LIPOPROTEIN LIPASE AND THE ATP BINDING CASSETTE TRANSPORTER ABCA1: TWO GENES REGULATING PLASMA HIGH DENSITY LIPOPROTEIN CHOLESTEROL AND TRIGLYCERIDE LEVELS AND RISK OF CORONARY ARTERY DISEASE

By

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Abstract

Elevated plasma triglyceride (TG) levels are independent risk factors for atherosclerotic coronary artery disease (CAD). In contrast, increased high density lipoprotein cholesterol (HDL-C), is associated with protection against CAD. These studies investigated the relationship between alterations in two genes involved in TG and HDL metabolism, lipoprotein lipase (*LPL*) and the ATP-binding cassette transporter *ABCA1*, plasma lipid levels and atherosclerosis.

Following initial studies validating the use of the mouse as an animal model in which to study the effects of LPL on atherosclerosis, data from apolipoprotein E deficient and cholesterol-fed C57BL/6 mice demonstrated that the role of LPL in atherosclerosis is dependent on the site from which it is expressed. Increased plasma LPL activity is anti-atherogenic, while increased LPL protein within the blood vessel wall is pro-atherogenic. Similar trends were demonstrated in a feline model of LPL deficiency.

Three common *LPL* polymorphisms (cSNPs) are associated with altered lipid levels and severity of CAD. These studies have shown that: the N291S variant is associated with decreased enzymatic activity, and an increased postprandial TG response; the D9N variant is associated with decreased LPL secretion, increased TG, and is in linkage disequilibrium with the g(-93)t variant (itself associated with decreased TG); and the S447X variant is associated with both decreased TG and blood pressure, which may account for its reduction in atherosclerosis independent of its effects on lipids.

Our identification of *ABCA1* demonstrated it is an important determinant of plasma HDL-C levels. Heterozygosity for *ABCA1* mutations is associated with decreased plasma HDL-C, increased TG, and a three-fold increased risk of CAD compared to unaffected relatives. The residual efflux activity in carriers of each mutation is a strong predictor of plasma HDL-C levels and CAD.

Several cSNPs in the *ABCA1* gene were identified, and were associated with plasma lipid levels and the severity of CAD. The R219K cSNP is associated with increased plasma HDL-C, decreased TG and decreased atherosclerosis, while others showed moderate effects on plasma lipid levels and/or the severity of atherosclerosis.

In conclusion, genetic variation in both *LPL* and *ABCA1* influences plasma TG and HDL-C levels, and significantly alters the severity of atherosclerosis.

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List of Abbreviations

Acyl CoA acetyltransferase (ACAT)
Adenosine triphosphate (ATP)
Analysis of variance (ANOVA)
Angina pectoris (AP)
Apolipoprotein (apo)
Area under the curve (AUC)
ATP binding cassette transporter (ABC)
ATP binding cassette transporter, subfamily A, 1 (ABC1, ABCA1)
Base pair (bp)
Body mass index (BMI)
Cerebrovascular accident (CVA)
Cholesterol (chol.)
Cholesterol efflux regulatory protein (CERP)
Cholesteryl ester (CE)
Cholesteryl ester transfer protein (CETP)
Chylomicron (CM)
Chylomicron retinyl palmitate (CRP)
Chylomicron triglyceride (CTG)
Coronary artery bypass graft (CABG)
Coronary artery disease (CAD)
Coronary heart disease (CHD)
Cytomegalovirus (CMV)
Density (d)
Deoxynucleotide triphosphate (dNTP)
Diastolic blood pressure (DBP)
Dulbecco's modified eagle medium (DMEM)
Familial hypoalphalipoproteinemia (FHA)
Familial HDL deficiency (FHD)
Familial hypercholesterolemia (FH)
Fast performance liquid chromatography (FPLC)
Fetal bovine serum (FBS)
Free fatty acids (FFA)
Gradient gel electrophoresis (GGE)
Heparan sulphate proteoglycans (HSPG)
Hepatic lipase (HL)
High density lipoprotein (HDL)
Hour (hr)
Intermediate density lipoprotein (IDL)
Kilo-basepair (kb)
Knockout (KO)
Lecithin:cholesterol acyl transferase (LCAT)
Lipoprotein (Lp)
Lipoprotein lipase (LPL)
Low density lipoprotein (LDL)
Low density lipoprotein receptor (LDLr)

Low density lipoprotein receptor related protein (LRP)
Myocardial infarction (MI)
Neomycin (Neo)
Non-chylomicron retinyl palmitate (NCRP)
Non-chylomicron triglyceride (NCTG)
Not statistically significant (NS)
Oil red O (ORO)
Online Mendelian Inheritance in Man (OMIM)
Optimal cutting temperature (OCT)
Parts per million (ppm)
Percutaneous transluminal coronary angioplasty (PTCA)
Peripheral vascular disease (PVD)
Phosphate buffered saline (PBS)
Phospholipid (PL)
Polymerase chain reaction (PCR)
Population attributable risk (PAR)
Restriction fragment length polymorphism (RFLP)
Retinyl palmitate (RP)
Reverse cholesterol transport (RCT)
Single nucleotide polymorphism (SNP)
Single nucleotide polymorphism within the coding region (cSNP)
Systolic blood pressure (SBP)
Tangier disease (TD)
Total cholesterol (TC)
Transgenic (Tg)
Transient ischemic attack (TIA)
Triglyceride (TG)
Triglyceride rich lipoproteins (TGRL)
Very low density lipoprotein (VLDL)
Very low density lipoprotein receptor (VLDLr)

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Chapter 1: Introduction

1.1 Atherosclerosis

Atherosclerosis, or the accumulation of lipid within the blood vessel wall, is the primary cause of heart attack and stroke. It is the leading cause of death in the Western world and soon to become the leading cause of death in developing nations. In 1990, heart disease was the first and cerebrovascular disease, or stroke, was the second leading cause of death, accounting for 6.3 million and 4.4 million deaths, respectively[1]. These findings are not expected to change over the next 20 years, during which time heart disease and stroke have been predicted to be the first and fourth leading causes, respectively, of disability worldwide, and to remain the first and second causes, respectively, in developed countries[2]. It has been estimated that cardiovascular disease accounts for 9.7% of disability on a global basis, including factors such as accidents, war and other diseases[3].

Atherosclerosis is a multifactorial disease[4,5]. Proteins implicated to have a role in its development include those involved in lipoprotein metabolism, thrombosis, and inflammation, amongst many others[6-8]. Environmental influences such as diet, exercise, smoking behaviour, or alcohol consumption are independent risk factors and also interact with numerous genetic factors in these pathways[5]. These proteins interact in a complex manner leading to the pathological consequences of the disease such as angina, heart attack (myocardial infarction, MI), or stroke.

Atherosclerosis is a cumulative, lifelong process, beginning from birth or even before[9]. It has been estimated that by approximately age 50, 30% of the intimal surface of the coronary arteries is covered by atherosclerotic lesions[10]. One of the earliest events in the atherosclerotic process is the accumulation of lipid within the arterial wall[8,11]. (A description of the cellular architecture of the blood vessel wall is provided in Figure 1.1 (A).) The arterial endothelium is permeable to proteins within the plasma[12]. In addition, substances may move through this cell layer either by transcytosis or through gap junctions between the endothelial cells[5]. This may be more likely to occur at certain sites within the arteries, such as those where blood flow is reduced, allowing a greater interaction time between lipoproteins and the endothelium[8], or sites where the endothelium may have increased permeability to substances from the plasma[5]. The concentration of these substances within the intima is generally proportionate with their concentrations in the plasma[12].

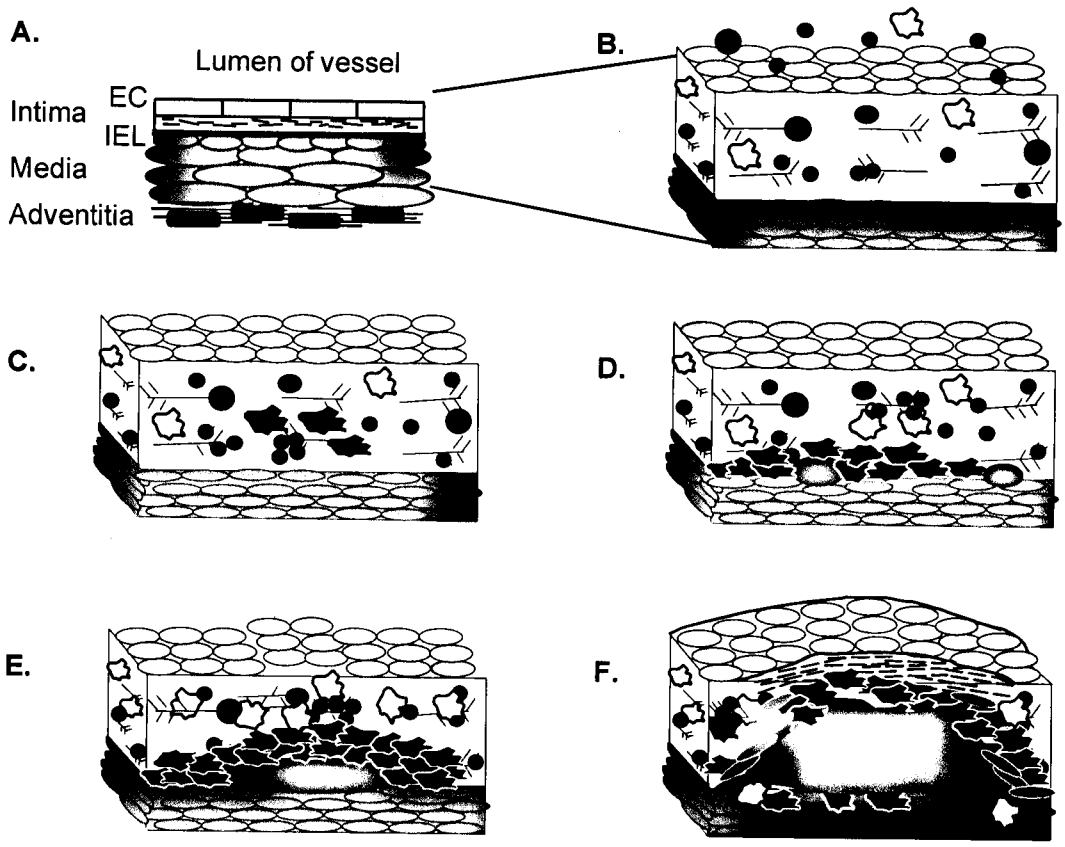

Figure 1.1. The development of atherosclerosis.
(A) A segment of a normal artery wall. The intima, or inner layer, is composed of a thin proteoglycan/extracellular matrix layer between a single endothelial cell (EC) layer on the luminal side and a musculoelastic layer composed of smooth muscle cells and elastic fibres (the internal elastic lamina, IEL). The medial layer is composed primarily of smooth muscle cells with small amounts of elastin, proteoglycans and collagen, separated from the intima by the IEL. The adventitia is the outer covering of the blood vessel and is composed primarily of fibroblasts and bundles of collagen and proteoglycans. It is separated from the media by the external elastic lamina. **(B)** An enlargement of the intima. Components of the plasma such as macrophages ( ), or lipoproteins ( ), may cross the endothelial layer and enter the intima. Lipoproteins may bind to the proteoglycans ( ) of the extracellular matrix and become "trapped" within the intima. **(C)** Lipoproteins accumulated within the intima may be taken up by macrophages, resulting in foam cells ( ). Accumulations of foam cells constitute a type I, or fatty streak, lesion. **(D)** Layers of foam cells form (type II lesion), followed by isolated extracellular lipid pools (grey pools with with centre, type III lesion) as the foam cells die. **(E)** Convergence of extracellular lipid pools results in a focal lipid core (type IV lesion). **(F)** Subsequent changes can include any or all of: formation of a fibrous/muscular cap, through smooth muscle cell migration (grey ovals) into the intima and proteoglycan deposition (, type V lesion); formation of a defect in the lesion surface resulting in a hematoma and/or thrombosis formation (type VI lesion, not shown); calcification (type VII lesion); or loss of the lipid core, resulting in predominantly fibrous tissue (type VIII lesion, not shown), which may result from regression of type IV-VI lesions. Lesions may cycle between these stages as lipid accumulates and is removed. Compiled from information in references[5,8,12,6,13,14].

Lipoproteins entering the intimal space, however, may be retained by proteoglycans[5,11], focally increasing their concentrations (Figure 1.1 (B)). Their rate of entry into the vessel wall

has been shown to be greater than the rate at which they leave, suggesting that lipoproteins may be retained within the vessel wall[11]. Such trapped lipoproteins are subject to numerous modifications, such as oxidation, fusion and aggregation[5,15,16], that render them more atherogenic. Modified lipoproteins may have many atherogenic effects on the cells of the vessel wall, including stimulating the production of adhesion molecules and growth factors[5], proteoglycans, lipoprotein lipase (LPL) and sphingomyelinase[11]. Lipoprotein retention within the vessel wall has been shown to be a critical early step of lesion formation[17].

Subsequent to lipid accumulation within the intimal space, monocyte-macrophages infiltrate into the intima[4,9]. Entry into the vessel wall occurs initially by binding of leukocytes to the endothelial surface through various endothelial adhesion molecules[5] followed by migration through cell junctions[7], and results in increased numbers of macrophages in the intimal space[8]. Growth factors produced by the endothelium, such as macrophage-colony stimulating factor, stimulate the growth, differentiation, and proliferation of monocytes and macrophages[5]. Macrophages express several receptors (scavenger receptors) that allow for the unregulated uptake of lipoproteins. Cholesterol loading of macrophages results in the upregulation and downregulation of several genes[18], including cytokines and several genes involved in lipid metabolism[8]. Included amongst these are apolipoprotein E and ABCA1[5], which could aid in cholesterol removal from these cells. These lipid filled macrophages are called foam cells and are a hallmark of the early atherosclerotic process (Figure 1.1 (C), a type I lesion[13], or fatty streak[14]), identifiable in even young children[8,9,11]. It has been estimated that by the age of 25, up to 50% of the aortic surface may be covered by these fatty streaks[14].

As the lesion progresses, foam cells continue to form in layers within the intima (type II lesion[13]). Small lipid droplets in the extracellular areas are also visible, which may in part result from the death of lipid filled cells[16], either by necrosis or apoptosis[19]. These lipid droplets may replace parts of the extracellular matrix, and disrupt the smooth muscle cell organization of the intima[8] (Figure 1.1 (D), a type III lesion[13]). Smooth muscle cells in the arterial wall may also take up lipid through various lipoprotein receptors. As the lesion grows, the lipid droplets grow and fuse with each other forming a large lipid core (Figure 1.1 (E), type IV lesion[13] or atheroma[14]). The growing lipid core of the atheroma results in a thickening of the artery wall, although not necessarily a narrowing of the lumen[6]. This does not mean that these lesions are without consequence, as lesions with a large lipid core without a protective covering or "cap"

may fissure or rupture[19], leading to the consequences described below. In fact, plaques that are more lipid rich are less stable[20].

Subsequently, the changes become heterogeneous, being present in some, but not all lesions. The specific causes of the observed changes are not well understood. Smooth muscle cell-produced fibrous tissue, predominantly collagen with some proteoglycans, increases in the area of the intima above the lipid core[6]. Smooth muscle cells may migrate from the media to the intima[21], proliferate and join the fibrous cap forming over the growing lesion (type V[13], or fibrous lesion[5,14]). Macrophages and foam cells may infiltrate the medial layer (Figure 1.1 (F)). In late stages, calcium deposits within the extracellular matrix may form (type VII[13]), and the core of the lesion becomes necrotic[6]. These features are characteristic of an advanced atherosclerotic lesion.

While a growing cap may occlude the vessel, the clinical consequences of atherosclerosis also arise when the plaque becomes damaged. The surface of the lesion may fissure or ulcerate, resulting in exposure and release of lipid from the core of the lesion. This stimulates the pathways involved in wound healing, and may generate a hematoma, inflammation and/or thrombosis (a type VI[13] or complicated lesion[14]). The stability of plaques is related to their constituents, not necessarily their size or the resultant luminal obstruction[5]. Characteristics of an unstable lesion that is more prone to rupture include an increased lipid content, an increased number of macrophages, and a decreased cap thickness[22]. The lipid content of lesions has been shown to correlate positively with the number of macrophages and negatively with the cap thickness[22]. Increased numbers of leukocytes lead to increased cytokine expression, which alters the expression of a number of genes within the cells of the lesion, including additional growth factors for both macrophages and smooth muscle cells[5]. This results in decreased extracellular matrix synthesis and increased matrix metalloproteinase expression from macrophages, which results in the degradation of the fibrous cap and lesion destabilization[5,19]. In fact, ruptures often occur on the edges of lesions where the foam cell content is high[5]. In addition, thrombi on the luminal surface of the lesion can break off, leading to occlusion of smaller vessels in the heart resulting in an MI, or in the brain resulting in stroke. Restricted blood flow to coronary arteries, or transient intermittent coronary obstruction due to thrombus formation or vessel spasm may result in angina[21]. Thrombi can also be incorporated into the growing lesion, resulting in further narrowing of the vessel[6], and perhaps ultimate occlusion of the vessel[21].

The development of atherosclerosis may be influenced at any of these stages. However, lipid infiltration and accumulation within the vascular wall is a key initiating event[11]. It has been estimated that over half of patients with premature CAD (before the age of 60) have a genetic lipoprotein disorder[23]. The importance of lipids in this process was recognized as early as the mid 1800's[21]. If lipid deposition and retention within the artery does not occur in the first place, the remaining events will not occur[11]. Furthermore, removal of lipid from lesions of type IV-VI, e.g. through lipid lowering[24], can result in a lesion which is predominantly fibrous (type VIII[13]), and presumably very stable. Thus, factors controlling the plasma lipoprotein levels and affecting their uptake into and retention within the vascular wall have been implicated as important initiators in the pathogenesis of atherosclerosis.

