comparing cells transfected with the plasmid expressing the 219K isoform with cells transfected with the plasmid expressing the wild-type (219R) isoform.

3.12 ABCA1 promoter assays

Transient transfection and reporter assays were performed by another member of the group to investigate whether the different ABCA1 promoter haplotypes had different promoter activity. The assays showed that the T-C-C-C-G-C, T-C-T-C-G-T and T-C-C-C-G-T haplotypes which contained the -565T, -407C and -278C alleles, had higher promoter activity than the wildtype haplotype C-G-C-G-G-C which contained the -565C, -407G and -278G alleles ($p<0.01$ comparing T-C-C-C-G-C and C-G-C-G-G-C, $p<0.05$ comparing T-C-T-C-G-T and C-G-C-G-G-C, $p>0.05$ comparing T-C-C-C-G-T and C-G-C-G-G-C) (figure 3.3).

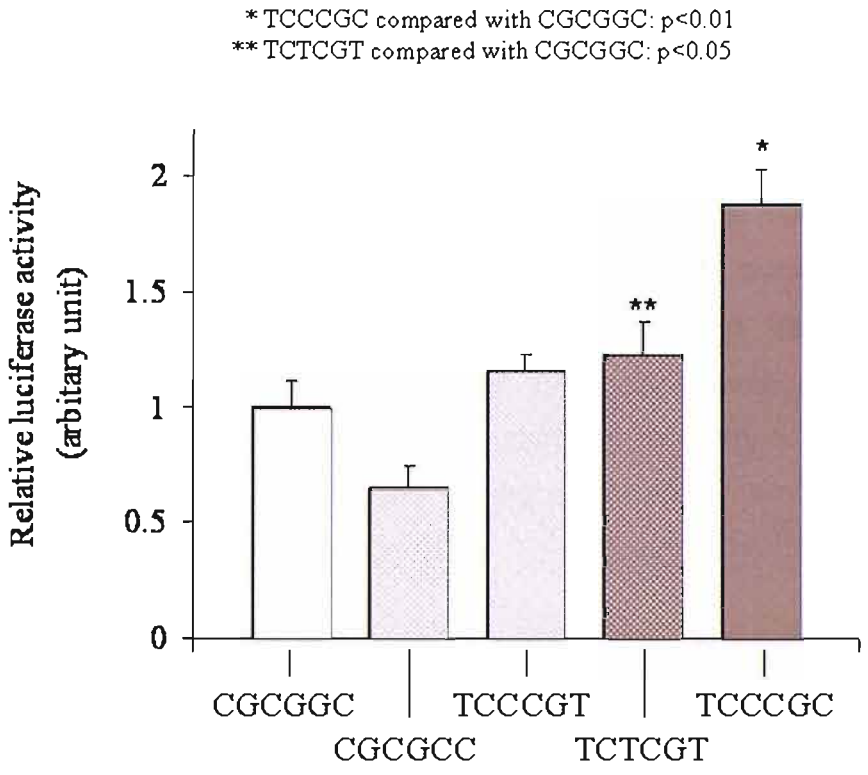

Figure 3.3 Relative promoter activity of ABCA1 gene promoter haplotypes

Data showed are from transient transfection and reporter assays. For each haplotype, the corresponding ABCA1 promoter (from position -588bp to +21bp relative to the transcriptional start site) was generated by PCR using genomic DNA as template and then inserted into a pGL3-basic vector which contains a firefly luciferase reporter gene. RAW264.7 cells were transfected with each of these constructs. A pRL-TK plasmid (Promega) containing a renilla luciferase gene under the control of a thymidine kinase promoter, was co-transferred into the cells to serve as a reference for transfection efficiency. At 36 hours after transfection, the cells were lysed, and the activities of firefly luciferase and renilla luciferase in the lysates were measured with the use of a dual-luciferase assay kit (Promega). ABCA1 gene promoter activity was determined by the ratio of firefly luciferase activity to renilla luciferase activity. Data shown in the figure are mean (standard error of mean) from three independent experiments in each of which transfection and luciferase assays were carried out in duplicate for each construct.

4 Discussion

4.1 Introduction to discussion

Genetic mutations in ABCA1 are responsible for the cause of Tangier disease and familial HDL deficiency. These findings have led to the hypothesis that common polymorphisms in this gene might be a genetic factor that influences lipid levels and phenotypes of coronary heart disease in the general population. We tested this hypothesis in this study. The study also examined the effects of common polymorphisms on plasma levels of total cholesterol, HDL-cholesterol and triglyceride, and the age of onset of symptoms in coronary artery disease. The study also examined the influence of the coding polymorphisms on cholesterol efflux and foam cell formation.

One of the key findings of the study is an association between HDL-cholesterol levels and three coding region polymorphisms (i.e. V825I, I883M and R1587K), with the 825I, 883M and 1587R alleles being associated with increased HDL-cholesterol levels. The study also detected an association between the V771M allele and reduced total cholesterol levels and a trend towards reduced HDL-cholesterol levels. The functional studies showed that the 825I and 883M alleles had increased cholesterol efflux, where the 771M allele had reduced cholesterol efflux.

Another major finding of the study is an association of -565 C>T, -407G>C and -278G>C polymorphisms with age of onset of symptom in coronary artery disease, with the -565T, -407C and -278C being associated with high age of onset. Functional assays showed that the promoter haplotypes containing the -565T, -407C and -278C alleles had higher promoter activity than the wildtype haplotype containing the -565T, -407C and -2738C alleles.

The associations observed might arise from one of the following possibilities:

1. The associations observed could be statistical type I errors (false positive findings). Statistical analyses were carried out to test the association of ABCA1 polymorphisms with HDL-cholesterol, total cholesterol, triglyceride and the age of onset of symptom in coronary artery disease. Most of the associations observed will not be statistically significant if applying Bonferroni correction for multiple testing which requires a

significance level at p=0.00045 [there were 110 tests, including 3 lipid traits each in 3 conditions (i.e. the sample as a whole, untreated group, and treated group) plus the age of symptom onset of CAD, all versus 11 polymorphisms]. However, Bonferroni correction might not be appropriate since the traits tested (HDL-cholesterol, total cholesterol, triglyceride and coronary artery disease) are not independent to each other, nor are the polymorphisms.