1.2 Lipoprotein metabolism

Owing to their hydrophobic nature, lipids (cholesterol, triglycerides, phospholipids) are not readily soluble in the plasma, posing difficulties in their transport. To facilitate their transport, lipid species are packaged into particles called lipoproteins. The core of the lipoprotein contains hydrophobic molecules such as cholesteryl esters (CE) and triglycerides (TG)[25,26]. The lipoprotein surface is covered by a single layer of the particles with a charged domain, such as phospholipids (PL) and unesterified (free) cholesterol (FC), and various proteins (apolipoproteins (apo), Figure 1.2), which provide structural integrity to the lipoprotein particle and serve as cofactors and ligands for the various enzymes and receptors involved in their metabolism[25-27]. The orientation of these surface components is such that their hydrophobic

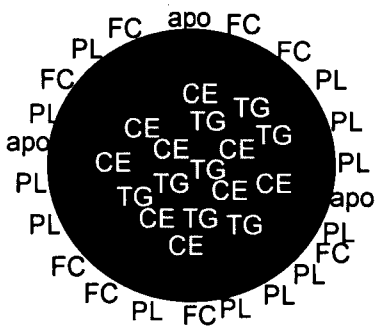

Figure 1.2. A hypothetical lipoprotein.
The surface of the particle contains unesterified (free) cholesterol (FC), phospholipids (PL) and apolipoproteins (apo). This surrounds a core composed of cholesteryl esters (CE) and triglycerides (TG).

domains face the core of the particle, whereas the charged, hydrophilic portions face the outside of the particle and its aqueous environment[28].

Lipoproteins are named and categorized primarily by their density, which varies inversely with their size and core lipid content[26]. Chylomicrons (CM) are rich in TG and are the least dense particles (d<0.94 g/mL)[26]. The very low density lipoproteins (VLDL) are also TG rich and have densities between 0.94-1.006 g/mL[25,26]. Intermediate density lipoproteins (IDL) have densities ranging from 1.006 g/mL to 1.019 g/mL, with cores consisting of a mixture of TG and CE[26]. Low density lipoproteins (LDL) are more CE rich and have densities less than 1.063 g/mL but greater than 1.019 g/mL[25,26]. High density lipoproteins (HDL), also CE-rich particles, have densities between 1.063 and 1.20 g/mL[25]. This class of lipoproteins can be further separated into two main subfractions: HDL_2 with densities between 1.063 and 1.125 g/mL, and HDL_3 with densities in the range $1.125 < d < 1.20$ g/mL[25].

The electrophoretic mobility of particles compared to the globulins has also been used to categorize lipoproteins. HDL have a mobility similar to alpha-globulin, and have been called the alpha-lipoproteins, while LDL have the mobility of beta globulin and have been called beta-lipoproteins[25,26]. VLDL and their remnants have pre-beta mobility, while chylomicrons remain at the origin[25,26].

The protein constituents of the lipoprotein particles are also key to their categorization. The main protein of HDL was initially called the apolipoprotein A, while the apolipoprotein of LDL and its precursors was identified as apolipoprotein B[25]. As it became recognized that there was more than one distinct protein of each type, they have been designated by roman numerals (e.g. apo AI, AII etc.). As additional apolipoprotein or apolipoprotein families were identified, they were called the C apolipoproteins, apoD, apoE and so forth[25].

While circulating in the plasma, lipoproteins undergo numerous changes. Lipoprotein metabolism is a complex process, involving numerous enzymes for the synthesis, secretion and interconversion of lipoproteins, as well as numerous receptors for their uptake in various tissues.

1.2.1 Intestinally-derived lipoproteins

Dietary lipids (TG, CE and PL), the bulk of which are TG, are emulsified and hydrolyzed to monoacylglycerol, unesterified cholesterol, lysophospholipids (respectively) and free fatty acids (FFA) by various digestive enzymes, allowing them to be absorbed by the intestine[27].

There the hydrolyzed lipids are re-esterified with the free fatty acids and packaged into large, TG rich particles called chylomicrons (CM)[26,27]. The chylomicrons are released into the lymphatics and enter the circulation through the thoracic duct[27]. Upon entering the circulation, the majority of the TG within the core of the CM are rapidly hydrolyzed[26,29,30]. The resultant lipoproteins are smaller in size, and are termed chylomicron remnants (Figure 1.3)[26].

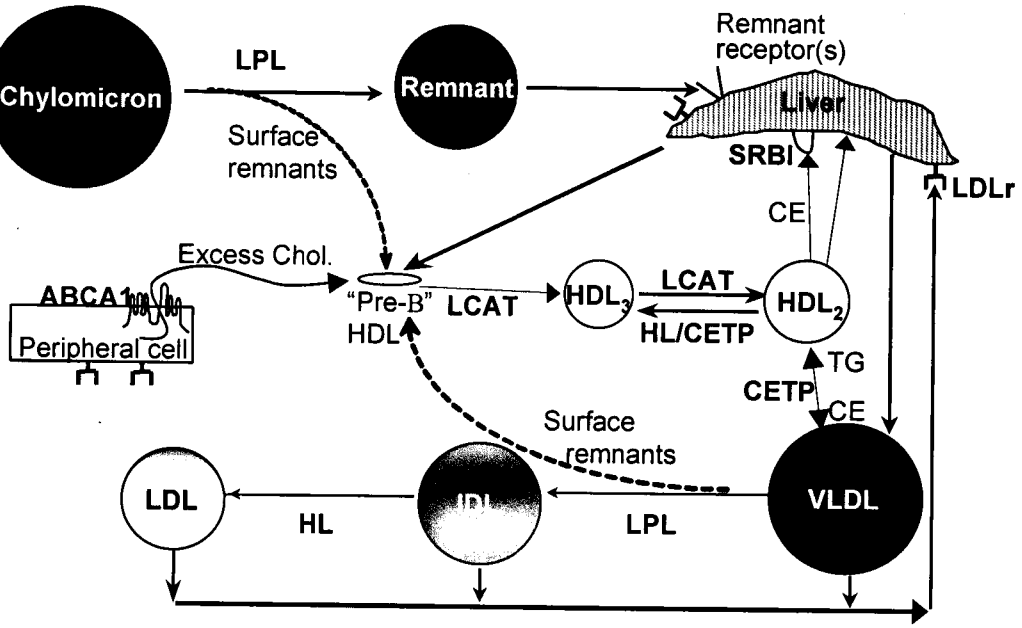

Figure 1.3. Lipoprotein metabolism.
Intestinally derived chylomicrons (CM) are hydrolyzed by lipoprotein lipase (LPL) to form chylomicron remnants, which are rapidly cleared from circulation by the LDL receptor (LDLr) and various remnant receptors in the liver. The liver packages lipid it receives in the form of very low density lipoproteins (VLDL) for distribution to the peripheral tissues. The triglyceride (TG) within VLDL are hydrolyzed by LPL, resulting in intermediate density lipoproteins (IDL), then in low density lipoproteins (LDL) by the actions of hepatic lipase (HL). All of these particles are cleared from the circulation by the LDLr. VLDL and IDL may also be removed from circulation by the remnant receptors. Liver and intestinally derived-apoAI particles which are complexed with the surface remnants generated by the LPL-mediated hydrolysis of the TG-rich lipoproteins, form a nascent (preB) HDL particle. This particle acts as an acceptor for excess cholesterol removed from peripheral cells in an ABCA1-dependent process, forming a mature HDL_3 particle. Further accumulation of cholesterol produces a larger HDL_2 particle. As cholesterol is picked up from cells, it is esterified by lecithin:cholesterol acyl transferase (LCAT), making it more hydrophobic, and allowing it to move into the core of the growing particle. HDL particles then deliver this cholesterol to the liver and other cells (such as those producing steroid hormones) by a variety of mechanisms. The cholesterol ester transfer protein (CETP) catalyzes the exchange of core lipids between lipoprotein particles. Cholesteryl esters (CE) are transferred to VLDL (and CM or CM remnants), while TG are transferred in the opposite direction. The cholesterol is then removed from circulation when these particles are taken up by the liver. The TG-enriched HDL may be hydrolyzed by hepatic lipase (HL) resulting in smaller HDL particles. The liver may also directly take up HDL particles (holoparticle uptake). Finally, CE may be selectively taken up from the HDL particle in a process called selective uptake, mediated by the scavenger receptor class B, type I (SRBI). Adapted from references[26-40].

The major structural apolipoprotein of chylomicrons is a truncated form of apolipoprotein B, which retains the N-terminal 48% of the protein, and has been named $apoB_{48}$. This version of apoB arises from editing of the apoB mRNA in the intestine[27,32], and is not recognized by the major lipoprotein receptors. Each CM particle contains a single molecule of $apoB_{48}$. During the synthesis of CM in the intestine, other apolipoproteins that are synthesized in the intestine are added to the CM particle. These include apoAI, apoAII, and apoAIV[26-28,33]. In the circulation, these non-structural apolipoproteins are exchanged with those on other lipoproteins. CM acquire apoC's (apoCII and/or apoCIII) and apoE in the circulation, through exchange with VLDL and HDL[26,28,31,41,42]. Following (or during) hydrolysis to CM remnants, the particles lose the apoC's and apoA's to VLDL and HDL, and acquire apoE[28,30,31].

CM remnants are cleared from the circulation by many potentially overlapping pathways in a multistep process[29,30,34]. CM themselves are not directly cleared by the liver[30], suggesting a need for some lipolysis before uptake can proceed[29]. $ApoB_{48}$ is not recognized by the major liver receptor, the LDL receptor (LDLr), and thus other mechanisms are required. The apoE on the CM remnant surface is a ligand for the LDLr. Its affinity for this receptor is even stronger than it's natural ligand, $apoB_{100}$, when multiple copies of apoE are present[30], allowing efficient clearance of these particles from the circulation by receptor-mediated endocytosis through the LDLr. Various so-called remnant receptors on the liver also recognize apoE, providing secondary mechanisms of clearance of these particles from circulation. The LDL receptor related protein (LRP) is most likely involved[34,35]. These processes are enhanced by apoE, lipoprotein lipase (LPL) and hepatic lipase (HL) which likely facilitate the initial sequestration of the lipoproteins in the space of Disse before uptake through the above processes[29,30,34]. Both LPL and HL have been suggested to be ligands for LRP[34], and thus enzyme-bound lipoproteins may be removed by binding of LPL or HL to LRP. Furthermore, LRP binds HSPG, and thus HSPG-lipoprotein complexes may also be cleared by LRP[34]. A fourth mechanism involves direct endocytosis of HSPG-bound lipoproteins[34,43]. The VLDL receptor (VLDLr) is expressed in both adipose tissue and muscle, and might be important in uptake of particles in these tissues[35], although its role is not yet well understood. Several additional receptors have been shown to bind apoE, however several of these are not expressed in the liver, and the significance of these molecules *in vivo* is unclear[30,34,35,44]. There has been some suggestion that lipoproteins

taken up by the LDLr may enter distinct cellular pools from those taken up by other mechanisms[35], although the significance of this remains poorly understood. This pathway involving lipids from the diet has been called the exogenous pathway.

1.2.2 Liver-derived apolipoprotein B containing lipoproteins

The liver is a major organ in lipoprotein metabolism. Lipid, either from de novo synthesis or from lipoproteins returning from the circulation, is packaged into and secreted as large TG-rich particles called VLDL (Figure 1.3)[26]. Each VLDL contains a single molecule of the full-length form of apoB (called $apoB_{100}$ to distinguish it from $apoB_{48}$) as its structural apolipoprotein. Other apolipoproteins include apoE and the apoC's, which can be readily exchanged with the other lipoproteins[26]. The $apoB_{100}$ on the liver-derived particles is recognized by the LDLr, allowing their clearance from the circulation. These endogenous lipoproteins follow a similar fate to the CM[26]. The TG within the core are hydrolyzed by LPL, generating smaller, remnant-like particles, the IDL. IDL are short-lived within the circulation, as further hydrolysis of core TG by HL results in the CE-rich LDL, the major cholesterol carriers in human plasma. IDL may also be directly removed from circulation through the mechanisms described for CM remnants[30,34], primarily direct uptake through the LDLr[36]. VLDL and IDL contain apoE in addition to $apoB_{100}$, but this is lost in LDL particles[36]. Thus, while VLDL and IDL can also be cleared from the circulation by the remnant receptors in addition to a high-affinity apoE-LDLr mediated process, LDL can only be cleared by the lower affinity $apoB_{100}$-LDLr mediated pathway[26,36]. Peripheral cells also express the LDLr, allowing them to take up cholesterol from the circulation as needed[26,45].

Cholesterol returning to the liver has several fates. It may be directly secreted in the bile, or used in bile acid synthesis, allowing net removal of cholesterol from the body[36,46], or, the cholesterol may be repackaged, and resecreted as lipoprotein particles[26]. Cellular cholesterol levels are tightly regulated. Increased cholesterol returning through the LDLr leads to a downregulation of endogenous cholesterol synthesis through feedback inhibition of HMG CoA reductase, the rate limiting step of cholesterol biosynthesis[36,45]. In addition, LDLr synthesis is downregulated to stem the influx of exogenously synthesized cholesterol[36,45].

1.2.3 High density lipoproteins

High density lipoproteins (HDL) have a different metabolic course than the apoB containing lipoproteins just described. Their metabolism is far less well understood, and several genes regulating their plasma levels remain to be identified[47]. The major core lipid of HDL is CE, and the primary structural apolipoprotein of HDL is apoAI[33]. Apo AI is produced in the liver and intestine[26,33], however, the route by which this becomes incorporated into the precursors of HDL is less certain.

ApoAI may be directly secreted from these cells as phospholipid-apolipoprotein-free cholesterol complexes known as nascent HDL[33,37,38]. These discoidal particles can be observed on electrophoresis as particles with pre-beta mobility, and thus have been called pre-β HDL. A part of the plasma apoAI may have initially been secreted on CM, or VLDL particles[38]. The hydrolysis of the TG within the core of these TG-rich lipoproteins (TGRL) generates excess surface components ("surface remnants"), containing apoAI, phospholipid, and free cholesterol, which can form discoidal complexes[28,33]. Surface remnants may also be transferred to HDL particles or lipid poor apoAI by the action of the phospholipid transfer protein (PLTP)[48,49]. It has been suggested that this is the major source of HDL precursors[26,33]. It is also possible that apoAI may be secreted directly, and then acquire PL at cell surface[33,50], either by diffusion/desorption from the plasma membrane and/or by active transport, or from the hydrolysis of TGRL[33].

A key step in the formation of mature, spherical HDL particles is the trafficking of cellular cholesterol to the plasma membrane and its subsequent transfer to nascent HDL particles[33]. Nascent HDL molecules are capable of acquiring excess cholesterol exported from peripheral cells, in a process dependant upon the ATP binding cassette transporter ABCA1[51-54]. The cholesterol acquired by these particles (unesterified, or free cholesterol) is esterified by the activity of lecithin:cholesterol acyl transferase (LCAT), producing cholesteryl esters (CE)[28,38,55,56]. This allows it to move to the core of the particle, so that more cholesterol can be accommodated. It also maintains a concentration gradient between the cells and the surface of the particle, which may be necessary to maintain the flux of cholesterol into the particle. This process may, however, may be more important in the subsequent enlargement of HDL particles ($HDL_3 \rightarrow HDL_2$) than in their initial generation[57]. Accumulation of CE within the core results in a spherical, "mature" HDL particle[33]. The subsequent enlargement of HDL particles likely occurs by passive diffusion of cholesterol from cell membranes to the HDL particle, not by

active efflux mediated by ABCA1[39,58]. This enlargement also requires a supply of phospholipids as a fatty acid source for cholesterol esterification, which may be provided through transfer from other lipoproteins by PLTP[59].

This cholesterol from HDL is then delivered to the liver for catabolism or other tissues requiring large amounts of cholesterol such as the steroid hormone producing cells. This can be accomplished in several ways. The cholesteryl esters (CE) within the HDL particle can be taken up by a poorly understood process called selective uptake, where CE are selectively removed from HDL[26,33,38], resulting in a smaller HDL particle without degradation of the HDL apolipoproteins. This process is mediated by the scavenger receptor, class B, type I (SRBI)[40,60]. Some evidence also suggests that HDL particles may be directly taken up by cells (holoparticle uptake)[33,38,49]. A specific apoAI or HDL receptor involved in this process remains to be identified[40,49], although some of this uptake may occur through the binding of apoE found on some HDL particles to the remnant receptors[26,38,61]. Finally, the CE within HDL may be transported to the liver indirectly, by first being transferred to TGRL[26,38]. This process is mediated by the cholesteryl ester transfer protein (CETP), which catalyzes the exchange of neutral lipids (CE, TG) between lipoprotein particles[38]. CE are transferred from HDL to TGRL, while TG are, in return, transferred from these particles to more cholesterol rich particles, such as HDL[38]. Once in the apoB containing lipoproteins, the CE can be removed from circulation as these particles are cleared through their various uptake paths. The TG-enriched HDL are substrates for hepatic lipase (HL), which hydrolyzes the TG, regenerating smaller HDL particles[26,33,38,62]. The relative contributions of these pathways *in vivo* are unknown. The entire HDL-mediated process whereby cholesterol is transported from the periphery to sites of catabolism such as the liver is referred to as reverse cholesterol transport (RCT)[63,64].

1.2.4 Lipids and atherosclerosis

High levels of plasma lipids (hyperlipidemia) is a major risk factor for CAD, a finding recognized for nearly a century[4]. Large, lipid rich plaques are more prone to rupture, and lipid lowering has been shown to increase the stability of atherosclerotic plaques[20], reducing clinical manifestations of the disease. Dyslipidemia is much more commonly found in patients with CAD than in unaffected individuals[23,65], providing further evidence of the importance of plasma lipid levels in determining the severity of atherosclerotic disease.