2. Because the study was carried out on unrelated individuals rather than in a collection of families, it is possible that the associations observed are due to population admixture, i.e. it is possible that the study sample contains groups of Caucasians from different origins, that the genotype frequencies of the ABCA1 polymorphisms differ among these groups, and that the distributions of HDL-cholesterol and the age of onset of symptom in coronary artery disease also differ among these groups. However, the subjects in this study were all recruited from the Southampton General Hospital which admits patients mainly from the Wessex region in Southern England, and studies have shown no evidence of significant genetic subdivision in Southern England, while there is evidence of subdivision in Central England marked by an excess of a Viking Y haplotype[219].

3. The associations might have arisen from linkage disequilibrium of the polymorphisms studied with certain unknown sequence variations that affect lipid levels and coronary artery disease.

4. The associations might have arisen from the direct effect of the polymorphisms on the activity or expression of ABCA1, and consequently on the HDL-cholesterol synthesis and atherogenesis.

Further studies in other samples and functional analyses could help resolve these issues.

4.2 The association of ABCA1 gene polymorphisms with HDL-cholesterol levels

In this study, the V825I polymorphism seems to have a co-dominant effect on HDL-C level, with the I allele homozygotes having highest, heterozygotes having intermediate and V allele homozygotes having lowest HDL-C levels. For the I883M

and R1587K polymorphisms, however, the allele associated with lower HDL-cholesterol level has a recessive effect, and the allele associated with the higher HDL-cholesterol level has a dominant effect. For example, the K allele (the minor allele) of the R1587K polymorphism is associated with a lower HDL-cholesterol level, and the K allele homozygotes had 10.4% and 13.2% lower HDL-cholesterol levels than heterozygotes and R allele homozygotes, respectively, whilst heterozyogtes had only a 3.1% lower HDL-cholesterol level than R allele homozygotes, which is consistent with a recessive effect for the K allele. This suggests that any effect of these amino acid substitutions on ABCA1 function is moderate, and an appreciable effect on HDL-cholesterol level only occurs when both copies of the ABCA1 gene contain the deleterious allele. In contrast, heterozygotes for severe ABCA1 gene mutations have been shown to have significantly lower HDL-cholesterol levels than individuals with a normal ABCA1 gene[220].

An association of the V825I, I883M and R1587K polymorphisms with HDL-cholesterol levels has been shown in some other studies reported either before or during the course of this project. Table 4.1 summarises these studies. The results of these studies including the present work are generally in agreement, with the I allele of the V825I polymorphism and the M allele of the I883M polymorphism being associated with higher HDL-cholesterol levels, and the K allele of the R1587K being associated with lower HDL-cholesterol levels. None of these studies shows an association between the R219K polymorphism and HDL-cholesterol level. The V771M SNP variant is documented to be associated with increased HDL-C in some studies. These generally consistent findings suggest that the association of the V825I, I883M and R1587K polymorphisms with HDL-cholesterol levels is not a statistical false finding.

SNP	studies	Genotype	HDL (mmol/L)	Allele Frequency	Number of sample	Type of sample	Age of sample	p-value
R219K	Clee *et al.* 2001	RR/RK/KK	0.93/0.94/0.93	0.25	804	Dutch men with CAD	53-57	n.s.
	Harada *et al.* 2003	RR/RK/KK	1.26/1.25/1.25	0.488	410	Japanese	65.4	n.s.
	Frikke-Schmidt *et al.* 2004	Male RR/RK/KK	1.40/1.37/1.39	0.26	9259-45%		57	n.s.
		Female RR/RK/KK	1.73/1.71/1.70		55%			n.s.
	Evans and Ulrich 2003	RR/RK/KK	1.32/ 1.35/1.27	0.25	813	Germany	45	-
		RR/RK/KK (after treatment)	1.22/ 1.24/1.17					
	Hodoglugil *et al.* 2005	Males RR/RK/KK	0.91/0.91/0.91	0.35	2700	Turkish	41	n.s
		FemalesRR/RK/KK	1.07/1.06/1.07					n.s
	Salaheen *et al.* 2005	RR/RK/KK/R/K	1.06/1.10/1.10/1.08/1.09	0.37	200	Pakistan	49	ns
	Yamakawa-Kobayashi *et al.* 2004	RR/RK/KK	1.41/1.35/1.47	0.29	327	Japanese children	9-15	n.s
	This study	RR/RK/KK	1.29/1.26/1.23	0.285	630	UK	63	n.s.

Table 4.1 Comparison of different studies

SNP	studies	Genotype	HDL (mmol/L)	Allele Frequency	Number of sample	Type of sample	Age of sample	p-value
V771M	Clee *et al.* 2001	VV/VM,MM	0.92/0.91	0.029	804	Dutch men with CAD	53-57	n.s.
	Frikke-Schmidt *et al.* 2004	Male			9259-45%		57	n.s.
		Female			55%			n.s.
	Hodoglugil *et al.* 2005	Males VV/VM/MM	0.91/0.96/1.18	0.051	2700	Turkish	41	p<0.01
		Females VV/VM/MM	1.05/1.12/1.00					n.s
	Salaheen *et al.* 2005	MM/VM/VV/M/V	1.09/0.98/1.07/1.04/1.07	0.111	200	Pakistan	49	n.s
	Yamakawa-Kobayashi *et al.* 2004	VV/VM/MM	1.38/1.47/1.49	0.009	327	Japanese children	9-15	n.s
	This study	VV/VM/MM	1.28/1.20/0.97	0.024				n.s.

SNP	studies	Genotype	HDL (mmol/L)	Allele Frequency	Number of sample	Type of sample	Age of sample	p-value
V825I	Clee *et al.* 2001	VV/VI + II	0.92/0.94	0.081	804	Dutch men with CAD	53-57	n.s.
	Frikke-Schmidt *et al.* 2004	Male VV/VI + II	1.39/1.28	0.06	9259-45%		57	p=0.09
		Female VV/VI + II	1.70/1.86		55%			p=0.008
	Hodoglugil *et al.* 2005	Males VV/VI/II	0.93/0.96/-	0.062	2700	Turkish	41	n.s
		Females VV/VI/II	1.05/1.01/1.09					n.s
	Salaheen *et al.* 2005	LL/LV/VV/L/V	0.89/1.06/1.07/1.07/1.00	0.26	200	Pakistan	49	LL , p 0.02 L p 0.05
	Yamakawa-Kobayashi *et al.* 2004	VV/VI/II	1.38/1.42/1.39	0.11	327	Japanese children	9-15	n.s
	This study	VV/VI/II	1.23/1.34/1.54	0.057	630	UK	63	p=0.05 (V/V vs V/I + I/I in untreated patients)

SNP	studies	Genotype	HDL (mmol/L)	Allele Frequency	Number of sample	Type of sample	Age of sample	p-value
I883M	Clee *et al.* 2001	II/IM + MM	0.92/0.92	0.136	804	Dutch men with CAD	53-57	n.s.
	Frikke-Schmidt *et al.* 2004	Male II/IM + MM	1.37/1.39	0.12	9259-45%		57	n.s.
		Female II/IM + MM	1.71/1.70		55%			n.s.
	Hodoglugil *et al.* 2005	Males II/IM/MM	0.91/0.93/0.92	0.21	2700	Turkish	41	n.s
		Females II/IM/MM	1.06/1.06/1.07					n.s
	Yamakawa-Kobayashi *et al.* 2004	MM/MI/II	1.40/1.39/1.43	0.36	327	Japanese children	9-15	n.s
	This study	II/IM/MM	1.20/1.40/1.35	0.144	630	UK	63	P=0.001 (I/I vs I/M + M/M in untreated patients)

SNP	studies	Genotype	HDL (mmol/L)	Allele Frequency	Number of sample	Type of sample	Age of sample	p-value
R1587K	Clee *et al.* 2001	RR/RK + KK	0.91/0.95	0.25	804	Dutch men with CAD	53-57	p=0.03
	Frikke-Schmidt *et al.* 2004	Male RR/RK/KK	1.40/1.35/1.33	0.24	9259-45%		57	p=0.07
		Female RR/RK/KK	1.73/1.67/1.62		55%			p=0.007
	Hodoglugil *et al.* 2005	Males RR/RK/KK	0.93/0.91/0.91	0.33	2700	Turkish	41	n.s
		Females RR/RK/KK	1.06/1.06/1.06					n.s
	Yamakawa-Kobayashi *et al.* 2004	RR/RK/KK	1.41/1.40/1.38	.18	327	Japanese children	9-15	n.s
	This study	RR/RK/KK	1.30/1.25/1.11	0.237	630	UK	63	p=0.028

ApoA1 is the main apolipoprotein in HDL. A study which related ABCA1 gene polymorphisms to ApoA1 levels rather than HDL levels showed that the 1587K allele was associated with reduced plasma apoA1 levels[221], which is consistent with the finding of this allele with reduced HDL-cholesterol levels in some other studies.

In contrast to the coding region polymorphisms, the promoter polymorphisms have been less intensively studied in relation to lipid levels. In this study, there was no association between any of the promoter polymorphisms with HDL-C levels. Zwarts *et al.* also found no association between ABCA1 promoter polymorphisms and lipid levels[222]. However, the -565C>T polymorphism has been reported to be associated with HDL-cholesterol level in a study by Lutucuta *et al.*[199] and with ApoA1 level in a study by Tregouet *et al.*[221], although it should be noted that in the study by Lutucuta *et al.*, it was found that heterozygotes had lower levels of HDL-cholesterol levels than homozygotes for the C allele and homozygotes for the T allele, which is not consistent with usually patterns of genetic association, raising the question whether this observed association is a false finding.

4.3 The association of ABCA1 polymorphisms with total cholesterol levels

Apart from the V771M polymorphism, this study found no association between the ABCA1 gene polymorphisms and total cholesterol levels. This is consistent with findings from many other studies (most papers do not show the total cholesterol data but just mention their findings, which makes it difficult to put together a table similar to the one for HDL-cholesterol in section 4.2, table 4.1).

For example, Frikke-Schmidt *et al.*[193] genotyped all common ABCA1 coding region polymorphisms including R219K, V771M, V825I, I883M and R1587K in a large Danish population sample and found no association between any of the polymorphisms with total cholesterol level despite some of these polymorphisms were found to be associated with HDL-cholesterol levels as discussed above. Clee *et al.* studied these polymorphisms a group of Dutch CAD patients and also did not find any association between these polymorphisms and total cholesterol levels[190].

Several studies examining only the R219K polymorphism also found no association between this polymorphism and total-cholesterol levels. These studies include a study from Garman[192], a Hungarian study[223], a Japanese study[224], and a study of individuals with familial hypercholesterolaemia[225]

In addition, Lutucuta *et al.* studied the -565C>T polymorphism (which is referred to as -477C>T in their paper) and found no association between this polymorphism and total cholesterol levels[199].

An association between the V771M polymorphism and total cholesterol levels was observed in this study but not in other studies. This raises that possibility that the association found in this study is a spurious finding, although a true association cannot be ruled out. Further studies in other samples will be required to further test these possibilities, given that this polymorphism has been less well studied than the R219K polymorphism. It is interesting to note that in this study, carriers of the 771M allele not only had lower total cholesterol level but also a trend towards lower HDL-cholesterol (statistically non-significant), and the cholesterol efflux assay showed that the 771M allele had reduced cholesterol efflux than the 771V (statistically non-significant).