Increased plasma total cholesterol and apoB levels have been associated with CAD in many studies[23,65-67]. Each 0.026 mmol/L (1 mg/dL) increase in total cholesterol (TC) has been associated with an approximately 1% increase in risk of MI[66]. Small increases in cholesterol for a long period of time can have a dramatic effect. Maternal cholesterol levels have even been correlated with fetal fatty streak formation[9]. As LDL is the major cholesterol carrier in humans, these findings for total cholesterol and plasma apoB predominantly relate to plasma LDL concentrations.

The negative role of LDL in atherosclerosis is well established[4], and thus LDL has been thought of as the "bad" cholesterol. This was dramatically illustrated in individuals with familial hypercholesterolemia (FH), through the pioneering work of Brown and Goldstein[36,45] and others[68]. This disorder arises from mutations in the LDLr gene[45]. Individuals with a complete absence of LDLr activity have severely increased LDL cholesterol (LDL-C, 6-10 fold), and often have heart attacks in childhood[36,45]. Individuals heterozygous for LDLr mutations have LDL-C that is increased two-fold from birth and have premature CAD, with onset often around their fourth decade of life[36,45,69]. Epidemiological studies have consistently shown increased LDL-C to be associated with CAD[67,70-72]. It has been estimated that the majority of men with CAD have increased LDL-C[73,74].

The majority of cholesterol entering the vessel wall is from LDL particles, and accumulation of LDL within the subendothelial layer is the initiating event in atherosclerosis[5]. LDL within the vessel wall can undergo oxidative modification[15,16]. Oxidized, in contrast to native LDL, is taken up in an unregulated manner by macrophages through the scavenger receptor[4], and results in increased foam cell formation. Oxidized LDL is present in very early lesions[9]. Increased LDL can also, presumably, lead to increased Lp(a), which is formed by the disulphide linkage of apo(a) to the $apoB_{100}$ molecule on LDL. Increased Lp(a) has also been associated with increased risk and severity of CAD[23,67,75].

The relationship between plasma TG and atherosclerosis has been less well demonstrated, primarily because it was believed that TGRL were too large to enter the vessel wall, and because TG are correlated with other plasma lipoproteins, making it difficult to demonstrate an independent role for them in CAD. However VLDL and the remnant lipoproteins have recently been identified within the vessel wall[4,31,76,77]. It has been recognized that 33% of men with CAD have increased TG[73], and TG levels have been shown to be a significant predictor of CAD[78-80].

Plasma TG are increased in CAD patients[67] and have been associated with increased progression of atherosclerosis[81-83]. Decreased clearance of TG following a fat-rich meal has also been identified as a risk factor[84], and remnant lipoproteins have been directly correlated with carotid atherosclerosis[85]. In fact, TGRL remnants have been suggested to be as atherogenic as LDL[31,86]. Furthermore, TG lowering from clinical trials has shown significant reduction in CAD[87], suggesting a direct relationship between TG levels and CAD.

In contrast to the apoB containing lipoproteins, *low* HDL cholesterol (HDL-C) levels are an important risk factor for CAD. HDL-C was first suggested to protect against the development of CAD, independent of LDL-C levels, over 25 years ago[88], giving it the reputation as the "good" cholesterol. Since then, a strong inverse relationship between plasma HDL-C levels and CAD has been confirmed in a large number of epidemiological studies[66,70,71,78,89-92]. Prospective and retrospective autopsy studies have shown that HDL-C levels are inversely correlated with coronary atherosclerosis[10]. Although low HDL-C is often seen in association with other lipid abnormalities, isolated low HDL-C is an independent risk factor for CAD[65,67,74,92,93]. Low plasma HDL-C is the most common lipoprotein disorder associated with premature atherosclerosis[23,67], and each 0.026 mmol/L (1 mg/dL) increase in HDL-C has been associated with a 3.5% reduction in the risk of MI[66]. A 10 mg/dL increase has been associated with a 50% decrease in risk[94]. Although many hypotheses have been suggested to explain the protective effects of HDL[64,95,96], the pivotal role HDL plays in reverse cholesterol transport (RCT)[63,64] is currently its most widely accepted antiatherogenic property.

Thus, genes involved in HDL and TG metabolism are good candidates for factors influencing the risk and severity of atherosclerosis. However, as TG have only recently become acknowledged as independent risk factors, the influence of variation in genes involved in TG metabolism on plasma lipid levels and severity of CAD was not well described. Furthermore, as many genes involved in HDL metabolism have only been recently identified, the role of their genetic variation in determining coronary disease risk had not been examined. The studies described in this thesis focus on two genes which influence both TG and HDL-C, namely *LPL* and the ABC transporter *ABCA1*, and their relationship to plasma lipid levels and CAD.

1.3 Lipoprotein lipase

Lipoprotein lipase (LPL, EC 3.1.1.34) is a key enzyme in lipoprotein metabolism. Its primary sites of synthesis are the parenchymal cells of adipose tissue, muscle and the heart. Other sites of synthesis include the lung, macrophages, adrenal tissue, the brain, lactating mammary cells, neonatal but not adult liver, and very low levels in the kidney and intestine[42,97].

The *LPL* gene contains 10 exons, spanning 30 kb on chromosome 8p22[42,98-100]. Transcription results in two mRNA species of 3.6 and 3.2 kb, which arise through alternate polyadenylation[101]. The significance of these two transcripts is still not fully appreciated, although it has been suggested that the longer form is translated better[101]. Most tissues express both transcripts, although the longer form is the predominant transcript in heart and muscle[101]. The full-length protein is 475 amino acids, which become processed to a mature protein of 448 amino acids following cleavage of a signal peptide[42,97].

Within the cell, LPL is synthesized in the ER then translocated to the golgi[100]. LPL undergoes several co- or post-translational modifications. Cysteine bonding occurs between four pairs of the ten cysteine residues[42,102]. Glycosylation occurs at asparagines 43 and 359, and is required for proper activity and secretion of the enzyme, although trimming and modification of the oligosaccharides is not[100]. The LPL protein is also sulphated, the significance of which is unknown[100]. The mature, active form of LPL is a non-covalently linked homodimer[42,102,103]. It is unclear at what stage dimerization occurs. Most evidence suggests that dimerization occurs at a similar time as the glycosylation process[100], resulting in an active enzyme that can be secreted from the golgi[42]. A large portion of LPL regulation occurs at the post-translational stage[104]. Much of the newly synthesized LPL may be degraded before it is secreted, although some evidence exists that a small intracellular pool is maintained and available for rapid secretion[100,103].

From its site of synthesis, LPL is transported through poorly identified mechanisms to the luminal surface of the vascular endothelium[42,105]. This movement may occur through transient binding of the LPL to heparan sulphate proteoglycans (HSPG)[105], followed by transcytosis to the luminal surface of the vascular endothelium. HSPG or heparan sulphate oligosaccharides may act as a chaperone in this process, as might the VLDL receptor[106,107]. It has also been suggested that factors secreted by the endothelium, perhaps a heparanase, may stimulate this process, allowing a rapid increase in the amount of LPL on the vascular endothelium, e.g. in response to

insulin[106,108]. LPL is anchored to the luminal surface of the vascular endothelium through non-covalent binding to HSPG[42]. This allows its displacement of the enzyme from the vessel wall into the plasma by intravenous injection of heparin[42]. In the plasma, the enzyme is only active in the presence of its activator, apolipoprotein CII (apoCII)[42,102].

Several structural domains of the LPL protein have been elucidated. While the enzyme has not been crystallized, a close family member, pancreatic lipase, has[109]. Much of what is known about the three-dimensional organization of LPL has been inferred from its similarity to pancreatic lipase. The catalytic core, residing in the N-terminal domain of the protein, is formed by three amino acids (Ser_{132}, Asp_{156}, and His_{241})[110], which are contained within a hydrophobic pocket. This pocket is covered by an amphipathic lid domain (amino acids 217-238), maintaining a hydrophobic area around the catalytic pocket, but allowing substrate access[109]. The regions between amino acids 126-135 and 245-252 have been proposed to be involved in lipid binding[42]. The amino acids lining the pocket and making up the lid help determine its substrate specificity. Amino acids 279-282 and 291-304 have been implicated in heparin binding[111], although several others have also been suggested to play a role[102]. The amino acids involved in the catalytic activity of LPL are all found in the large N-terminal globular "head" of the protein. The C-terminus forms a smaller globular structure, referred to as the "tail". The site of binding of its activator, apoCII, has been localized to the C-terminal domain[42,112], as have some regions involved in heparin binding[102]. The initial lipoprotein binding sites are also suggested to reside in the C-terminus[102]. Recent studies suggest that the arrangements of the subunits within the active dimer is a head-to-tail orientation[113].

1.3.1 Lipoprotein lipase and lipoprotein metabolism

Situated on the vascular endothelium, LPL binds circulating TGRL, where its primary enzymatic role is to hydrolyze the core TG in circulating TGRL, converting these into remnant particles[42] (Figure 1.4). This process generates FFA that may be taken up and used for energy, as in muscle, or for storage, as in adipose tissue[42]. In fed states, LPL in adipose tissue is upregulated, delivering FFA to adipose tissue for storage, whereas in times of increased energy demand, such as in fasting states, LPL in muscle is upregulated to provide increased FFA to be used as an energy source[42]. As such, LPL has been suggested to act as a "gatekeeper", partitioning FFA to sites of utilization[42]. Lipolysis of TG in these large TGRL results in the

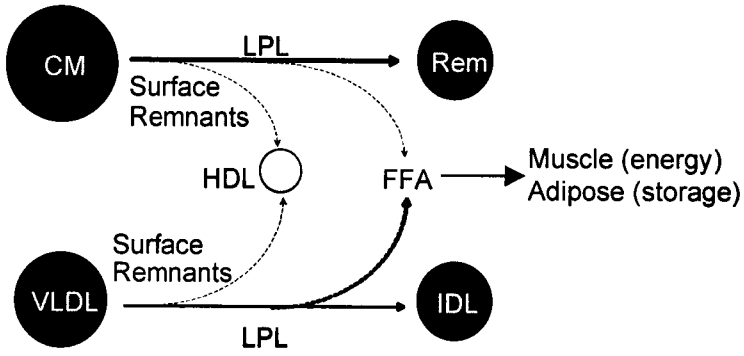

Figure 1.4. Enzymatic activity of lipoprotein lipase.
LPL hydrolyzes the core TG of CM and VLDL producing free fatty acids (FFA) that can be used for energy or stored. This process results in the formation of chylomicron remnants (Rem) and IDL, respectively. A by-product of this hydrolysis is the generation of surface remnants, which are key components of HDL.

formation of denser remnant particles. The hydrolysis of TGRL also results in the generation of surface remnants which form the basis of HDL[42].

The remnants (CM remnants and IDL) are taken up primarily by the liver via cellular receptors. This process has generally been considered to be a three step process[30,34]. The remnants are first cleared from the plasma into the space of Disse in the liver, in a process known as sequestration[34]. This process traps lipoproteins in the space adjacent to hepatocytes, allowing their subsequent uptake. HSPG, apoE, HL and LPL have all been suggested to serve as ligands important in this tethering step[29,34]. The second step may involve further processing of these remnants by HL and/or LPL[34]. The third step of this process is uptake of the remnants into the hepatocyte[34]. This may occur directly through the binding of $apoB_{100}$ or apoE to the LDLr or LRP, as discussed above.

LPL has been reported to function in this uptake process in many ways. It has been suggested LPL may aid uptake by anchoring these remnants to cell surface components prior to their uptake[114]. LPL has been shown to increase the binding to and degradation of lipoproteins in cells *in vitro*[115,116], independent of binding to the LDLr[115,117]. LPL may also serve directly as a ligand to LRP[118-121], and serve as a cross-linker or "bridge" between the lipoproteins and this receptor[119,121-124], possibly through binding to HSPG[125,126]. This process occurs through a C-terminal fragment of LPL, independent of catalytic activity[125,127-129]. Direct uptake through internalization of HSPG may also be facilitated by binding of lipoproteins to HSPG via LPL[122,130]. The source of this LPL is presumably that found attached to circulating lipoproteins,

as the adult liver does not itself synthesize LPL. These processes may depend on the presence of dimeric LPL[119,131]

Thus LPL has been suggested to have a triple-function in the metabolism of TGRL: it attaches them to the vascular endothelium temporarily removing them from circulation so that lipolysis may proceed, it hydrolyzes the core TG of the particles, then LPL mediates the clearance of the remnants by aiding in their binding to liver receptors for clearance[41].

Following hydrolysis, perhaps because of displacement from the vascular endothelium by fatty acids, the LPL may become attached to the lipoprotein remnants[132-134]. In fasting plasma the majority of LPL found free in the circulation is inactive, likely monomeric, and attached to lipoproteins[132]. In post-prandial plasma, however, the LPL may be present on the TGRL remnants as dimers. This is further strengthened by the finding that LPL in preheparin plasma (i.e. that not bound to the HSPG of the arterial endothelium) is correlated to LDL-C and FFA and negatively correlated to TG, suggesting that displacement of LPL from HSPG is related to the conversion of VLDL to LDL[135-137]. LPL in the circulation is rapidly taken up by the liver and degraded, perhaps as a part of the lipoprotein uptake described above. Degradation may occur by uptake through LRP, the VLDLr, or direct endocytosis of HSPG[119,122,138].

Complete absence of LPL activity in humans is rare (approximately 1 in a million, worldwide), although mutations are present at a higher frequency (1 per 5000) in certain founder populations such as Quebec[42,102]. Our group was the first to identify mutations in this gene underlying familial chylomicronemia[139]. Complete absence of LPL activity results in a drastic elevation of CM and a concomitant marked decrease in LDL and HDL-cholesterol concentrations[42,140]. Chylomicrons accumulate in the plasma, giving it a milky appearance[42,140]. Clinical symptoms often present in early childhood with symptoms including abdominal pain and pancreatitis, hepatosplenomegaly, eruptive xanthomas, lipemia retinalis and a general failure to thrive[42]. The expression of these symptoms is somewhat dependent on the TG levels reached[42]. To date, the only treatment available is maintenance on a diet very low in fat[42]. In contrast to complete LPL deficiency, partial LPL deficiency, whereby enzymatic activity is reduced but not completely abolished, is common (3-5%) in general populations of Caucasian descent. This may result from heterozygosity for mutations in the gene, or from either heterozygosity or homozygosity for some of the polymorphisms within the gene. Partial loss of LPL activity predisposes to an altered lipoprotein profile, including high TG and low HDL-C,

which is compatible with an increased atherogenic risk[141-145]. Genetic variation affecting LPL activity thus occurs frequently in the general population, highlighting the importance of understanding the role of this key enzyme in the atherosclerotic process.

Over 70 mutations and functional polymorphisms in the coding region of the *LPL* gene have now been described[102,146]. The majority of mutations occur within exons 4-6, which house the catalytic domain[102,147]. *LPL* variants, cumulatively, may be present at carrier frequencies approaching 20% in populations of European descent. Thus, understanding this enzyme is of key importance in furthering our understanding of atherogenesis.

1.3.2 Lipoprotein lipase and atherosclerosis

Various lines of evidence have suggested that LPL may confer either increased or decreased risk for atherosclerosis depending on its site of expression (reviewed in reference[148]). These roles may depend on the tissue from which LPL is being expressed. They also may vary depending on whether the LPL is acting enzymatically, or playing a structural role.

In plasma, the role of LPL in atherosclerosis is generally anti-atherogenic. Heterozygotes for LPL deficiency have increased TG and decreased HDL cholesterol levels[143,145,149], a profile associated with increased atherogenic risk[80,83,150]. Figures 1.2 and 1.4 demonstrate the role of LPL in lipoprotein metabolic pathways, leading to decreased plasma TG. A side product of the hydrolysis of chylomicrons by LPL is the production of surface remnants, which can go on to form HDL[42], a particle which is generally understood to have an inverse relationship with the development of atherosclerosis[66,70,71,78,89-92]. Furthermore, increased plasma concentrations of TGRL are now themselves being shown to be atherogenic[67,81-84], suggesting an anti-atherogenic role for LPL. Decreased LPL activity has been demonstrated in a CAD population, and was associated with increased TG and decreased HDL-C[151]. The LPL protein may also play a structural role in the bridging and uptake of lipoproteins in the liver, and has been hypothesized to aid hepatic clearance of remnant lipoproteins[116,117,127,129]. These data suggest that increased plasma LPL activity is associated with protection against atherosclerosis. Figure 1.5 (A) summarizes the anti-atherogenic roles of plasma LPL.

However, the chylomicron remnants and IDL particles that result from lipolysis of TGRL may themselves be atherogenic[81,84]. While TGRL have been thought to be too large to enter the vessel wall, particles the size of these remnants have been detected in the vessel wall and in

lesions[76,77]. IDL can also be further processed to form LDL particles, which have been long recognized to be atherogenic. This would suggest that LPL in the plasma might play a more atherogenic role.