4.4 The association of ABCA1 polymorphisms with triglyceride levels

The study showed a weak association between the -278 G>C polymorphism and triglyceride levels in patients not on lipid-lowering treatment. None of the other SNPs studied was found to be associated with triglyceride levels.

Most publications have not reported any association between ABCA1 gene SNPs and triglyceride levels. However, Clee *et al.* showed an association of the R219K SNP with triglyceride levels in a group of Dutch patients with coronary artery disease[190]. In that study, the 219K allele was associated with lower triglyceride levels.

The mechanisms underlying the association between ABCA1 SNPs and triglyceride levels are unclear, but there is generally an inverse relationship between HDL-cholesterol and triglyceride levels.

4.5 The association of ABCA1 gene polymorphisms and age of onset of symptomatic CAD

This study showed that age of onset of symptomatic CAD in the general population is associated with common polymorphisms of the ABCA1 gene. In families of Tangier disease patients with severe *ABCA1* mutations, it has been reported that age of onset of CAD is approximately 20 years earlier in *ABCA1* mutant homozygotes and about 10 years earlier in heterozygotes as compared with mutation non-carriers. The study found the mean age of onset being 38.0, 48.9 and 60.4 years respectively[220]. Our study showed that the age of onset of symptoms in CAD patients in the general population was approximately 2.5 years earlier in homozygotes of "deleterious" alleles (i.e. -565C, -407G, and -278G) of several common polymorphisms in the *ABCA1* promoter than in homozygotes of "protective" alleles (i.e. -565T, -407C, and -278C). The heterozygotes had intermediate mean age of onset of approximately 59, 60 and 61.5 years respectively. The finding gives a new outlook of relationships between *ABCA1* variations and inter-individual variability of CAD in the general population. This is consistent with the idea that the phenotypic effects of common genetic variations are moderate as compared with that of rare, severe mutations.

Several previous studies have encountered a situation where ABCA1 SNPs are associated with CAD in the absence of an association with plasma HDL level[199,220]. In this study, we observed that the -565C>T, -407G>C and -278G>C SNPs were associated with age of onset of symptomatic CAD without an association with plasma HDL level. It has been suggested that subtle changes in cellular cholesterol efflux in the vascular wall could have an impact on atherogenesis, without an apparent effect on plasma HDL level[199,220]. In contrast with the traditional "reverse cholesterol transport" theory in which HDL is thought to originate from peripheral tissues and subsequently transferred to the liver, recent studies have revealed that the major source of plasma HDL is actually the liver[226,227], and that cholesterol efflux from macrophages accounts for only a very small portion of the total plasma HDL but nevertheless is very important with regard to the development of atherosclerosis[228-230]. As discussed in the Introduction section, the human ABCA1 gene contains two promoters, one being located upstream of exon 1 and the other situated within intron[166]. It has been shown that in macrophages, ABCA1 expression is driven only

by the promoter upstream of exon 1, whereas in liver cells, ABCA1 expression is controlled also by the internal promoter located in intron[231]. The -565C>T, -407G>C and -278G>C SNPs reside in the promoter upstream of exon 1, and therefore it would be expected that these SNPs would have a greater effect on ABCA1 transcription in macrophages than ABCA1 transcription in the liver. This could potentially be another explanation for the finding that these SNPs were associated with CAD but not with plasma HDL level.

This study also showed a trend towards higher age of onset of symptomatic CAD in 825I and 883M carriers. As discussed above, there is a relationship between plasma HDL level and the V825I and I883M SNPs, with the 825I and 883M alleles being associated with higher HDL levels. In addition, functional assays demonstrated that the rate of cholesterol efflux was higher in cells expressing the 825I and 883M isoforms than in cells expressing the 825V and 883I isoforms. There was also a trend towards lower rates of foam cell formation. Thus, it is possible that a trend towards higher age of onset of symptomatic CAD in 825I and 883M carriers is a result of increased cellular cholesterol efflux and reduced foam cell formation.

4.6 ABCA1 promoter haplotypes and promoter activity

The promoter assays showed that the ABCA1 gene promoter haplotypes containing -565T, -407C, and -278C alleles had higher promoter activity than the haplotypes containing the -565C, -407G, and -278G alleles. This could potentially explain association of the haplotype containing -565T, -407C, and -278C alleles with higher age of onset of symptomatic CAD as increased ABCA1 expression would be expected to have a protective effect against atherosclerosis[117,232].

Studies of SNPs in the promoter of several other genes by other researchers have showed that the effect of an SNP can be dependent on haplotype context and that a particular allele of a SNP can reduce promoter activity in the context of one haplotype but increase promoter activity in the context of another haplotype[233-235]. In previous work by our group examining the effect of the -565C>T polymorphism in the C-407-C-302-C-278-G-99-C-14 haplotype background, it was found that the -565T allele had a lower promoter activity than the -559C allele. However, it was subsequently found that in the G-407-C-302-G-278-G-99-C-14 haplotype background, the -565T allele

had higher promoter activity than the -559C allele. This suggests that the effect of the ABCA1 -565C>T SNP is dependent on the context of the other ABCA1 promoter SNPs. The T-565-C-407-C-302-C-278-G-99-C-14 and the C-565-G-407-C-302-G-278-G-99-C-14 haplotypes have much higher frequency in the population than the C-565-C-407-C-302-C-278-G-99-C-14 and T-565-G-407-C-302-G-278-G-99-C-14 haplotypes (0.277 and 0.105 compared with 0.0007 and 0.001 respectively). Therefore, in the common haplotype background, the -565T allele has higher promoter activity than the -565C allele.

4.7 Cholesterol efflux and foam cell formation similarity

In this study, cells transfected to express the 825I isoform had a significantly higher rate of apoAI-mediated cholesterol efflux than cells transfected to express the 825V isoform. There was also a trend towards a lower rate of cholesterol laden foam cells in cells expressing the 825I isoform compared with cells expressing the 825V isoform. There has been no reported functional study of this SNP.