In the vessel wall, however, increased LPL protein mass and/or activity may be pro-atherogenic. Macrophages are the primary source of LPL within the vessel wall[152,153] and higher levels of macrophage LPL have been correlated with increased susceptibility to atherosclerosis in mice[154]. Addition of LPL to macrophage cultures *in vitro* has been shown to increase their uptake of VLDL and CE accumulation within the cells[155]. As described, LPL is capable of binding proteoglycans of the extracellular matrix and lipoproteins simultaneously, and has been proposed to act as a bridge, linking lipoproteins to the proteoglycans, and trapping lipoproteins sub-endothelially[124,156]. *In vitro*, LPL has been shown to increase the binding of lipoproteins to arterial matrix proteoglycans[116,124,157] and to increase the retention of lipoproteins within the endothelial cell matrix[158], as well as in vessels *in situ*[156]. Furthermore, LPL within the vessel wall increases lipoprotein retention within the subendothelial cell matrix[124,157-159] and in aortic

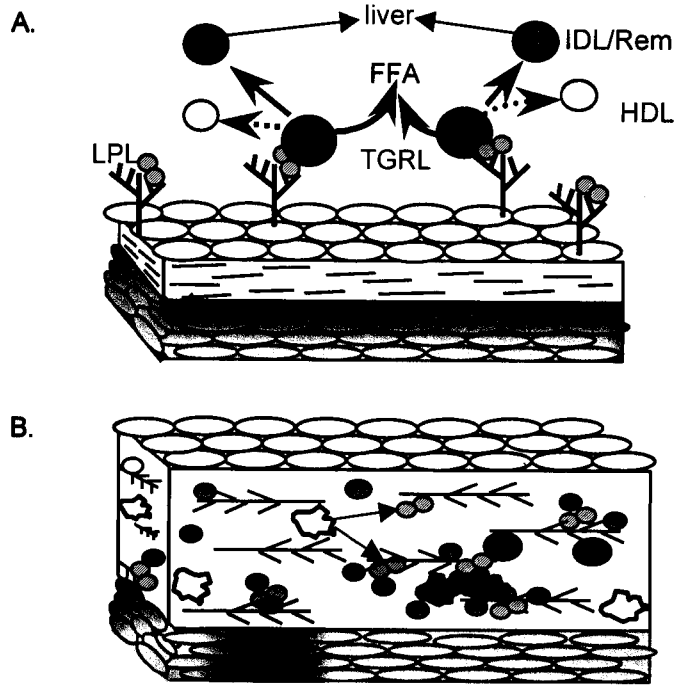

Figure 1.5. Roles of LPL in atherosclerosis.
(A) Plasma LPL ( ) activity decreases the plasma TG concentrations, and increases HDL-C. In addition, the LPL protein aids uptake of the remnant lipoproteins in the liver. **(B)** In the vessel wall, LPL protein (likely from macrophages) may aid retention of lipoproteins by acting as a "bridge" between the lipoproteins and proteoglycans. LPL-mediated hydrolysis may increase uptake of lipoproteins. Both these roles may lead to increased foam cell formation ( ).

segments[156,160]. Such trapped lipoproteins are more susceptible to atherogenic modification, and may be more rapidly taken up by macrophages, aiding foam cell formation[161-164]. Particles thus retained may be taken up more easily by macrophages[162,164-166], leading to increased foam cell formation[11]. Macrophage LPL itself is capable of this increased binding and uptake[167]. The above roles only require the presence of the LPL protein, and do not depend on its catalytic activity. Thus, these effects should occur independently of catalytic activity and only require the presence of the protein. Indeed, roles have been shown for both dimeric and monomeric LPL, both of which occurred independent of LPL activity[168] (Figure 1.5 (B)).

In addition, macrophage uptake of lipoproteins in the vessel wall may be enhanced by LPL lipolytic activity, via the local generation of smaller remnants that are more amenable to uptake[164,166]. Aortic LPL has been positively correlated with cholesterol uptake in the aorta of cholesterol fed rabbits[169]. In addition, synergistic effects between LPL and sphingomyelinase have been shown to increase retention, aggregation, and uptake of LDL particles in vitro[161]. It has been further suggested that LPL may also enhance interactions between the lipoproteins and receptors responsible for their uptake, through conformational changes of the ligands for the receptors mediated by partial hydrolysis of the lipoproteins[164]. These mechanisms imply the necessity of catalytic activity in the process (Figure 1.5 (B)).

Furthermore, it was shown recently that LPL may act as a monocyte adhesion protein[170,171], and may play a role in the recruitment of monocytes into the vessel wall and their differentiation to macrophages[172]. LPL may also increase cytokine production by macrophages[172]. This data suggests that increased LPL activity and/or protein within the vessel wall may promote atherosclerosis. Thus, in the vessel wall, LPL seems to have a pro-atherogenic potential through a variety of mechanisms. Studies in this thesis illustrate both the pro- and anti- atherogenic roles of LPL.

1.4 ATP Binding Cassette Transporter A 1

Glomset first proposed that the primary anti-atherogenic function of HDL might be related to its key role in the transport of cholesterol from peripheral cells to the liver nearly 30 years ago[56]. However, until recently, little has been understood about the initial step of reverse cholesterol transport, namely the removal of cholesterol from peripheral cells. Both genetic and environmental factors can contribute to decreased levels of HDL, or hypoalphalipoproteinemia.

Complete or near-complete absence of HDL-C can result from rare genetic disorders, including mutations in the genes for apoAI, LPL, CETP, and LCAT[140,173].

Tangier disease (TD), was originally described by Fredrickson et al. in 1961, and is a rare genetic disorder, diagnosed in approximately 60 patients worldwide. It is associated with a near absence of HDL-C and apoAI, hepatosplenomegaly, neuropathy, and marked CE deposition within tissues[174]. Biochemically, TD is associated with decreased cellular cholesterol and phospholipid efflux[175,176]. Studies described in this thesis led to the identification of ABCA1 (ABC1) as the underlying cause of TD and as a key genetic factor in lipid metabolism and atherosclerosis. ABCA1 is a member of the large family of ATP binding cassette transporters, known to be involved in the energy dependent transport of a variety of substrates[177]. We have also shown that familial hypoalphalipoproteinemia (FHA) with decreased cholesterol efflux[178,179] is allelic to TD and due to heterozygosity for mutations in the *ABCA1* gene[52].

The *ABCA1* gene is comprised of 50 exons covering a distance of approximately 149 kb[180] on the long arm of human chromosome 9 (9q31)[181,182]. *ABCA1* is expressed in the placenta, liver, lung, small intestine, brain and adrenal glands[183]. Lower expression has been noted in several other tissues[183]. The mature protein is made up of 2261 amino acids[184]. Like other ABC A subfamily members, ABCA1 possesses two transmembrane domains, each composed of 6 membrane spanning segments, followed by two ATP binding cassettes[185].

Very little is known about the post-translational processing of this protein, or about its exact biological function. ABCA1 was originally suggested to play a role in the phagocytosis of apoptotic cells[186-188] and to be involved in the secretion of interleukin 1β[189]. Given the severe defect in cholesterol efflux in TD, ABCA1 is obviously a key protein in HDL metabolism. However, its subcellular localization has not been confirmed, nor has the identity of the substrate(s) it transports. It is assumed that its major function occurs at the plasma membrane[190], but it is unknown whether ABCA1 transports cholesterol, PL or both. It is also unknown whether ABCA1 plays a role in the intracellular trafficking of cholesterol and/or PL to the plasma membrane. ABCA1 mRNA levels are correlated with cholesterol efflux[191], and increased ABCA1 expression results in increased cholesterol and PL efflux and in increased apoAI binding to the cell[190]. ABCA1 has also been suggested to regulate intestinal absorption of cholesterol[192].

Many studies have examined the regulation of *ABCA1*. This gene has been shown to be upregulated by cholesterol in macrophages[53,183]. This upregulation has been shown to be mediated by the LXR/RXR family of transcription factors[192-195]. *ABCA1* is also upregulated by cAMP, a known inducer of efflux[196]. PPARα and PPARγ activators have also been shown to upregulate *ABCA1*, although this may occur secondary to their upregulation of LXR[197]. The only molecule shown to downregulate *ABCA1* to date is interferon γ[198].

Mice deficient in ABCA1 have been shown to have an almost complete absence of HDL-C, phospholipids and apoAI[199-201]. The mice had an enlarged spleen, abnormalities of the small intestine, and CE accumulation in the adrenal glands and lungs[199,201]. The effects on plasma LDL-C have been mixed[199,201]. ApoAI-mediated cholesterol efflux was reduced in fibroblasts from these mice[199]. One line of mice also showed severe placental malformations[200]. In general, these findings are very similar to what is seen in TD.

The studies described in this thesis have contributed to what we know about its role in human lipid metabolism. We, and others, have shown that individuals heterozygous for mutations in *ABCA1* have decreased HDL-C and apoAI, increased TG, and reduced cholesterol efflux[202,203]. Efflux was strongly correlated with HDL-C[202,203]. Furthermore, heterozygotes have a three-fold increased risk of CAD[202]. The discovery that mutations within this gene are associated with a marked impairment of cholesterol efflux and severely decreased HDL-C, suggest that ABCA1 is a key component of the RCT pathway. By assessing the role of variation in this gene on the development of atherosclerosis, we have been able to directly demonstrate that alterations in RCT influence atherosclerosis.

1.5 Objectives

Clearly, several genes are involved and play an important role in lipoprotein metabolism and the development of atherosclerosis[204,205]. The goals of the studies described in this thesis were to relate genetic variation in *LPL* and *ABCA1*, two genes involved in TG and HDL-C metabolism, to dyslipidemia and atherosclerosis. Specifically, the objectives of the studies on LPL were to examine whether variation in LPL is associated with alterations in atherosclerosis, and whether these alterations are dependent upon the site of *LPL* expression. With the identification of ABCA1, the goals of the studies described were to determine how loss of

ABCA1 activity, or how common variation in *ABCA1*, contributes to plasma lipid levels and the risk of CAD.

Chapter 2: Validation of the mouse as a model for studying the role of LPL in HDL metabolism

The work presented in this chapter has been published as

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and in abstract form as

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Preface

I designed and performed all of the experiments and analyses presented within the chapter, with the exception of that noted below. Li Miao provided technical assistance with the LPL activity and plasma lipoprotein measurements. Nagat Bissada, our animal technician, performed the DNA extractions and assisted with the PCR genotyping and blood sampling. The CETP transgenic mice were a kind gift from Dr. R. LeBoeuf. The CETP activities were measured by Dr. G. Shen in the group under the direction of Dr. A. Angel.

2.1 Introduction

Lipoprotein metabolism is a dynamic system of interactions between the lipoprotein particles and several enzymes and receptors. This makes the study of one isolated component *in vitro* difficult, and perhaps less relevant to the *in vivo* situation. In contrast, *in vivo*, fluctuations in several other parameters may affect the metabolism of the components under study. Thus the use of genetically defined inbred animal model systems to control other factors has greatly facilitated the study of lipoprotein metabolism[118,206]. In such model systems, not only is the genetic background of the animals controlled, environmental factors such as diet, climate, and exercise can also be more adequately controlled.

Several proteins involved in lipid metabolism have been studied using lines of transgenic and gene-targeted knockout mice, including many apolipoproteins, receptors and enzymes[207-209]. These have yielded many insights into the effects of increasing or decreasing expression of individual components. However, the resultant lipoprotein profile is governed by many complex interactions, and thus dissection of these interactions is needed to understand the relevance of altering expression of any one component. Crosses between the various genetically altered mouse lines allow the examination of the joint effects of altering the expression of two of these components, and thus provides a powerful tool for examining gene-gene interactions and genetic pathways in lipoprotein metabolism. However due to differences in lipoprotein metabolism between mice and humans, animal models must be validated in order to understand the resulting phenotype.

Positive correlations between LPL activity and HDL-C concentrations have been observed by numerous investigators, in many different normo- and dyslipidemic human populations[42]. Individuals with complete LPL deficiency, due to homozygosity or compound heterozygosity for mutations in the *LPL* gene, typically manifest with lipid abnormalities that include markedly decreased HDL-C concentrations[42]. Heterozygous carriers have also been shown to have increased plasma TG and decreased HDL-C levels[142,143,145]. Correlations between LPL activity and HDL cholesterol have further been extended, and are still valid in populations with very low (hypoalphalipoproteinemia) or very high (hyperalphalipoproteinemia) HDL-C, in both men and women[210]. Thus, alterations of LPL catalytic activity clearly affect plasma HDL-C concentrations in humans.

These correlations have not, however, been detected in the genetically engineered mouse models of LPL. In transgenic mouse models with widespread overexpression of LPL, while the predicted effects of increased LPL activity on decreasing TG have been evident, significant increases in HDL-C have not been observed[211-213]. Similarly, in the two *LPL* gene targeted models, while decreasing LPL activity was associated with increased TG, no significant changes in HDL-C were noted in the heterozygous offspring[214,215]. Additionally, no consistent relationships between LPL activity and HDL-C have been observed in any of the subsequent tissue-specific LPL mouse models[216-220]. Given that these relations are well documented in humans, the mild effect of LPL on HDL-C in these mouse models may be attributable to species differences in lipid metabolism.

Several differences in lipoprotein metabolism between mice and humans have been documented, some of which are of direct importance to HDL metabolism (Table 2.1). HDL is the predominant cholesterol carrying particle in mice. Mice possess higher circulating HL activities compared with humans[221]. Furthermore, mice lack plasma CETP activity[206,222]. These differences between the species might therefore be of particular importance to consider when examining models of human HDL metabolism in mice.

CETP is found in the plasma, carried to a large extent on HDL particles[222,223], primarily particles containing only apoAI (LpAI)[223]. It binds to lipoproteins ionically through negatively charged residues on the lipoprotein surface, and catalyzes the exchange of neutral lipids between lipoprotein fractions[222] (Figure 1.3). Transfer often occurs down the concentration gradient of each component[222,224], resulting in an equilibration of components between particles[222]. Thus,

Table 2.1. Differences in lipoprotein metabolism between mice and humans

	Humans	Mice
Main Cholesterol Carrier	LDL	HDL
Plasma CETP	Present	Absent
Circulating HL	Low	High
Apo (a)	Present	Absent
ApoB editing in liver	Absent	Present

CE are transferred from relatively CE rich-TG poor particles, such as HDL, to TG rich-CE poor acceptors such as VLDL, while TG may be exchanged in the opposite direction, from VLDL to HDL[223,224]. One-way transfer of CE without reciprocal TG exchange, and transfer of CE to LDL may also occur[223,224]. Individuals with genetic CETP deficiency have lipoprotein profiles that more closely resemble those of CETP deficient animals, including increased HDL, and decreased VLDL, IDL and LDL[38], and have been shown to possess large apoE rich HDL[222], similar to that found in mice[225]. Thus, a significant portion of the differences in lipoprotein profiles between humans and mice may be attributable to the absence of plasma CETP activity in mice.

Evidence has accumulated that interactions between LPL and CETP may coordinately regulate plasma levels of HDL *in vivo*. LPL and CETP are expressed in a similar tissue distribution pattern[226], and have common substrates in TGRL and HDL. *In vivo*, in the absence of plasma LPL activity, CETP activity in the plasma is very low[227]. This occurs despite increased plasma transfer activity towards exogenous substrates[228], suggesting a need for lipolysis of endogenous TGRL before transfer activity can proceed *in vivo*. VLDL lipolyzed by LPL have indeed been shown to be better acceptors of CE than non-lipolyzed particles *in vitro*[38]. However, beyond this initial requirement, it is unclear how alterations in LPL activity may modulate the transfer process, affecting HDL-C concentrations.

The goal of this study was to further examine the relationship between LPL and CETP in regulating HDL-C levels *in vivo*. These studies would serve as the validation of the mouse as an animal model for our ultimate goal of assessing the role of LPL in atherosclerosis. Specifically, this study was designed to address the question of whether the lack of effect of LPL on HDL-C in mice was due to the lack of CETP in mice, or whether other potential mechanisms exist.

2.2 Methods

2.2.1 Animal housing and diets

Transgenic mice overexpressing human LPL previously created by our group[211] were used in this study to obtain a group of mice expressing a wider range of LPL activities than obtained with only non-transgenic mice. This line of transgenic mice containing the human LPL cDNA driven by the CMV promoter have been shown to express LPL at high levels in heart, muscle and adipose tissues, as well as stomach, and at lower levels in the kidney[145], and provides a constitutive increase in LPL, above that from the endogenous mouse gene, which maintains its

natural regulation. These mice were bred with mice expressing the simian CETP gene[229], resulting in mice expressing CETP with a broad distribution of LPL activities. Under these conditions, the relationship between plasma LPL activity and HDL-C levels was examined.

The parent LPL and CETP transgenic strains were bred for at least five generations onto the C57BL/6 strain by successive backcrossing. The mice were housed individually or in small groups in microisolator cages, with 12 hour light and dark cycles in an environmentally controlled facility. Mice were fed a standard rodent chow (Laboratory Rodent Diet, PMI Feeds, 5001) consisting of 23.4% protein, 4.5% fat, with no more than 270 parts per million (ppm) cholesterol, and were provided free access to food and water. For high fat/high carbohydrate feeding studies, male mice were fed a semi-synthetic diet consisting of 50% sucrose, 15% corn oil, and 21.9 % protein, with no added cholesterol (Harlan Teklad, TD 96202).

Blood samples of approximately 500 μL were withdrawn retro-orbitally following light anaesthetic (Halothane, MTC Pharmaceuticals, Cambridge ON). Samples for plasma LPL and HL measurements were withdrawn 10 minutes following an intravenous (tail vein) bolus of heparin (Liquemin, Hoffman-La Roche, 49800), at a standard dose of 100 U/kg. Lipoprotein assessment was performed on samples collected without the administration of heparin into tubes containing 1 mM EDTA. For chow diet measurements, pre- and post-heparin samples were taken on two separate occasions spanning at least two weeks. For measurements in male mice consuming the high fat, high carbohydrate diet, pre- and post-heparin samples were measured on the same day, with the total blood volume withdrawn not exceeding 500 μL. All samples were taken following an overnight fast of approximately fourteen hours (6pm – 8 am), and placed immediately on ice. Plasma was removed following centrifugation at 14000 rpm (Eppendorf, 5415C) for ten minutes, and immediately frozen at -70°C until analysis. All work was approved by the University of British Columbia Animal Care Committee.

2.2.2 Identification of genotypes by polymerase chain reaction

The presence of each cDNA transgene was identified by polymerase chain reaction (PCR) using primers spanning more than one exon, such that endogenous genes would not amplify. Both sets of reactions contained 1.5 mM Mg^{2+}, and 200 μM of each dNTP. For determination of the LPL transgene, 20 pmol of the upstream primer LPL65 (5'GTGGGACAGGATGTGGC), located in exon 3, and the downstream primer LPL55

(5'AAGTCCTCTCTCTGCAATCAC), in exon 5 were used in each 50 μL reaction. Thermal cycle conditions were 5 minutes at 96°C, followed by 30 cycles of 96°C for 1 minute, 58°C for 1 minute, 72°C for 45 seconds, and a final 5 minute extension at 72°C. The CETP transgene was assessed using 15 pmol each of primer CETP1 (5'CCTGAAGTATGGCTACACCAC) in exon 3, and primer CETP2 (5'GTGGAAGACTTGCTCGGAGAAC) in exon 9, with cycle conditions consisting of 5 minutes at 96°C followed by 30 cycles of 96°C for 1 minute, 51°C for 30 seconds, 72°C for 45 seconds, with a final extension of 5 minutes at 72°C. Products, approximately 400 bp for LPL and 525 bp for CETP, were visualized following electrophoresis on a 1% agarose gel.

2.2.3 Post-heparin lipase activities

Due to its non-covalent interaction with HSPG on the vascular endothelium, LPL can be released into circulation by a bolus injection of heparin. Total plasma lipase activity was measured in duplicate using a radiolabelled ^{3}H-tri-olein emulsion according to the method of Nilsson-Ehle and Schotz[230]. Ten microlitres of post-heparin plasma was incubated with 100 μL of the radioactive substrate for 1 hour at 37°C. Human heat-inactivated pre-heparin serum is included in the substrate as a source of apoCII. The free fatty acids generated were extracted with a mixture of methanol, chloroform and heptane (1.41:1.25:1), and quantified by liquid scintillation counting. HL activity in the sample was measured following inhibition of LPL with 1 M NaCl[231] (30 minutes, 4°C, in duplicate), and LPL was measured by subtraction of the HL activity from the total lipase activity. One milliunit (mU) of activity is defined as the amount, which hydrolyzes 1 nmol free fatty acids per minute at 37°C, and plasma activities are expressed as mU per millilitre of plasma.

2.2.4 Cholesteryl ester transfer activity

LDL (density 1.024-1.063 g/mL), HDL_3 (density 1.125-1.210 g/mL) and density >1.125 g/mL fractions of human plasma were isolated by sequential ultracentrifugation. The density >1.125 g/mL plasma fraction was incubated with ^{14}C-cholesterol (Amersham, Oakville, Ont.) at 37°C overnight[232]. The remaining free ^{14}C-cholesterol was removed by incubation with excess

LDL. Over 95% of the radioactivity was found in the chemical form of CE on thin layer chromatography, as previously described[233].

Aliquots of sample plasma were incubated with 20 μg of ^{14}C-CE-HDL$_3$ and 100 μg of LDL at 37°C for 16 hours in a final volume of 0.7 mL. After the incubation, LDL in the incubation mixture was precipitated by 50 mM sodium phosphate (pH 7.4) and 16 mM $MnCl_2$ as previously described[233]. Pellets containing LDL following centrifugation (9000xg for 3 minutes) were washed using the buffer and then precipitated again by the same procedure. The radioactivity in the pellets following the second precipitation was counted using a liquid scintillation system. The amount of ^{14}C-CE in LDL indicated CETP activity. The values for CETP activity were corrected by subtracting the incubation blank. The CETP activity in plasma was expressed in nmol CE/h/mL.

2.2.5 Lipid and lipoprotein assessment

Plasma total cholesterol (TC) and TG were measured in duplicate by enzymatic colorimetric procedures using commercially available kits (Boehringer Mannheim, Numbers 1442350 and 450032, respectively). Cholesterol is measured in a single assay whereby CE are converted to free cholesterol, which is then oxidized, generating hydrogen peroxide. The peroxide, in turn, reacts with the substrate to produce a red-coloured product (4-(p-benzoquinone-monoimino)-phenazone), which can be quantified by measuring the optical density (OD) at 500 nm. Sample concentrations are interpolated from the ODs of the samples from a standard curve included in each assay.

TG are quantified in a two-step process. Any existing glycerol in the sample is converted to an oxidized by-product, then TG in the sample are converted to glycerol and FFA. The glycerol (equivalent to TG on a mole to mole basis) is then measured in a three-step enzymatic reaction, generating peroxide and ultimately resulting in the same end product as the cholesterol assay. HDL-C was measured using the cholesterol assay, following precipitation of apoB containing lipoproteins with an equal volume of a 20% polyethylene glycol solution, as previously described[211]. Non-HDL cholesterol (nonHDL-C) is obtained by subtraction of the HDL-C value from the total cholesterol measurement.

2.2.6 Fast performance liquid chromatography (FPLC) analysis of plasma samples

FPLC separation of plasma lipoproteins was performed using two Superose^{TM} 6 (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) columns in series, as previously described[211]. This method utilizes gel filtration to separate the lipoproteins on the basis of their size[25]. Larger lipoproteins pass into the gel particles less, and are thus eluted from the column earlier. The smaller lipoproteins migrate through the gel, and are thus eluted in later fractions.

Equal volumes of plasma from each mouse in each group were pooled, and filtered through a 0.22 μm filter. Filtered plasma (200 μL) was loaded onto the columns, and eluted at a flow rate of 0.5 mL/min in a buffer consisting of 0.15 M NaCl, 1mM EDTA, and 0.02% NaN_3, pH 8.2. The cholesterol and TG content in each 0.5 mL fraction was assessed using commercially available enzymatic kits (Boehringer Mannheim, Numbers 1442350 and 701904, respectively).

2.2.7 Statistical analysis

Males and females were analyzed separately. Mice were grouped according to LPL activity in the highest (high) or lowest (low) tertiles (thirds), and the presence or absence of plasma CETP activity, creating four study groups for each sex. Values are presented as mean ± standard deviation. Between group differences were measured using an analysis of variance (ANOVA). As there was a wide distribution of ages within each group, age was included as a covariate within all analyses, except those noted in the tables, where covariate analysis was not possible. Individual pairwise comparisons between groups were made using the Tukey procedure. Correlations reported are Pearson correlation coefficients. P-values less than 0.05 were considered statistically significant. All analysis was performed using the Systat analysis program (Systat for Windows, version 5.0, SPSS).

2.3 The involvement of CETP

2.3.1 Baseline characteristics

Transgenic mice overexpressing human LPL[211] were bred with transgenic mice expressing simian CETP[229], producing mice either hemizygous for the CETP transgene, or lacking the CETP transgene and thus CETP activity. As the LPL transgenic mice have activities

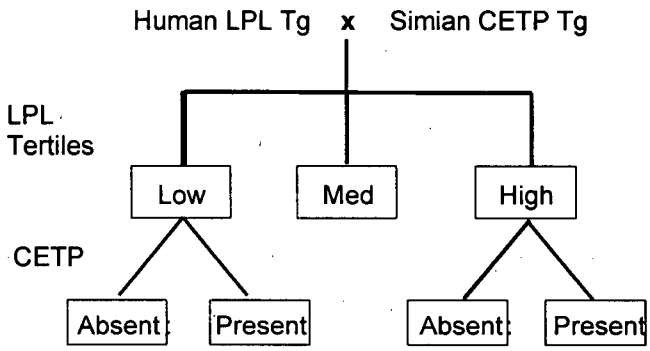

Figure 2.1. Generation of study groups.
Human LPL transgenic mice were bred with simian CETP transgenic mice. The resultant offspring (males and females separately) were divided into tertiles of plasma LPL activity. Within each tertile, the presence or absence of the CETP transgene, and thus plasma CETP activity, was assessed.

that overlap with their non-transgenic littermates, this resulted in mice with a broad distribution of LPL activities. Thus, mice were divided into tertiles of LPL activity, and those in the highest and lowest groups were chosen for study. This resulted in four study groups: those within the lowest and highest tertiles of LPL activity, either expressing CETP or littermate controls without CETP, respectively (Figure 2.1).

Baseline group characteristics are presented in Table 2.2. Within both male and female groups, the presence of the CETP transgene was associated with significant levels of plasma CETP activity ($p<0.001$), and did not differ with LPL status. Similarly, plasma LPL activity of mice in the highest tertile was increased approximately 1.8 fold over those in the lowest tertile ($p<0.001$), and was unaffected by the presence of the CETP transgene. Thus the activity of either enzyme, as measured towards exogenous substrates, does not appear to be directly influenced by the activity of the other. There were no other differences in baseline characteristics except that male mice with CETP tended to have lower HL activities than those not expressing CETP.

2.3.2 Lipoprotein profiles

Lipoprotein profiles were then examined for each of the groups, and are presented for males and females in Tables 2.3 and 2.4, respectively. In males, the presence of plasma CETP activity had the predicted effects on the lipoprotein profile when compared with mice matched for LPL activity within the lowest tertile, but lacking CETP (Table 2.3, first vs. third columns).

Table 2.2. Baseline characteristics of male and female mice

	CETP Present		CETP Absent	
	LPL low	**LPL high**	**LPL low**	**LPL high**
Males				
n	9	17	16	11
Age (weeks)	29.2±6.1	28.5±6.4	26.7±1.3	30.8±6.9
CETP activity (nmol/h/mL)	13.9±4.8[a]	13.0±7.3[a]	0.5±1.0	0.7±1.2
LPL activity (mU/mL)	469±97	888±92[a]	478±61	868±96[a]
HL activity (mU/mL)	90±22[b,c]	105±20[d]	115±20	128±21
Females				
n	10	9	14	17
Age (weeks)	27.4±1.8	27.2±5.3	28.8±2.7	31.9±7.3
CETP activity (nmol/h/mL)	15.4±5.9[a]	13.1±8.7[a]	0.4±0.8	0.8±1.1
LPL activity (mU/mL)	394±122	878±70[a]	398±109	893±78[a]
HL activity (mU/mL)	115±20	136±23	135±26	142±36

All statistics were performed on values corrected for age except female CETP activities.
[a] All comparisons for low vs. high activity are significant at p<0.001
[b] P-values CETP Present-LPL low vs. CETP Absent-LPL low=0.04
[c] P-values CETP Present-LPL low vs. CETP Absent-LPL high=0.001
[d] P-values CETP Present-LPL high vs. CETP Absent-LPL high=0.03
To convert mg/dL to mmol/L, divide cholesterol by 38.7, TG by 88.6 and PL by 75.0.

HDL-C was significantly decreased in mice expressing CETP (31±4 vs. 61±16 mg/dL, p=0.005, CETP present vs. absent), contributing to decreased plasma total cholesterol levels. This led, overall, to a significantly increased total cholesterol to HDL-C ratio (1.45±0.20 vs. 1.17±0.23, p=0.008, CETP present vs. absent). TG and non-HDL cholesterol were not significantly affected by the addition of CETP.

When LPL activity was increased from the lowest to highest tertile in the mice expressing CETP, HDL-C levels were restored to values not significantly different from those seen in the absence of CETP (51±29 mg/dL) (Table 2.3, second column). This increase nearly reached significance compared to those in the lowest tertile of LPL activity (p=0.07, likely due to the large standard deviation), and contributed to elevated total cholesterol levels. Furthermore,

Table 2.3. Lipoprotein profiles of male mice consuming standard rodent chow

	CETP Present		CETP Absent	
	LPL low	**LPL high**	**LPL low**	**LPL high**
n	9	17	16	11
Age (weeks)	31.9±7.0	29.2±5.1	31.3±7.5	32.5±6.9
Total chol. (mg/dL)	45±7[c]	65±35	69±15	76±14
TG (mg/dL)	60±13	39±16	57±21	56±26
HDL-C (mg/dL)	31±4[a,d]	51±29	61±16	65±12
Non-HDL chol. (mg/dL)	14±5	14±9	8±7	11±8
Total/HDL-C	1.45±0.20[b,c]	1.32±0.20	1.17±0.23	1.18±0.13

All statistics were performed on values corrected for age.
P-values CETP Present-LPL low vs. CETP Absent-LPL low: [a]0.005, [b]0.008
P-values CETP Present-LPL low vs. CETP Absent-LPL high: [c]0.02, [d]0.003

in the presence of CETP, LPL activity and HDL-C were significantly correlated (Figure 2.2, r=0.43, p=0.006). In contrast to the results in mice expressing CETP, in the absence of CETP there were no significant differences in lipoprotein profiles of mice between the lowest or highest tertiles of LPL activity (Table 2.3, third and fourth columns). No correlation between LPL activity and HDL-C was evident (Figure 2.2, r=0.15, p=0.36).

Similar profiles to the males were seen in the female mice (Table 2.4). The addition of the CETP transgene had the predicted effects on the lipoprotein profiles (Table 2.4, first and third columns). Comparing mice within the lowest tertile of LPL activity with and without CETP, total and HDL-C levels were significantly decreased (46±14 vs. 63±12 mg/dL, p=0.02 and 33±12 vs. 53±12 mg/dL, p=0.002, respectively, in the presence vs. absence of CETP), while the total cholesterol to HDL-C ratio was significantly increased (1.42±0.19 vs. 1.21±0.12, p=0.03). Increasing LPL within the CETP expressing group (Table 2.4, second column) was again associated with increased HDL-C levels (33±12 vs. 45±14 mg/dL, lowest vs. highest tertiles of LPL activity), although the correlation between the two parameters did not reach significance (r=0.29, p=0.12, data not shown). Additionally, increased total cholesterol and

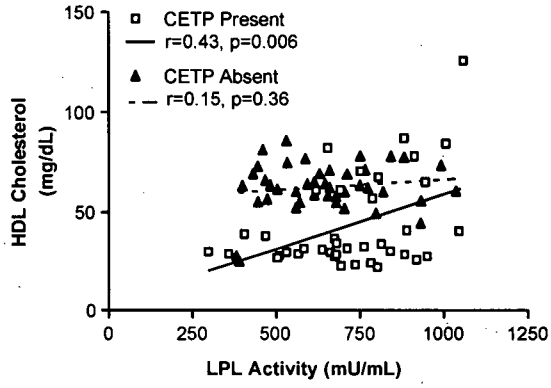

Figure 2.2. Correlations between LPL activity and HDL-C concentrations in male mice fed a standard rodent chow diet.
Mice expressing CETP are represented by open squares and the solid line. Mice not expressing CETP are represented by solid triangles and a dashed line.

Table 2.4. Lipoprotein profiles of female mice consuming standard rodent chow

	CETP Present		CETP Absent	
	LPL low	**LPL high**	**LPL low**	**LPL high**
n	10	9	14	17
Age (weeks)	27.4±7.7	28.1±3.5	28.3±7.7	30.4±7.6
Total chol. (mg/dL)	46±14[a]	59±17	63±12	58±12
TG (mg/dL)	42±13	36±11	33±11	35±11
HDL-C (mg/dL)	33±12[b,d]	45±13	53±12	49±12
Non-HDL chol. (mg/dL)	13±6	14±12	10±5	9±5
Total/HDL-C	1.42±0.19[c,d]	1.34±0.27	1.21±0.12	1.21±0.14

All statistics were performed on values corrected for age.
P-values CETP Present-LPL low vs. CETP Absent-LPL low: [a]0.02, [b]0.002, [c]0.03
P-values CETP Present-LPL low vs. CETP Absent-LPL high: [d]0.02

a decreased total cholesterol to HDL-C ratio were noted. As in the males, in the absence of CETP, increasing LPL activity had little effect on the HDL-C levels (Table 2.4, third and fourth columns), and no correlation was observed.

2.3.3 FPLC analysis

These differences in lipoprotein profiles are also clearly illustrated in the FPLC profiles of these animals (Figures 2.3 and 2.4, for males and females, respectively), where the major effects of the genetic manipulations manifest in the HDL peak. In mice expressing CETP (Figure 2.3, top left), the HDL peak is characterized by a shift towards smaller particles (towards the right) compared to mice without CETP (top right). Furthermore, the addition of CETP was associated with apparently increased HDL and LDL triglyceride, and VLDL-C, as would be predicted by the cholesteryl ester transfer process.

Increasing LPL within this group (bottom left panel) led to a shift towards larger sized particles, resulting overall in dramatically elevated plasma HDL-C levels. HDL-TG and LDL-TG levels also appeared reduced. As these lipoproteins are not generally substrates for LPL, this suggests an inhibition of the CE transfer process within the plasma by increasing LPL and decreasing transfer of TG into these particles. In the absence of CETP (Figure 2.3, right panels) the HDL peak is large, unimodal, and consists of particles relatively large in size. This profile does not change with increasing LPL activity (bottom panels compared to top).

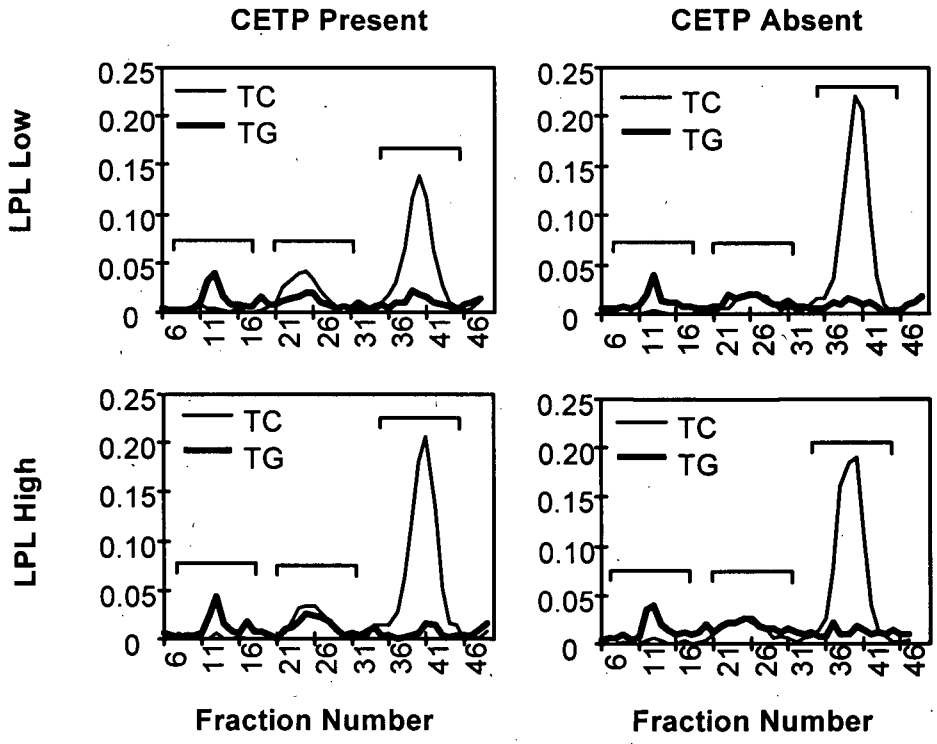

Figure 2.3. FPLC profiles of male mice fed a standard rodent chow diet.
Cholesterol in each fraction is shown by the thin line, and triglycerides in each fraction are shown by the thick line. From left to right, peak areas depicted represent VLDL, LDL and HDL, respectively.