Similarly, the 883M isoform had a higher rate of apoAI-mediated cholesterol efflux from the cell as well as a lower rate of the foam cell formation compared with the 883I isoform. In contrast to this study, Brunham *et al.* have reported that cells transfected to express the 883M isoform were found to have a lower rate of cholesterol efflux[236]. However, the disparate findings between these two studies could potentially be due to different experimental designs. In the study by Brunham *et al.*, stable transfection of cells was performed. A drawback of the stable transfection technique is that the "exogenous" gene, the ABCA1 gene in this case, can be incorporated into different locations of the genome and, due to a positional effect, the expression levels of the "exogenous" gene can vary significantly depending on the locations where the "exogenous" gene is incorporated. Therefore, the results of the Brunham study could potentially be confounded by such positional effects. In our study, transient transfection was performed. Although in transient transfection, the expression of the "exogenous" gene could be influenced by the transfection efficiency, in this study, the rate of cholesterol efflux was standardised by fluorescence activated cell sorting.

Cells expressing the 219K isoform had a slightly higher rate of apoAI-mediated cholesterol efflux and a significantly lower rate of the foam cell formation than cells

expressing the 219R isoform. Native cholesterol was used in the cholesterol efflux assays, whereas acetylated cholesterol was used in the foam cell formation assays. It is possible that the R219K polymorphism has a greater effect on efflux of acetylated cholesterol than efflux of native cholesterol. Similar to this study, Clee *et al.* found increased cholesterol efflux in cells expressing the 219K allele compared with cells expressing the 219R allele[190]. The slightly higher rate of cholesterol efflux and significantly lower rate of foam cell formation in cells expressing the 219K isoform compared with cells expressing the 219R isoform could potentially explain the reported association between the 219K allele and a protective effect against CAD without a significant effect on plasma HDL-cholesterol[190].

Cells expressing the 771M allele had lower rate of cholesterol efflux and lower rate of foam cell formation than cells expression the 771V isoform, but neither was statistically significant.

In both the apoAI-mediated cholesterol efflux and foam cell formation assays, the 1587K SNP did not have a significant effect.

4.8 Linkage disequilibrium between SNPs

The study noted that the SNPs in the ABCA1 promoter are in strong linkage disequilibrium (LD), which is not surprising given the close proximity of these polymorphisms. There is also some LD between the polymorphisms in the coding region, but the LD here is weaker than the LD between the polymorphisms in the promoter. This is also not surprising as these coding region polymorphisms spread out in a larger genomic region than the promoter polymorphisms. There is little LD between these two blocks, which suggests that there have been considerable recombination events in the sequence between these two blocks.

The linkage disequilibrium between the ABCA1 SNPs might explain some of the relationships of ABCA1 SNPs with lipid levels and CAD phenotypes. For example, both the V825I and I883M SNPs are associated with plasma HDL-cholesterol levels, and it is possible that only one of these polymorphisms is functional whereas the other is marking the effect of the functional polymorphism through linkage disequilibrium, although it is also possible that both polymorphisms are functional.

The cholesterol efflux assays showed that cholesterol efflux was significantly higher in cells expressing the 825I isoform than in cells expressing the 825V isoform, suggesting that the V825I polymorphism has a functional effect. However, the assays also showed that there was a trend towards higher cholesterol efflux in cells expressing the 883M isoform than cells expressing the 883I although the difference was statistically significant. Therefore, it is possible that the I883M SNP might also have a functional effect.

This study and the study by Frikke-Schmidt *et al.*[193] showed an association between lower HDL-C and the 1587K allele. The cholesterol efflux assays and foam cell formation experiments did not show a significant effect of this SNP on cholesterol efflux. However, the possibility that this SNP has a functional effect can not be precluded. As the major source of HDL is the liver as discussed in the Introduction section, functional studies using liver cells would be more informative in addressing whether this SNP has a functional effect that could explain the association of this SNP with HDL-C levels. An alternative explanation of the association would be that the association arises from linkage disequilibrium of this SNPs with functional SNPs in the ABCA1 gene. However, this seems an unlikely explanation, as there is no strong linkage disequilibrium between this SNP and any other SNPs studied.

Similarly the associations of the promoter SNPs, -565C>T, -407C>G and -278C>G with age of onset of symptom in CAD could result from linkage disequilibrium, i.e. it is possible that some of these SNPs are functional and some of them are functionally neutral, and the associations of these SNPs with age of onset of symptom in CAD are the results of linkage disequilibrium between these SNPs. However, it is also possible and perhaps plausible that these SNPs together exert a haplotype effect on ABCA1 promoter activity.

4.9 The association of ABCA1 gene polymorphisms and coronary stenosis

In this study we have demonstrated an association between patients homozygous for the V allele of the V771M polymorphism, and the tendency of having greater mean number of coronary arteries with >50% stenosis (p=0.004). This association remained significant even with multivariate analysis with other factors and

interventions implicated in atherosclerosis (p=0.031). The finding was exclusively observed in this homozygote for V771 of all the ABCA1 SNPs tested in this study.

As we have shown in this thesis the 771V allele is functional with effects on both cholesterol efflux and foam cell formation, both of which lead to atherogenesis. Thus, the effect of the 771V allele on these process is the most likely the explanation for the observed increase in the mean number of coronary arterieswith >50% stenosis. However, other possibilities may also exist. The observation may also have arisen as a result of linkage disequilibrium of the 771V allele with certain unknown sequence variations. These variations may influence lipid levels and coronary artery disease. Another possibility is that this SNP has additional functionality altering responses of human cells or enhancement of functional effects by an as yet unidentified mechanism.