In females, increasing LPL activity in mice expressing CETP was associated with a restoration of the HDL-C peak and decreased HDL-TG (Figure 2.4, left panels). In contrast, in the absence of CETP, few differences in the profiles between the lowest and highest LPL tertiles were evident (Figure 2.4, right panels).

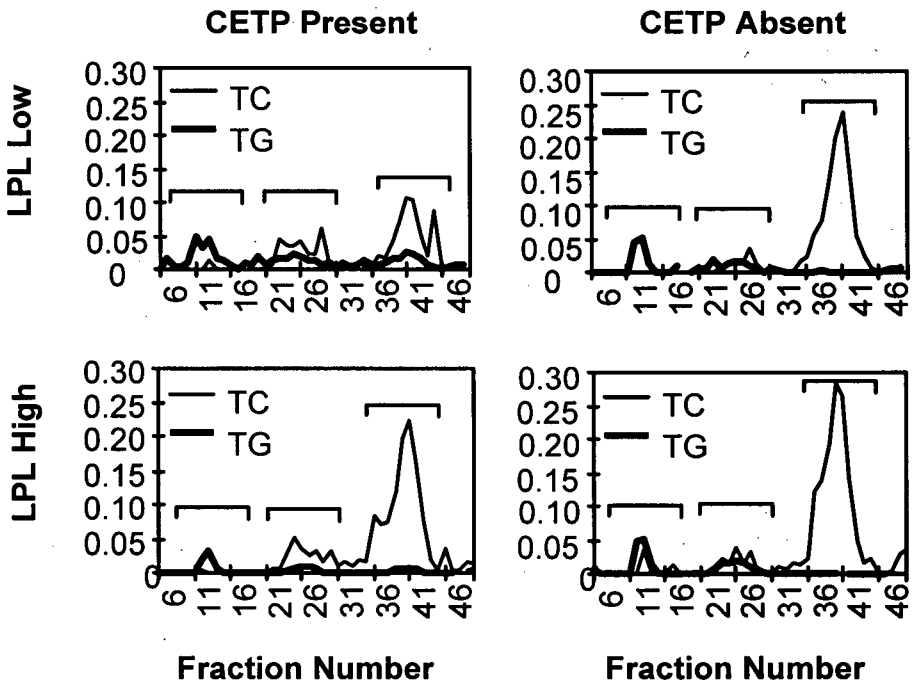

Figure 2.4. FPLC profiles of female mice fed a standard rodent chow diet. Cholesterol in each fraction is shown by the thin line, and triglycerides in each fraction are shown by the thick line. From left to right, peak areas depicted represent VLDL, LDL and HDL, respectively.

2.4 Implications of dietary fat content

As the concentration of plasma TG rich acceptor particles, such as VLDL, has been suggested to be an important modulator of the transfer process[38,222], the relation between LPL and CETP was further explored under dietary conditions designed to favour VLDL synthesis. Male mice were fed a high fat, high carbohydrate diet (15% corn oil, 50% sucrose) for a period of five weeks, and lipoprotein profiles were examined. Following high fat, high carbohydrate feeding, plasma LPL activities increased dramatically in all groups, consistent with results seen in humans following a fat-rich meal[134] and in LPL transgenic mice fed a high fat, cholesterol enriched diet[234], such that there were no longer significant differences between the groups

(716±161 vs. 918±203; 1048±479 vs. 968±519 mU/mL for "lowest" vs. "highest" tertiles on the chow diet, with and without CETP, respectively). Plasma TG increased by approximately 50%, and total and HDL cholesterol increased by approximately 130%, in all groups compared with prediet values (data not shown). Non-HDL cholesterol increased, though due to large standard deviations, not significantly in all groups.

However, when levels of LPL activity are examined without grouping by tertiles, an association between LPL and HDL-C is evident. Following high fat, high carbohydrate feeding, plasma LPL activity is positively correlated with HDL-C levels in both the presence (Figure 2.5, $r=0.45$, $p=0.03$) and absence (Figure 2.5, $r=0.73$, $p<0.001$) of CETP. While consuming a standard low fat, low carbohydrate rodent chow, the relationship between LPL activity and HDL-C is not evident in the absence of CETP. However, the effects of LPL on HDL-C levels may become apparent when the system is challenged by a high fat, high carbohydrate diet, irrespective of the presence or absence of CETP.

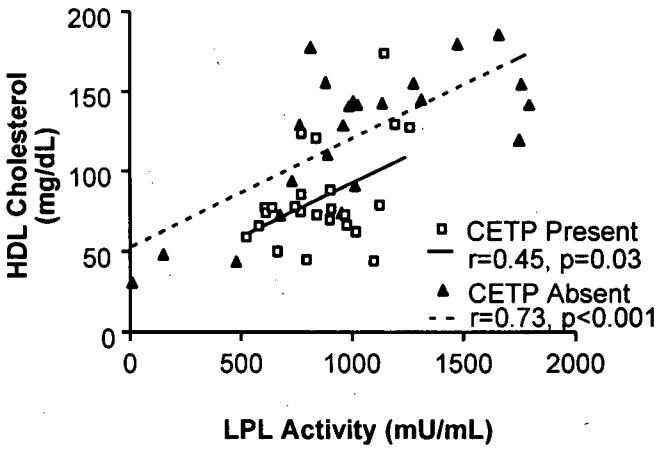

Figure 2.5. Correlations between LPL activity and HDL-C concentrations in male mice following high fat, high carbohydrate feeding.
Mice expressing CETP are represented by open squares and the solid line. Mice not expressing CETP are represented by solid triangles and a dashed line.

Representative post-diet FPLC profiles of groups of mice matched for LPL activity, with and without CETP are shown in Figure 2.6. Following high fat, high carbohydrate diet feeding, changes in the lipoprotein profiles were evident, compared with those seen on the rodent chow diet (Figure 2.3). In the absence of CETP, an accumulation of small LDL particles, which were overlapping with the HDL-C range, was evident, while the addition of CETP resulted in a much

clearer distinction between the LDL and HDL fractions. This likely results from the transfer of cholesterol from HDL to LDL, resulting in larger LDL and smaller HDL.

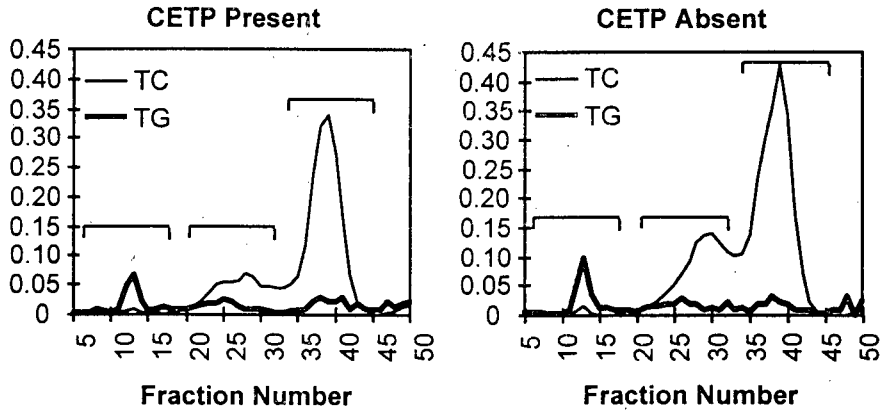

Figure 2.6. Post-diet FPLC profiles of male mice.
Groups were matched for LPL activity (918±203 and 968±519 mU/mL for CETP present and absent, respectively.) Cholesterol in each fraction is shown by the thin line, and triglycerides in each fraction are shown by the thick line. From left to right, peak areas depicted represent VLDL, LDL and HDL, respectively.

2.5 Discussion

Plasma levels of HDL-C have generally been inversely correlated with an individual's risk of developing CAD[38]. As such, understanding factors regulating the concentrations of this lipoprotein are of prime importance. Several genetic and metabolic factors have been implicated in this process[38]. Four key enzymes in lipoprotein metabolism, HL, LPL, LCAT and CETP, have been shown to account for almost 50% of the variability of HDL-C levels within a hypertriglyceridemic cohort[235]. However, while correlations between plasma LPL activity and HDL-C have been observed in humans[142,143,145,210], such relations have not been observed in the genetically altered mouse models[211,212,214,215,234]. Thus, for mice to be useful models in which to study the effects of LPL on lipids and atherosclerosis in relation to those seen in humans, these differences must be better understood. In this study we have examined whether species differences in lipid metabolism, such as the absence of plasma CETP activity in mice, might be contributing to these findings. We show on a standard rodent chow diet that an increase in LPL activity is associated with increased HDL-C levels only in the presence of CETP activity.

Two possible mechanisms may account for the interaction between LPL and CETP in regulating HDL-C levels. One factor which plays a major role in determining the rate and extent of lipid transfer between particles is the concentration of TG-rich acceptor particles[38,236]. Cholesteryl ester transfer from HDL to VLDL and LDL fractions is correlated with plasma cholesterol concentrations in apoB containing lipoproteins, and with plasma TG levels[237]. Furthermore, VLDL-TG levels have also been shown to be a major predictor of CE transfer rate[238], and increased transfer of CE from HDL to TGRL is seen in hypertriglyceridemic individuals[38,239]. The size of the TG rich acceptor pool has thus been suggested to be rate limiting for transfer of CE from HDL to VLDL[222]. Therefore, a decreased VLDL concentration, due to increased hydrolysis by LPL, leads to a decreased concentration of TG rich acceptors. This decreased acceptor concentration is likely to impair the ability of CETP to transfer CE from HDL to this fraction, resulting in increased HDL-C concentrations. This is the probable mechanism of interaction between LPL and CETP in the regulation of HDL-C levels.

However, another possible mechanism linking lipolysis and CE transport, is that altered surface composition of VLDL or HDL, resulting from increased lipolysis, may affect the binding of CETP to, and interactions with, these particles[38]. Lipolyzed VLDL may be a better acceptor of CE than non-lipolyzed VLDL[227,236], due to enhanced binding of CETP to the particles, through an increase in charged products on their surface[240]. These data suggest the surface alterations induced by lipolysis may directly affect the ability of CETP to interact with its target lipoproteins. It could thus also be suggested that large increases in lipolysis may alter surface composition to the extent that CETP may bind less effectively, providing another potential mechanism underlying the interaction between LPL and CETP seen in this study.

Previous studies have shown that an interaction between hypertriglyceridemia and CETP in mice further decreases HDL concentrations[241]. To further examine the relation between LPL and CETP under a TG challenge, mice were fed a high fat, high carbohydrate diet intended to increase plasma TG, and thus CE acceptor concentrations. Interestingly, feeding of this diet increased both LPL activities and HDL-C concentrations, and resulted in striking correlations between LPL and HDL-C, independent of CETP status.

Several alterations in lipoprotein metabolism may occur upon high carbohydrate and high fat feeding[242,243] which may help to explain the correlations between LPL and HDL regardless of CETP status. Consumption of a high sucrose diet increases hepatic secretion of TGRL[242], while

high fat feeding increases apoAI concentrations[243]. Taken together with the changes observed in this study, these metabolic alterations may be sufficient to account for the relation between LPL and HDL-C, independent of CETP status. We have observed an accumulation of small LDL (Figure 2.6), which may result from increased hepatic secretion of TGRL, combined with the increased LPL activities, observed in this study. This suggests that lipolysis is increased in response to the dietary challenge. Therefore, we propose that consumption of a normal chow diet results in insufficient hydrolysis of TG rich particles and formation of surface remnants, such that a strong correlation between LPL activity and HDL-C levels is not evident in the absence of CETP. However, following high fat, high carbohydrate feeding, increased lipolysis generates sufficient surface remnants, perhaps in conjunction with increased apoAI concentrations to aid HDL formation, such that the association between HDL-C and LPL activity independent of CETP status becomes apparent.

A potential confounding factor in this study is the significant difference in HL activities between the male mice at baseline. Hepatic lipase is a heparin-releasable enzyme whose main function is the hydrolysis of TG and PL in the less TG-rich lipoprotein particles such as IDL, LDL and HDL[244]. One prime role for this enzyme is in the HDL conversion process, where it hydrolyzes core TG in HDL_2, which, in conjunction with loss of CE through CETP action, leads to the formation of HDL_3[38,210,244]. Thus, HL activity has been shown to correlate negatively with plasma HDL-C[38,210,244]. Differences in HL levels between groups could therefore affect comparisons of HDL. However, both groups of mice expressing CETP, which resulted in decreased HDL-C concentrations, had the lowest HL activities. Decreased HL activity would be expected to increase HDL-C within these groups. In addition, the effect of increasing LPL within the CETP expressing group, which was associated with increased HDL, would be opposed by the higher HL activity in this group. Furthermore, in the female mice, there were no significant differences in HL activities between groups, yet the same HDL trends were maintained. Thus, it seems unlikely that the findings of this study could be attributed to altered HL activities between the groups.

Interestingly, while there were no consistently significant differences between male and female mice, females tended to have both increased HL activities, and decreased plasma TG compared to males. The combination of increased hydrolysis of TG in the less TG rich particles,

and decreased TG rich acceptor pool concentrations may partly explain the milder effects of CETP in the females compared to the males.

Surprisingly, no differences in plasma TG levels with increasing LPL activity at baseline were observed in mice lacking CETP. This may be due to the altered timing of the fasting period in this study. Specifically, as the initiation of fasting in this study coincided with the onset of the dark cycle, the effective fasting time may have been longer if mice did not consume much during their less active light cycle period. Such prolonged fasting may have been of sufficient duration for most TGRL to be hydrolyzed, even with lower LPL activities. Thus, a failure to measure differences in plasma TG may not necessarily indicate that there was no difference in LPL activities between the groups.

In summary, we have shown that under normal metabolic conditions in both male and female mice, plasma LPL activity was only correlated with HDL-C in the presence of CETP, providing additional *in vivo* evidence of the interaction between LPL and CETP in the regulation of HDL-C levels, likely due to changes in TG rich acceptor pool concentrations. Following the metabolic alterations induced by high fat, high carbohydrate feeding, strong correlations between LPL and HDL were demonstrated regardless of the CETP status. These data highlight the importance of gene-diet interactions in lipoprotein metabolism, and suggest dietary factors also regulate the relationship between LPL and HDL-C. The insights into the relationship between LPL and HDL-C in mice gained here provide validation for the use of such mouse models in understanding the role of LPL in lipid metabolism and atherosclerosis. Furthermore, in the presence of CETP, as in many species including humans, irrespective of dietary status, LPL activity is an important predictor of HDL-C levels, capable of overcoming some of the detrimental effects of CETP. Thus measurements of LPL activity are strong predictors of HDL-C levels, and may therefore be important determinants of atherosclerosis susceptibility.

Chapter 3: Plasma and vessel wall lipoprotein lipase have different roles in atherosclerosis

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Preface

I designed all experiments described herein, and performed all of the work except as described subsequently. Technical support for the project was provided by Li Miao, Fudan Miao and Nagat Bissada. Pieternel Steures, a summer student under my supervision, assisted with the DNA extractions and genotyping. Ms. Julie Chow performed the sectioning and staining of the aortas. Dr. Bruce McManus and Janet McManus provided access to and instruction on the digital system used in measuring the lesions and a detailed pathological description of "typical" lesions of each genotype. The FPLCs in this chapter were performed in the lab of Dr. Renee LeBoeuf at the University of Washington by Ms. Cynthia Vick. The gradient gel electrophoresis was performed by Bharti Ratanjee in the lab of Dr. David Marais at the University of Cape Town, South Africa.

3.1 Introduction

As described in Chapter 1, LPL has been hypothesized to have differing roles in atherosclerotic lesion formation, depending on its site of expression. While LPL activity in the plasma compartment is generally considered to be anti-atherogenic, in the vessel wall, however, increased LPL protein has been proposed to be pro-atherogenic. Human studies are complicated by many potential confounding factors, such as diet, alcohol consumption, smoking behaviour, exercise level and other genetic factors, which are more easily controlled in inbred animal models. Thus, studies in animal model systems where such factors can be controlled and where specific genetic manipulation can occur are of extreme relevance. Furthermore, animal models allow for tissue-specific manipulation of the expression of the desired gene.

Previous work studying the role of LPL in atherosclerosis in mice has shown that widespread high level overexpression of LPL was associated with decreased atherosclerosis in a cholesterol fed LDLr deficient model[245]. This is consistent with the effects observed when rats and rabbits were treated with the compound NO-1886, which increases LPL activity[246,247]. But, the LPL transgenic mice have been shown to express LPL in the aorta as well as other tissues contributing to plasma LPL, and it remains unclear how tissue LPL expression is altered by NO1886. No studies have examined the relative effects of vessel wall versus plasma LPL expression on susceptibility to atherosclerosis.