In support of our findings, Clee et al.[190] in their study found that male subjects with the 771M allele had reduced focal atherosclerosis, (although without alterations in plasma lipid levels). By implication these patients would be expected to have a smaller degree of coronary stenosis than subjects carrying the 771V allele, in line with the significant finding in our study. In a small case-control study from Hungary by Andrikovics et al.[237] was also shown that the 771M allele was associated with a protective role against CHD and possibly cerebrovascular disease. Furthermore, Yamakawa-Kobayashi et al.[197] demonstrated that M771 allele appears to be an anti-atherogenic allele, despite the observation both the M771 and V771 alleles were noted to have similar levels of HDL-C in the study. Singaraja et al.[238] recognized V771M as putative anti-atherogenic SNP associated with increased HDL-C and reduced triglycerides while M771 was characterized by increased HDL-C and ApoA-I.

In summary, there appears to be a consistent observation that either the 771M allele confers protection from cardiovascular disease, or, as in our study, the V771 allele increases severity. Whether this effect is mediated through effects on plasma lipid levels, foam cell formation or some other biological mechanism remains to be establsihed.

4.10 Summary of discussion

In summary, the results of this study support the hypothesis that common polymorphisms in the ABCA1 gene could influence HDL-cholesterol levels and the development of atherosclerosis in the population. The study also suggests that the ABCA1 gene polymorphisms have only moderate effects on these traits which are thought to have a multifactorial aetiology.

The functional assays suggest that the SNPs in the ABCA1 promoter exert a haplotype effect on ABCA1 promoter activity, that the V825I SNP has an effect on cholesterol efflux, and that the R219K SNP has an effect on acetylated cholesterol laden foam cell formation. These functional effect could potentially explain some of the associations of ABCA1 gene SNPs with lipid levels and CAD.

4.11 Further studies

Further genetic epidemiological studies and functional studies would be required to address the following questions.

1. Whether the findings of the association of the ABCA1 SNPs with age of onset in CAD patients could be replicated in other population samples and other ethnic groups.

To address this question will require further studies of the ABCA1 SNPs in other large CAD cohorts. The associations of the coding region SNPs with lipid levels have been shown in a number of studies, and therefore replications studies for these associations will be less important than for the association with age of CAD onset.

2. Whether any SNPs located in the second promoter, introns and 3' untranslated regions of the ABCA1 gene are associated with lipid levels and CAD traits. This study only examined SNPs in the first promoter and non-synonymous coding SNPs in the ABCA1 gene. It is possible that lipid levels and CAD traits could also associated with SNPs in other regions, such as the second promoter (located in intron 1), introns and 3' untranslated regions, as these regions could play a role in the regulation of ABCA1 transcription, RNA splicing, and/or RNA stability. Genetic epidemiological studies and functional studies of SNPs in these regions would be warranted.

3. Whether any of the ABCA1 SNPs exert functional effects in other cell types, such as liver cells, that have not been examined in this study. The functional studies of the promoter SNPs were carried out in RAW264.7 cells which are murine macrophage-like cells, and the cholesterol efflux assays and foam cell formation experiments of the coding region SNPs were performed in COS7 cells which are African green monkey kidney cells, partly due to practical reasons as discussed in the Methods section, i.e. transfection of HepG2 cells and RAW264.7 cells with the ABCA1 expressing plasmids proved to be difficult. Since the liver is the major source of HDL and some of the coding region SNPs (i.e. V825I, I883M and R1587K) were associated with HDL levels, it would be interesting to study whether these SNPs have functional effects in liver cells such as HepG2 cells. Other transfection methods that had not been used in this study, such as electroporation, could be tested, and if high

transfection efficiency could be achieved, cholesterol efflux assays could be performed. Similarly, if high transfection efficiency in macrophages such as RAW264.7 cells with the ABCA1 expressing plasmids could be achieved, foam cell formation experiments could be performed.

4. Whether any of the ABCA1 SNPs exert synergistic or epistatic functional effects. To address this question, cholesterol efflux assay and foam cell formation assays could be performed using plasmids that contain different combinations of the alleles of the coding region SNPs.

Appendix 1 Optimization of transfection

1. Optimization of transfection of Fugene 6

Make 6 tubes

1st add
- **100μL** Serum free medium
- **6μL** (3μL x 2) Fugene 6
- 1μg DNA (plasmid) **10μL**

2nd add
- **100μL** Serum free medium
- **12μL** (3μL x 2) Fugene 6
- 1μg DNA (plasmid) **10μL**

3rd add
- **100μL** Serum free medium
- **20μL** (3μL x 2) Fugene 6
- 1μg DNA (plasmid) **10μL**

4th add
- **100μL** Serum free medium
- **6μL** (3μL x 2) Fugene 6
- 1μg DNA (plasmid) **20μL**

5^{th} add
- **100μL** Serum free medium
- **12μL** (3μL x 2) Fugene 6
- 1μg DNA (plasmid) **20μL**

6th add
- **100μL** Serum free medium
- **20μL** (3μL x 2) Fugene 6
- 1μg DNA (plasmid) **20μL**

Then incubate these 6 tubes at room temperature for 15min

Then add the mixture to the over night cell.

2. Optimization of transfection of gene carrier

Optimization for transfection by GenCarrier (DNA/liposome complex)

1st tube
100 µL of low protein or serum- free and antibiotics- free medium.
Add 2µg DNA per 35 mm well 20 µL
Add 6 µL geneCarrier-2

2nd tube
100 µL of low protein or serum- free and antibiotics- free medium.
Add 1µg DNA per 35 mm well 10 µL
Add 8 µL geneCarrier-2

3rd tube
100 µL of low protein or serum- free and antibiotics- free medium.
Add 1µg DNA per 35 mm well 20 µL
Add 8 µL geneCarrier-2

*incubate these mixtures tubes for at least 15 (not more than 45 min) min at room temperature.

Transfection by GenCarrier-2

1. Wash the cell layer twice with **1 to 2 ml** amounts of low protein or serum-free and antibiotics- free medium.
2. Add **0.9 ml** of low protein or serum- free and antibiotics- free medium to each well.
3. Add the **110µl** (all) amount of the DNA/liposome complex to each well in a dropwise (drop by drop) and gently mix the solution by swirling the plate to ensure the entire layer is immersed in solution.
4. Incubate the cell for **5 to 18 hours** under conditions normally used to culture the cell.

Next day

5. Following incubation, add a **1 ml** amount of medium containing 2 times the normal serum and antibiotics concentration.
6. Incubate the cell an additional **18-24 hours** under conditions normally used to culture the cell.
7. Aspirate (remove and change) the medium and replace with fresh 1x medium containing the additives normally used to culture the cell.
8. Assay the cells using the appropriate protocol 24-72 hours after the addition of fresh medium in step 7.

Appendix 2 Hardy-Weinberg Law (HWL) (HW eq.)

It is one of the basic principles of population genetics. Some conditions are presumed in this law:

1. The population size is indefinite or large enough to ignore sampling error
2. Random mating
3. No genotype has any kind of selective advantage, i.e. all of them have equal chance of living and fertility
4. Confounding factors like mutation, migration and random genetic drift are absent.

Suppose that in such a population a locus has two alleles M and m. The frequency of dominant allele M is represented as p and that of recessive one, m, as q. Obviously, p + q = 1. A punnett square shows the possible combinations of gametes:

	M(p)	m(q)
M(p)	MM p^2	Mm pq
M(q)	mM qp	Mm q^2

We see that the probability of the occurrence of MM is p × p = p2. It is the same as other genotypes:

Mm and mM = pq × qp = 2pq
mm q × q = q2
Above explanation implies that genotype frequencies are determined by allele frequencies. The distribution of genotypes in the next generation will be:

p2 + 2pq + q2 = 1

Another concept of HWL is that the frequencies of alleles remain constant from one generation to the next. A population in such a condition is said to be in a state of genetic equilibrium.

Appendix 3 ABCA1 sequence and SNP

Promoter SNPs

1 cctggagatc ctgttgactg tagcatggag ggggcttgtg cagctgaatg tctgcatgca

61 ggtggtggga gttctggaat atgatggagc tggaggtggg aagagaagta ggcttggggc

121 agctctctca tgccacctca ttctggccaa aactcaggtc aaactgtgaa gagtctaaat

181 gtgaatctgc ccttcaaggt ggctacaaag gtatctttgt caaggtagga gaccttgtgg

241 cctccacgtg cacttccagg gcctgcttgg gcctcttcta cgggtctgtc ctgagtcttc

301 tatgaatctg tccttcaggg cagattcata tttagactct tcacagtttg acctgagttt

361 tggccagaat aaggtgacat ttagtttgtt ggcttgatgg atgacttaaa tatttagaca

421 tggtgtgtag gcctgcattc ctactcttgc ctttttttt gcccctccag tgttttgggt

481 agttttgctc ccctacagcc aaaggcaaac agagaagttg gaggtctgga gtggctacat

541 aattttacac gactgcaatt ctctggctgc acttcacaaa tgtatacaaa ctaaatacaa

601 gtcctgtgtt tttatcacag ggaggctgat caatataatg aaattaaaag ggggctggtc

661 catattgttc tgtgtttttg tttgtttgtt ttgtttgttt ctttttttgt ttttgtggcc

721 tccttcctct caatttatga agagaagcag taagatgttc ctctcgggtc ctctgaggga

781 cctggggagc tcaggctggg aatctccaag gcagtaggtc gcctatcaaa aatcaaagtc

-564T

841 caggtttgtg gggggaaaac aaaagcagcc cattacccag aggactgtc **C** gccttcccct

901 caccccagcc taggcctttg aaaggaaaca aaagacaaga caaaatgatt ggcgtcctga

961 gggagattca gcctagagct ctctctcccc caatccctcc ctccggctga ggaaactaac

-407C

1021 aaaggaaaaa aaaattgcgg aaagca **G** gat ttagaggaag caaattccac tggtgccctt

1081 ggctgccggg aacgtggact agagagtctg cggcgcagcc ccgagcccag cgcttcccgc

-302T -278C

1141 gcgtcttagg c **C** ggcgggcc cgggcggggg aaggg **G** acgc agaccgcgga ccctaagaca

1201 cctgctgtac cctccacccc caccccaccc cacccacctc cccccaactc cctagatgtg

1261 tcgtgggcgg ctgaacgtcg cccgtttaag gggcgggccc cggctccacg tgctttctgc

-99C

1321 tgagtgactg aactacataa acagaggccg ggaa **G** ggggc ggggaggagg gagagcacag

-14T

1381 gctttgaccg atagtaacct ctgcgctcgg tgcagccgaa tctataaaag gaactagtc **C**

1441 cggcaaaaac cccgtaattg

Exon 1 and 2 SNPs (117) and Ins G 319

cccgtaattg cgagcgagag tgagtggggc cgggacccgc agagccgagc

1501 cgacccttct ctcccgggct gcggcagggc agggcgggga gctccgcgca ccaacagagc

+117G/C

1561 cggttctca **G** ggcgctttgc tccttgtttt ttccccggtt ctgttttctc cccttctccg

1621 gaaggcttgt caaggggtag gagaaagaga cgcaaacaca aaagtggaaa acaggtaaga

1681 ggctctccag tgacttactt gggcgttatt gttttgtttc gaggccaagg aggcttcggg

1741 aagtgctcgg tttcggggac tttgatccgg acccacat ccccaccact tgcaactcag

1801 atgggaccgg aggcggtgtt aaatggggag acgatgtcct agtacgagct ctggtgaccc

1861 caggactctg cgctgctgcg cttggggctt gcccgacggt ggagaccggg gagcatctct

Exon 7 SNP R219K (G/A)