Mice do not naturally develop atherosclerosis. Early studies demonstrated that feeding C57BL/6 mice a cholesterol-containing diet induced lesion formation in this strain[248-250]. With the subsequent development of gene-targeting techniques, two other mouse models have emerged, the apolipoprotein E deficient ($E^{-/-}$)[251-254] and LDL receptor deficient ($LDLr^{-/-}$)[255,256] models[208]. Both these models develop lesions spontaneously, that is, in the absence of cholesterol feeding. However, the process is much slower in the $LDLr^{-/-}$ model, thus cholesterol is often added to the diet of this model to accelerate lesion formation[255].

In humans, plasma clearance of VLDL is mediated through the actions of two of its apolipoproteins, $apoB_{100}$ and apoE, both of which bind to the LDL receptor[28,30,36]. In addition, apoE can also bind to remnant receptors in the liver, providing a second mechanism of removal of apoE containing particles[28,30,34]. As lipolysis proceeds and VLDL are converted to smaller particles (IDL and LDL), apoE is lost, leaving solely the $apoB_{100}$ moiety to mediate clearance of LDL through the LDL receptor (Figure 1.3)[30,36]. In humans, $apoB_{48}$ (which cannot bind the

LDLr[118]) is synthesized only in the intestine, and incorporated into chylomicrons[28], however in mice it is also synthesized in liver, and incorporated into VLDL[30,118,257]. Thus, up to 70% of mouse VLDL contains $apoB_{48}$ instead of $apoB_{100}$[257]. In the absence of $apoB_{100}$, VLDL and their derivatives retain apoE[118],[257] and are thus dependent on apoE binding to the LDL receptor and remnant receptors for their removal from circulation. Thus, in the absence of apoE, these particles cannot be cleared by receptor-mediated uptake, and must rely upon secondary mechanisms of removal.

In the absence of functioning LDL receptors, in mice, most particles may still be taken up and cleared from circulation through binding of apoE to remnant receptors. $E^{-/-}$ mice therefore have a much more severe accumulation of IDL and LDL than $LDLr^{-/-}$ mice[257], as clearance of a large portion of VLDL and its derivatives through both the LDL receptor and remnant receptors is impaired. LPL and apoE have been shown to have independent, complementary effects on the binding of lipoproteins to proteoglycans[117,258], and LPL may also aid receptor independent uptake[130]. Thus, by blocking a larger portion of clearance through the absence of apoE, LPL aided clearance mechanisms may be more important in these mice.

We have examined the effects of decreased LPL expression on atherosclerotic lesion formation in the three mouse models of atherosclerosis: $E^{-/-}$, cholesterol-fed C57BL/6, and cholesterol-fed $LDLr^{-/-}$, and have compared these results with the effects of overexpression of LPL exclusively in the plasma on atherogenesis in these same model systems. We have sought to directly address the question of the relative atherogenicity of vessel wall versus plasma LPL *in vivo*, using all three mouse models of atherosclerosis. By comparing the atherosclerosis susceptibility of mice with decreased LPL expression in both plasma and vessel wall, with mice that overexpressed LPL in tissues contributing solely to plasma LPL excluding the vessel wall, we have been able to test the effects of tissue differences in LPL expression on the development of atherosclerotic lesions.

3.2 Methods

3.2.1 Animals

Decreased LPL expression was examined in a line of mice heterozygous (+/-) for a targeted (null, "-") *LPL* allele, a kind gift from Drs. T. Coleman and C. Semenkovich[214], and compared to their normal $LPL^{+/+}$ siblings. For the model of increased plasma LPL expression,

the transgenic mice (LPL^{Tg}) that overexpress human LPL in heart, skeletal muscle, adipose tissue, kidney and stomach described in Chapter 2 were used[211]. Macrophages, the primary sources of vessel wall LPL[152],[153] do not express the human LPL transgene, although the mice do maintain the wildtype mouse LPL (+/+) background. No LPL expression has been detected from peritoneal macrophages of *LPL* knockout mice rescued with this CMV-human LPL transgene. The mean LPL activity in media following a 24 hour culture of these macrophages was 1.59±0.33 in macrophages from the rescued knockouts (n=3), compared to 8.54±0.44 mU/mL in normal mice (n=3; media alone gave a measurement of 2.4 mU/mL). Also, no human LPL has been detected in lesions of these mice by immunohistochemistry with the 5D2 monoclonal antibody (R.C. LeBoeuf, unpublished data), indicating that LPL is not expressed in the vessel wall of these mice. Although we have shown that there is some overlap in LPL activity between the transgenic and non-transgenic mice (Chapter 2) that might lead to increased variability in the measurements between groups, these mice have the well-defined expression patterns crucial to this study, and were readily available.

In addition, the above three groups of mice were bred onto the $E^{-/-}$ or $LDLr^{-/-}$ mouse line obtained from the Jackson Laboratory (Bar Harbor, ME), producing mice that were deficient in apoE or the LDLr, and either +/- or +/+ at the mouse *LPL* locus, or containing the human LPL cDNA transgene (Tg, +/+ at mouse *LPL* locus). As all mice either have endogenous hypertriglyceridemia and/or were fed high fat-cholesterol rich diets, the CETP transgene was not

Table 3.1. Nomenclature of the genotypes studied

Designation	*LPL* genotype	ApoE genotype	LDLr genotype
$LPL^{+/-}E^{-/-}$	+/-	-/-	+/+
$LPL^{+/+}E^{-/-}$	+/+	-/-	+/+
$LPL^{Tg}E^{-/-}$	transgenic[a]	-/-	+/+
$LPL^{+/-}$	+/-	+/+	+/+
$LPL^{+/+}$	+/+	+/+	+/+
LPL^{Tg}	transgenic[a]	+/+	+/+
$LPL^{+/-}LDLr^{-/-}$	+/-	+/+	-/-
$LPL^{+/+}LDLr^{-/-}$	+/+	+/+	-/-
$LPL^{Tg}LDLr^{-/-}$	transgenic[a]	+/+	-/-

[a]CMV-human LPL transgenic mice are +/+ at the mouse LPL locus

included within these crosses. All mice in the study are estimated to contain a greater than 90% C57BL/6 genetic background. Their nomenclatures are described in Table 3.1. Our study groups comprised only female mice.

Animals were housed in microisolator cages in groups of 3-4 mice per cage, in an environmentally controlled facility, with 12 hour light and dark cycles (7 am-7 pm). Animals had free access to food and water, except immediately prior to lipid measurements, as indicated below. All procedures were approved by the University of British Columbia Committee on Animal Care.

3.2.1.1 Diets

Mice were fed a standard mouse chow (Purina Laboratory Rodent Diet, 5001, PMI Feeds) that contained approximately 4.5% fat and 23.4% protein, with no more than 270 ppm cholesterol. The BL/6 and LDLr$^{-/-}$ mice (E$^{+/+}$) were also fed an atherogenic diet (Harlan Teklad #88051)[250] containing approximately 15% cocoa butter, 6.2% carbohydrate, and 20.6% protein, with 1.25% cholesterol and 0.5% cholate for a period of 12 weeks, as described below.

3.2.1.2 Animal procedures

At 10 weeks of age, mice were fasted overnight (from approximately 10 pm-8 am) prior to withdrawing blood samples for lipid measurements. At 12 weeks of age the BL/6 and LDLr$^{-/-}$ mice were placed on the atherogenic diet for a period of 12 weeks. Blood samples were withdrawn following an overnight fast (as above) at 22 weeks of age (after 10 weeks on the diet) for lipid analysis, and mice were sacrificed at 24 weeks of age.

Following exsanguination, mice were perfused with 4% paraformaldehyde in phosphate buffered saline for approximately 5 minutes at a flow rate of 3.5 mL/min. Hearts and upper aortae were then removed and fixed in the same solution prior to embedding and sectioning.

3.2.2 Genotyping

All genotypes were determined by PCR. The presence of the LPL transgene was determined as described in Chapter 2. The presence of the *neo* insertion in exon 8 of the mouse *LPL* gene was assessed using multiplex PCR including 3 primers. The forward primer is located complementary to the junction of intron 7 and exon 8 (LPLK3, 5'GAAATTTTCACCCAGGCC

GGAGG), while there are two reverse primers, one in the neomycin resistance insertion as described[214] (Neo, 5'TCGCCTTCTATCGCCTTCTTGAC) and one at the 3' end of exon 8 distal to the insertion site (LPLK1, 5'CCTCTCGATGACGAAGCTGG). In the absence of the insertion, primers LPLK3 and LPLK1 amplify a band of approximately 150 bp of mouse LPL exon 8. In the presence of the *neo* insertion, the fragment (>1.5 kb) between LPLK3 and LPLK1 does not amplify under the given conditions, but LPLK3 and Neo produce a 600 bp product. A mouse heterozygous for the *neo* insertion thus will display both the 600 and 150 bp products, while a mouse wildtype at the *LPL* locus will only display the 150 bp band. The genotype at the mouse *LPL* locus can thus be unambiguously identified. PCR was carried out using 2 mM Mg^{2+}, 12 pmol LPLK1, 20 pmol LPLK3 and 24 pmol Neo for 35 cycles under the following thermocycling conditions: 94°C for 1 minute, 56°C for 1 minute and 72°C for 2 minutes.

A similar scheme was used to genotype the mouse LDLr and apoE loci. For the LDLr gene, upstream primers LDLRK3 (24 pmol, exon 4, 5'GACTTCCGATGCCAGGATGG) and Neo (20 pmol), and a single downstream primer LDLRK2 (14 pmol, exon 4, 5'GCTGCGATG GATACACTCACTG) were used in the presence of 1.6 mM Mg^{2+}, for 35 cycles consisting of: 94°C for 1 minute, 56°C for 45 seconds, 72°C for 1 minute. Genotyping at the apoE locus used 20 pmol of each primer (E1: forward, exon 3, 5'GATGCCTAGCCGAGGGAGAGC; E2: reverse, intron 3, 5'GAATTGCAGAGCCTTCGAAGC; and Neo2: forward, 5'TGGCGGACC GCTATCAGGAC) and 2 mM Mg^{2+}, with cycles consisting of 96°C for 1 minute, 51°C for 30 seconds and 72°C for 45 seconds.

All PCR reactions were carried out in 50μL volumes, in the presence of 200 μmol each dNTP. Each had an initial denaturation of 5 minutes at 96°C and a final extension of 5 minutes at 72°C.

3.2.3 Lipid and lipoprotein analysis

Plasma was separated by microcentrifugation for 10 minutes at 4°C, aliquoted, and stored at -70°C until analysis. TG, total cholesterol, HDL-C and nonHDL-C were measured as described in Chapter 2.

3.2.3.1 FPLC separation of plasma lipoproteins

Plasma lipoproteins were separated by FPLC gel filtration using a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech LKB Biotechnology, Uppsala, Sweden). This procedure is a slight modification of the one described in Chapter 2. Plasma (100 μL) was loaded onto the column and eluted with phosphate buffered saline (PBS) at a constant flow rate of 0.2 mL/minute at 4°C. Sixty fractions, 500 μl each, were collected using a Frac 100 fraction collector (Pharmacia). Cholesterol and triglyceride concentrations were determined colorimetrically using 100 μl of each fraction (Diagnostic Chemicals Limited, Prince Edward Island, Canada and Boehringer Mannheim Corp., Indianapolis, IN, respectively) and adjusted to reflect plasma total cholesterol and TG levels.

3.2.3.2 Gradient gel electrophoresis (GGE)

GGE was performed on plasma samples obtained at mouse sacrifice. In brief, plasma (50 μL) was pre-incubated with Sudan Black (25μL of 1% (w/v) in ethylene glycol) at 4°C for 1 hour. Samples were centrifuged at 10000 x g for 20 minutes, an aliquot was mixed with an equal volume of saturated sucrose, and volumes equivalent to 4 μL plasma were loaded into a 2-8% gradient polyacrylamide gel. The samples were electrophoresed at 130 V for 18-24 hours at 4°C. Lipoprotein species were identified and named according to their migration relative to the corresponding human species. The procedure allows the identification of the following lipoproteins: $VLDL_1$ (S_f 60-400), $VLDL_2$ (S_f 20-60), IDL (S_f 12-20), and LDL (S_f 0-12), within which size heterogeneity can be observed in humans. The retardation factors (R_f) for the above classes of lipoprotein are <0.45, 0.45-0.7, 0.7-0.85, and 0.85-1.0 respectively, when small human LDL is used as a reference. Analysis was performed by counting the number of mice of each genotype that displayed the various lipoprotein species.

3.2.4 Lesion assessment

The ventricular apex of fixed hearts was transected below the atria and discarded. The upper portion of the hearts was transferred to a 1:1 solution of Optimal Cutting Temperature (OCT™, Tissue Tek, 4583) media and buffered saline overnight. Hearts were then embedded with the cut surface down in OCT, flash frozen, and stored at -70°C until sectioning.

Sectioning was performed as described[248]. Serial 10 μm sections were obtained working from the apex of the heart towards the aortic origin, beginning to mount sections from the point where all 3 aortic valve cusps became clearly visible. Every fourth section was placed on a slide for Oil red O (ORO) staining of neutral lipid (counterstained with hematoxylin), such that each slide had sections 40 μm apart. Sections immediately prior to those used for ORO staining were saved on a separate slide and stained with Movat's pentachrome for the identification of elastin, collagen, glycosaminoglycans and smooth muscle cells. Sectioning and staining were performed by Ms. Julie Chow in the University of British Columbia University Hospital Morphological Services Laboratory.

Atherosclerotic lesion areas were measured using the Bioview Color Image Analysis system (M^{c}Donald Research Laboratories-UBC, Infrascan Inc., 1993), by spectral analysis (amount of red-staining area) in the user-defined area of the image (the vessel itself, excluding surrounding tissue). Areas are reported as the average ORO staining area per section in the first 5 such sections for each mouse. Lesions in the aortic root were examined rather than those from the whole aorta to produce results which would be comparable for both model systems.

3.2.5 Statistical analysis

Statistical analyses were performed using Systat (version 7.0, SPSS Inc.). Data are reported as mean $\pm$ standard deviation. Between group comparisons were made using Student's *t*-tests ($LPL^{+/-}$ vs. $LPL^{+/+}$ and LPL^{Tg} vs. $LPL^{+/+}$).

3.3 The apoE deficient model

3.3.1 LPL-induced alterations in lipid profiles of $E^{-/-}$ mice

To examine the effects of decreased LPL on lipid profiles and atherogenesis, heterozygous LPL deficient ($LPL^{+/-}$) mice[214] were bred with $E^{-/-}$ mice, resulting in mice lacking apoE and either $LPL^{+/-}$ or $LPL^{+/+}$ ($LPL^{+/-}E^{-/-}$ and $LPL^{+/+}E^{-/-}$, respectively). Mice heterozygous for LPL deficiency had an approximately two-fold increase in TG (213$\pm$92 vs.118$\pm$54 mg/dL, $LPL^{+/-}E^{-/-}$ vs. $LPL^{+/+}E^{-/-}$, $p<0.001$; Table 3.2). Total cholesterol was mildly (20%) increased ($p=0.03$), caused by both an increase in HDL-C (55$\pm$41 vs. 34$\pm$25 mg/dL, $p=0.04$) and a smaller increase in nonHDL cholesterol (Table 3.2).

Table 3.2. Lipid levels in apoE deficient mice by *LPL* genotype

	LPL +/- (n=25)	LPL +/+ (n=23)	LPL Tg (n=18)	P-Values +/- vs. +/+	P-Values Tg vs. +/+
TG (mg/dL)	213±92	118±54	80±37	**<0.001**	**0.01**
TC (mg/dL)	515±155	433±102	363±92	**0.03**	**0.03**
HDL-C (mg/dL)	55±41	34±25	10.9±10.9	**0.04**	**<0.001**
Non-HDL chol. (mg/dL)	460±156	399±90	352±87	0.10	0.10
	(n=24)	**(n=21)**	**(n=18)**		
Lesion area (μm^2)	62792±38391	87636±40218	56877±29533	**0.04**	**0.01**

Lipid levels were measured at 10 weeks of age, and lesion areas were measured at 16 weeks of age. Mice were fed a standard rodent chow diet for the duration of the study.

To examine the effects of increasing LPL in plasma, but not within the vessel wall, transgenic mice (LPL^{Tg}) containing a transgene driven by the CMV promoter[211] and overexpressing human LPL in several tissues excluding macrophages (the primary source of vessel wall LPL[152,153]), were bred with the $E^{-/-}$ mice. The anti-atherogenic lipid profiles seen with increasing LPL were mirrored in the LPL transgenic mice. TG were further reduced versus the $LPL^{+/+}E^{-/-}$ (80±37 vs. 118±54 mg/dL, p=0.01; Table 3.2), as was TC (363±92 vs. 433±102 mg/dL, p=0.03). This was contributed to by decreased HDL-C (11±11 vs. 34±25 mg/dL, p<0.001) and nonHDL cholesterol, (Table 3.2).

3.3.2 LPL expression and atherosclerotic lesion formation in $E^{-/-}$ mice

Examination of atherosclerotic lesion areas revealed that $LPL^{+/-}E^{-/-}$ mice (mice with decreased LPL in plasma and the vessel wall) displayed a 30% reduction in mean ORO staining area compared with their $LPL^{+/+}E^{-/-}$ littermates (62792±38391 vs. 87636±40218 μm^2,p=0.04), despite pro-atherogenic plasma lipid profiles (Table 3.2; Figure 3.1, A and B). This suggests that the loss of LPL protein in the vessel wall resulted in less atherosclerosis, and had a greater effect on reducing susceptibility to atherosclerosis than the atherogenic lipid changes caused by low plasma LPL activity.

In contrast, in $LPL^{Tg}E^{-/-}$ mice overexpressing LPL only in the plasma and not in macrophages, atherosclerotic lesion areas were approximately 35% smaller (56877±29533 vs.