70741 atatgaagtt ctccaaaaga cttcaaggac ccagcttcca atcttcataa tcctcttgtg

70801 cttgtctctc tttgcatgaa atgcttccag gtatttttgc aaggctacca gttacatttg

70861 acaagtctgt gcaatggatc aaaatcagaa gagatgattc aacttggtga ccaagaagtt

1051A

70921 tctgagcttt gtggcctacc aa **G**gagaaa ctggctgcag cagagcgagt acttcgttcc

70981 aacatggaca tcctgaagcc aatcctggtg agtagacttg ctcactggag aaacttcaag

71041 cactaatgct ttcggaatgt gaggcttttc cttggacagc atgactttgt tttgtagaaa

Exon 17 SNP V825I (G/A) 2826

103441 ggttacaaat ggaatcattt tatatgttac ttggtagccc accactcccc taaagggact

103501 ctataggtaa atactacttc tgcaccttat gattgatcca ttttgcaaat tcaaatttct

103561 ccaggtataa tttacactag aagagataga aaaatgagac tgaccaggaa atggataggt

103621 gactttgcct gtttctcaca garcctgctg tctcctgtgg cttttgggtt tggctgtgag

103681 tactttgccc tttttgagga gcagggcatt ggagtgcagt gggacaacct gtttgagagt

2826A

103741 cctgtggagg aagatggctt caatctcacc acttcg **G** tct ccatgatgct gtttgacacc

103801 ttcctctatg gggtgatgac ctggtacatt gaggctgtct ttccaggtac actgctttgg

103861 gcatctgttt ggaaaatatg acttctagct gatgtccttt ctttgtgcta gaatctctgc

103921 agtgcatggg cttccctggg aagtggtttg ggctatagat ctatagtaaa cagatagtcc

103981 aaggacaggc agctgatgct gaaagtacaa ttgtcactac ttgtacagca cttgtttctt

Exon 18 SNP I883M (A/G) 3044

104821 ctttccccac atccattcaa atctgttcct tctccaaagg atgtgtcaag gaggaaatgg
104881 acctggctgg gaaaccctca gaatactggg atgatgctga gcttggctca tacctgtgct
104941 ttgctttcag gccagtacgg aattcccagg ccctggtatt ttccttgcac caagtcctac
105001 tggtttggcg aggaaagtga tgagaagagc caccctggtt ccaaccagaa gagaat A tca
105061 gaaagtaagt gctgttgacc tcctgctctt tctttaacct agtgctgctg cctctgctaa
105121 ctgttggggg caagcgatgt ctcctgcctt tctaaaagac tgtgaaacca ctccaggggc
105181 agagaaatca catgcagtgt ccctttccaa atcctcccat gccatttatg tccaatgctg
105241 ttgacctatt gggagttcac ggtctcgatc cctgagggac attttctttg ttgtcttggc
105301 ttctagaaga gtatctttta cttgcccccc cccaaacaca catttcatgg tctcctaaca
105361 agctagaaga aagaggtaaa gacaagcgtg attgtggaac catagcctcg ctgcctgcct

Exon 35 SNP R1587K (G/A) 5255

128821 agtgaacaag gtagtggcat tgctcttcac agggccgtcc tgttgtccac aggttccaga
128881 ttgactgttg ccccttatct atgtgaacag tcacaactga ggcaggtttc tgttgtttac
128941 aggacagttc tgcagatcga tttctcaaca gcttgggaag atttatgaca ggactggaca
129001 cca G aaataa tgtcaaggta aaccgctgtc tttgttctag tagctttttg atgaacaata
129061 atccttatgt ttcctggagt actttcaact catggtaaag ttggcagggg cattcacaac
129121 agaaaagagc aaactattaa ctttaccagt gaggcagtac ggtgtagtgt agtgattcag
129181 agaatttgct ttgccaccag acataccagg taaccttgac taagttactt aacctatcta
129241 aacctcagtt tcctcatctg tgaaatggag acagtaatca tagctatttc caaactgttg

Appendix 4 Publication

Functional polymorphism in ABCA1 influences age of symptom onset in coronary artery disease patients[239].

Human molecular genetics

Kyriakou, T., Pontefract, D.E., Viturro, E., Hodgkinson, C.P., Laxton, R.C., Bogari, N., Cooper, G., Davies, M., Giblett, J., Day, I.N., Simpson, I.A., Albrecht, C. and Ye, S.

Human Genetics Division, School of Medicine, University of Southampton, UK.

ATP-binding-cassette-transporter-A1 (ABCA1) plays a pivotal role in intracellular cholesterol removal, exerting a protective effect against atherosclerosis. ABCA1 gene severe mutations underlie Tangier disease, a rare Mendelian disorder that can lead to premature coronary artery disease (CAD), with age of CAD onset being two decades earlier in mutant homozygotes and one decade earlier in heterozygotes than in mutation non-carriers. It is unknown whether common polymorphisms in ABCA1 could influence age of symptom onset of CAD in the general population. We examined common promoter and non-synonymous coding polymorphisms in relation to age of symptom onset in a group of CAD patients (n=1164), and also carried out *in vitro* assays to test effects of the promoter variations on ABCA1 promoter transcriptional activity and effects of the coding variations on ABCA1 function in mediating cellular cholesterol efflux. Age of symptom onset was found to be associated with the promoter -407G>C polymorphism, being 2.82 years higher in C allele homozygotes than in G allele homozygotes and intermediate in heterozygotes (61.54, 59.79 and 58.72 years respectively, p=0.002). In agreement, patients carrying ABCA1 haplotypes containing the -407C allele had higher age of symptom onset. Patients of the G/G or G/C genotype of the -407G>C polymorphism had significant coronary artery stenosis (>75%) at a younger age than those of the C/C genotype (p=0.003). Reporter gene assays showed that ABCA1 haplotypes bearing the -407C allele had higher promoter activity than haplotypes with the -407G allele. Functional analyses of the coding polymorphisms showed an effect of the V825I substitution on ABCA1 function, with the 825I variant having higher activity in mediating cholesterol efflux than the wild-type (825V). A trend towards higher symptom onset age in 825I allele carriers was observed. The data indicate an influence of common ABCA1 functional polymorphisms on age of symptom onset in CAD patients.

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