87636±40218 μm^2, p=0.01, Table 3.2) as compared to the $LPL^{+/+}E^{-/-}$ mice (Figure 3.1C). This suggests that increasing LPL expression specifically in plasma, without increasing vessel wall LPL concentrations, is associated with decreased lesion formation. Thus, increasing plasma LPL activity is associated with alterations in lipid levels, which in turn convey lowered susceptibility to atherosclerosis.

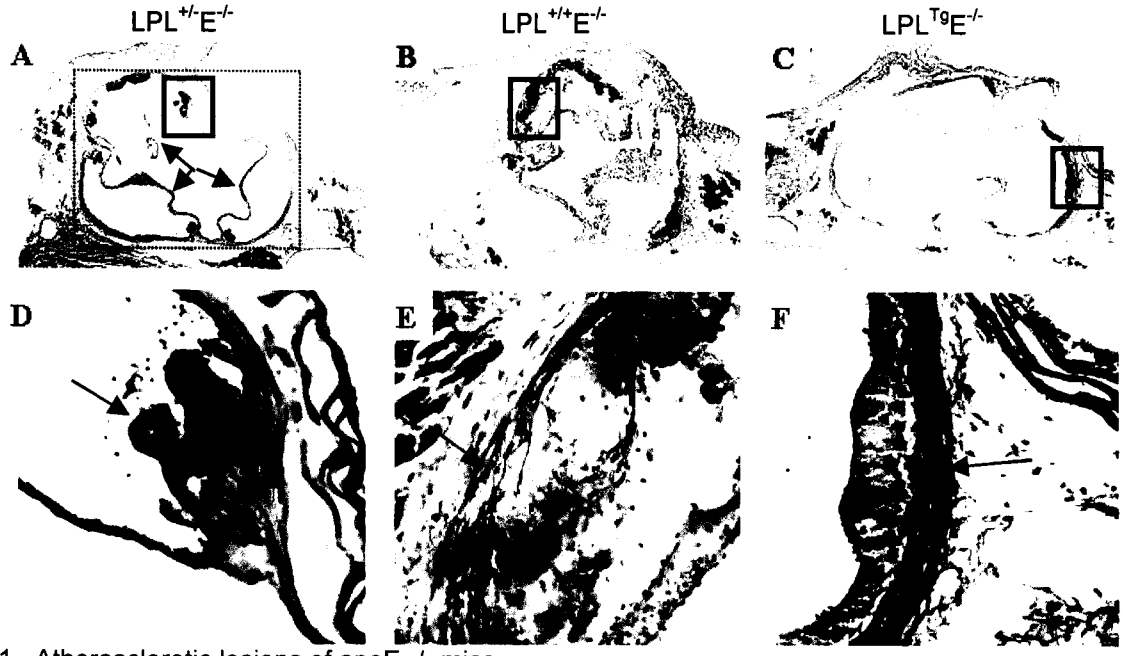

Figure 3.1. Atherosclerotic lesions of apoE -/- mice.
Panels A-C display representative aortic cross-sections (e.g. dashed box in A), displaying the three aortic valve cusps (e.g. tri-arrow in A). Lipid deposits are stained with oil red O (red), and sections were counterstained with hematoxylin (blue), 15x magnification. Panels D-F depict magnified (50x) images from the boxed region on adjacent serial sections, stained with Movat's pentachrome which stains muscle red, nuclei and elastin black, proteoglycans aqua, and collagen yellow. Lesions were predominantly foam cells (purple granular cytoplasm with small dark nuclei, colocalized with red ORO staining; e.g. arrow in D) with some matrix deposition (aqua), and tended to be more prominent and slightly more complex in the $LPL^{+/+}E^{-/-}$ mice (more smooth muscle cell (red) infiltration and disruption of the elastic layer (black); compare arrows in E,F).

In addition to differences in mean area of ORO positivity, differences in lesion complexity were noted between the *LPL* genotypes (Figure 3.1 D-F). In the $LPL^{+/-}E^{-/-}$ mice, lesions included numerous foam cells in clusters covered by an elastic membrane and endothelium. Small clusters of smooth muscle cells were observed focally. A small amount of matrix was present in some of the lesions, which tended to co-localize with neointimal smooth muscle cells. In the $LPL^{+/+}E^{-/-}$ mice, lesions were more prominent and had a somewhat altered cellular composition. Foam cells were more numerous and readily visible, however they constituted a lower total percentage of the lesion, and were generally found deeper within the neointima, in association with the matrix and smooth muscle cells. Extracellular cholesterol

clefts were visible, found adjacent to the foam cells. In addition, the aortic valve cusps appeared slightly more glycosaminoglycan-rich than normal. Lesions in the $LPL^{Tg}E^{-/-}$ mice were smaller, with a less well developed matrix and a less prominent smooth muscle cell content. They were predominantly foam cell rich, flat lesions. Thus, the differences noted in lesion areas were somewhat paralleled by differences in lesion complexity, with the $LPL^{+/+}E^{-/-}$ mice having the most complex lesions.

3.3.3 Detailed lipoprotein analysis

It could be hypothesized that the decreased atherosclerosis susceptibility in the mice with decreased LPL activity in the vessel wall and plasma ($LPL^{+/-}$) was due to the presence of larger, TG rich apoB containing lipoproteins that had a decreased ability to enter the vessel wall and/or be taken up by macrophages in these mice, and not due to decreased LPL in the vessel wall.

As an initial attempt to examine the lipoprotein distribution within the various genotypic groups, FPLC of pooled plasma was performed. As shown in Figure 3.2, $LPL^{+/-}E^{-/-}$ mice have a somewhat altered FPLC profile compared to their $LPL^{+/+}E^{-/-}$ and $LPL^{Tg}E^{-/-}$ counterparts. These mice have a tremendous increase in the amount of cholesterol rich remnant-type particles, but not in the TG-rich, VLDL fraction. Thus more large TG rich particles are not apparent in the animals heterozygous for *LPL* compared to those homozygous or transgenic.

To further address whether individual mice had an altered composition or size distribution of nonHDL lipoproteins that was masked by the use of pooled plasma for the FPLC, gradient gel electrophoresis (GGE) of plasma from each mouse was performed. Mouse lipoproteins were analyzed as TG-rich ($VLDL_0$, $VLDL_1$, and $VLDL_2$) and LDL-like (IDL, LDL). Compared with fasted human lipoprotein patterns, the mouse lipoproteins displayed a larger lipoprotein species (termed $VLDL_0$) and lipoproteins similar to $VLDL_1$, $VLDL_2$ and IDL. No lipoproteins similar in size to human LDL were seen. The patterns are summarized in Table 3.3. Very little difference in the distribution of lipoprotein species within each genotype is evident.

The majority of $LPL^{+/-}E^{-/-}$ and $LPL^{+/+}E^{-/-}$ mice (60-70%) typically displayed a broad band incorporating both particles similar in size to human $VLDL_1$ and $VLDL_2$. No IDL-sized species were detected in either the $LPL^{+/-}E^{-/-}$ or $LPL^{+/+}E^{-/-}$, while 4 $LPL^{Tg}E^{-/-}$ had lipoprotein species of a size similar to human IDL. Thus the representation of $LPL^{+/-}E^{-/-}$ and $LPL^{+/+}E^{-/-}$ mice in the various lipoprotein classes was almost identical. The $LPL^{Tg}E^{-/-}$ mice had more polarization of

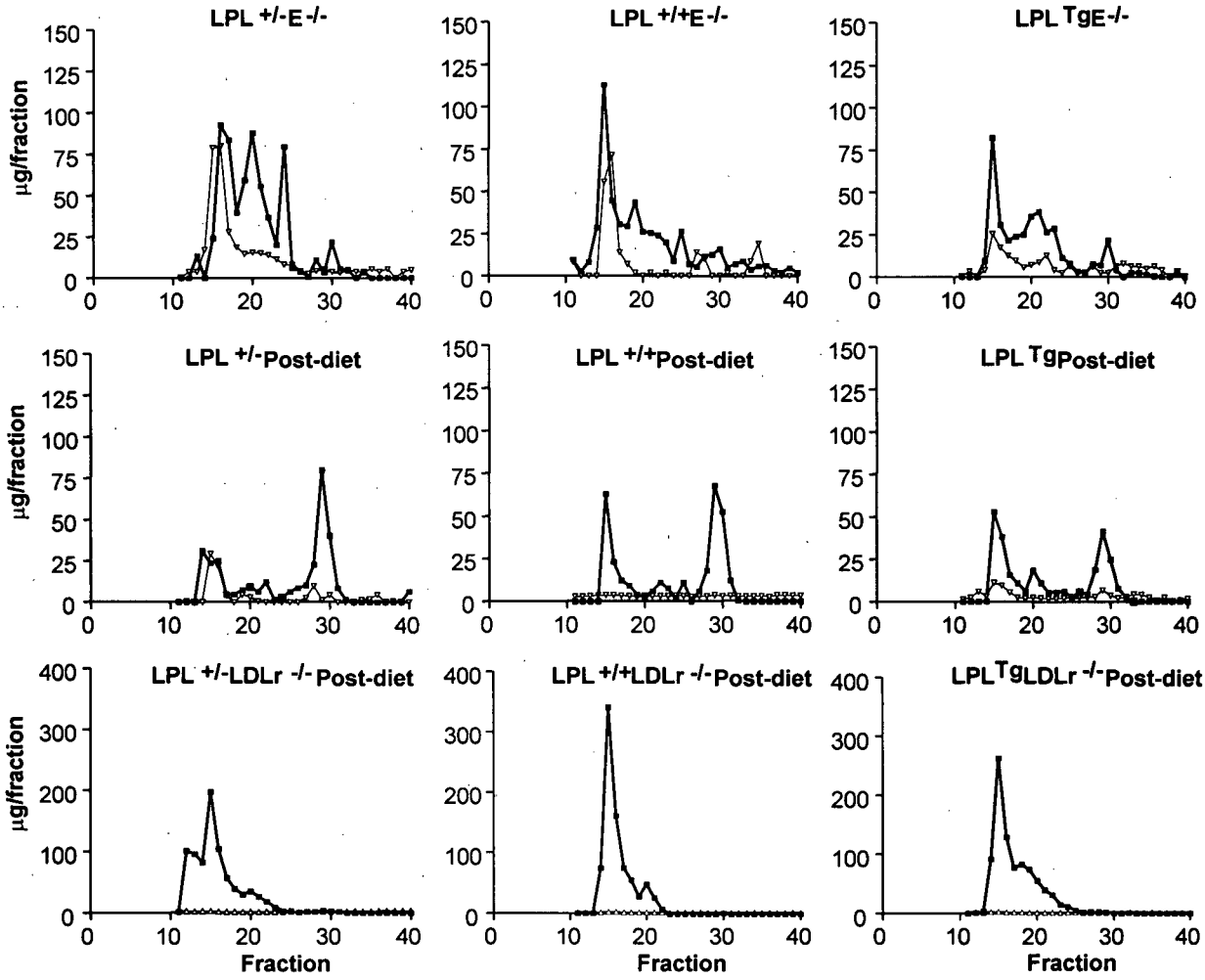

Figure 3.2. FPLC profiles of pooled plasma samples from each genotype.
Total cholesterol (black squares) and triglycerides (white triangles) in each fraction are plotted for each *LPL* genotype within the $E^{-/-}$ and BL/6 and $LDLr^{-/-}$ mice following cholesterol feeding. Note the scale in the $LDLr^{-/-}$ mice is increased above those in the other two models. From left to right peaks are: VLDL, IDL, LDL, HDL.

lipoproteins to either larger or smaller lipoproteins and less commonly displayed the broader range of both $VLDL_1$ and $VLDL_2$. While there was a trend towards larger particles, these mice also displayed an IDL-like species which could have penetrated the vessel wall more easily than the VLDL-like species.

There was very little difference in the lipoprotein species betweeen the *LPL* genotypes, either comparing the lipoprotein species present within each genotype (Table 3.3), or comparing the genotype distribution within each lipoprotein class (data not shown). Thus, the increased TG (and TC) seen in the $LPL^{+/-}E^{-/-}$ mice in all likelihood reflects an increased number of particles,

Table 3.3. Gradient gel electrophoresis characterization of nonHDL cholesterol fractions

Group		% of mice with					% of mice with	
	n	$VLDL_0$	$VLDL_1$	$VLDL_{1\&2}$	$VLDL_2$	None	IDL	no IDL
$LPL^{+/-}E^{-/-}$	24	12.5	4.2	70.8	12.5	0	0	100
$LPL^{+/+}E^{-/-}$	21	14.3	9.5	61.9	14.3	0	0	100
$LPL^{Tg}E^{-/-}$	17	23.5	29.4	17.6	29.4	0	23.5	76.5
$LPL^{+/-}$	18	11.1	77.8	0	11.1	0	11.1	88.9
$LPL^{+/+}$	17	0	70.6	0	29.4	0	0	100
LPL^{Tg}	20	0	65.0	10.0	25.0	0	0	100
$LPL^{+/-}LDLr^{-/-}$	19	10.5	26.3	36.8	26.3	0	0	100
$LPL^{+/+}LDLr^{-/-}$	25	16.0	4.0	56.0	24.0	0	0	100
$LPL^{Tg}LDLr^{-/-}$	16	93.8	6.3	0	0	59.0	0	100

The TG-rich lipoproteins of all mice were classified as lipoproteins resembling $VLDL_1$, $VLDL_2$, and a larger category resembling $VLDL_0$.
IDL-like lipoproteins were analyzed separately.
The data for BL/6 and LDLr-/- mice is based on animals consuming an atherogenic diet for 12 weeks.

rather than altered particle composition. This is supported by the FPLC data which depicts increased amounts of the remnant-like species, but no obvious shifts in size. The decreased susceptibility to atherosclerosis in these mice , therefore, is clearly not due to the presence of larger, less penetrant TG rich particles in the $LPL^{+/-}E^{-/-}$ mice.

3.4 Under and over-expression of LPL and atherosclerosis in wildtype C57BL/6 mice

3.4.1 Lipoprotein analysis

The effect of *LPL* genotype on lipid profiles in the BL/6 model was less pronounced than in the $apoE^{-/-}$ model. Prior to the initiation of high fat/high cholesterol feeding (while mice were consuming the standard chow), $LPL^{+/-}$ mice had a trend toward increased TG as compared with $LPL^{+/+}$ mice ($p=0.07$, Table 3.4). There were no significant differences in total cholesterol or any specific cholesterol fraction. Following 10 weeks consumption of the high fat/high cholesterol diet, $LPL^{+/-}$ mice displayed over two-fold higher TG as compared with their $LPL^{+/+}$ sibs (27 ± 20 vs. 12.5 ± 11.0 mg/dL, $p=0.01$, Table 3.4). Consistent with what was seen in the $E^{-/-}$ mice, $LPL^{+/-}$ mice also had increased HDL-C (47 ± 11 vs. 36 ± 13 mg/dL, $p=0.02$ vs. $LPL^{+/+}$).

Similar trends were seen in the mice overexpressing LPL (LPL^{Tg}), although no comparisons reached statistical significance (Table 3.4).

Table 3.4. Lipid levels in C57BL/6 mice, by *LPL* genotype

	LPL +/- (n=22)	LPL +/+ (n=18)	LPL Tg (n=23)	P-Values +/- vs. +/+	P-Values *Tg* vs. +/+
On Chow (pre-diet)					
TG (mg/dL)	100±49	73±46	57±24	0.07	NS
TC (mg/dL)	57±17	50±17	49±10	NS	NS
HDL-C (mg/dL)	46±13	41±15	42±9	NS	NS
Non-HDL chol. (mg/dL)	11±6	9±5	7±3	NS	NS
Post Atherogenic Diet Feeding					
	(n=20)	**(n=17)**	**(n=20)**		
TG (mg/dL)	27±20	12.5±11.0	9±6	**0.01**	NS
TC (mg/dL)	199±44	186±50	195±45	NS	NS
HDL-C (mg/dL)	47±11	36±13	31±14	**0.02**	NS
Non-HDL chol. (mg/dL)	152±43	149±45	163±43	NS	NS
	(n=18)				
Lesion area (μm^2)	2016±2801	4125±4122	1921±2132	NS	0.06

Lipid levels at 10 weeks of age, consuming a standard chow diet, and at 22 weeks of age following 10 weeks consumption of an atherogenic diet. Lesion areas were measured following 12 weeks consumption of the atherogenic diet.
NS= not significant

In the cholesterol fed BL/6 animals, no obvious differences in FPLC profiles were evident aside from changing relative amounts of the various lipoprotein species (Figure 3.2). As with the $apoE^{-/-}$ mice, there were no differences in lipoprotein sizes between the gentoypes. The predominant nonHDL particle in the BL/6 mice on the atherogenic diet was $VLDL_1$-like in all *LPL* genotypes (Table 3.3). $VLDL_0$ and IDL were found only in a small percentage of the $LPL^{+/-}$ mice. The $LPL^{+/+}$ mice had only $VLDL_1$. $VLDL_2$ was only seen in the LPL^{Tg} mice. This data is consistent with the predicted plasma lipolytic activities of these animals: those with less LPL had more larger particles, while those with the most plasma LPL had the smallest particles.

3.4.2 Atherosclerosis

Small foam cell lesions, the equivalent of fatty streaks, were observed in these mice (Figure 3.3). Similar trends in lesion areas were noted as with the $E^{-/-}$ mice. Thus, mean lesion areas in the $LPL^{+/-}$ mice, with decreased vessel wall and plasma LPL, were reduced as compared with $LPL^{+/+}$ mice (2016±2801 vs. 4125±4122 μm^2). However, due to the large variation in lesion areas, the difference did not reach statistical significance (Table 3.4). Lesion areas were also decreased in the mice overexpressing human LPL in plasma but not in the vessel wall (1921±2132 μm^2 vs. 4125±4122 μm^2 in $LPL^{+/+}$ mice, p=0.06). A few cholesterol clefts were noted in the $LPL^{+/+}$ mice. Otherwise there were no significant differences in lesion morphology between the *LPL* genotypes.

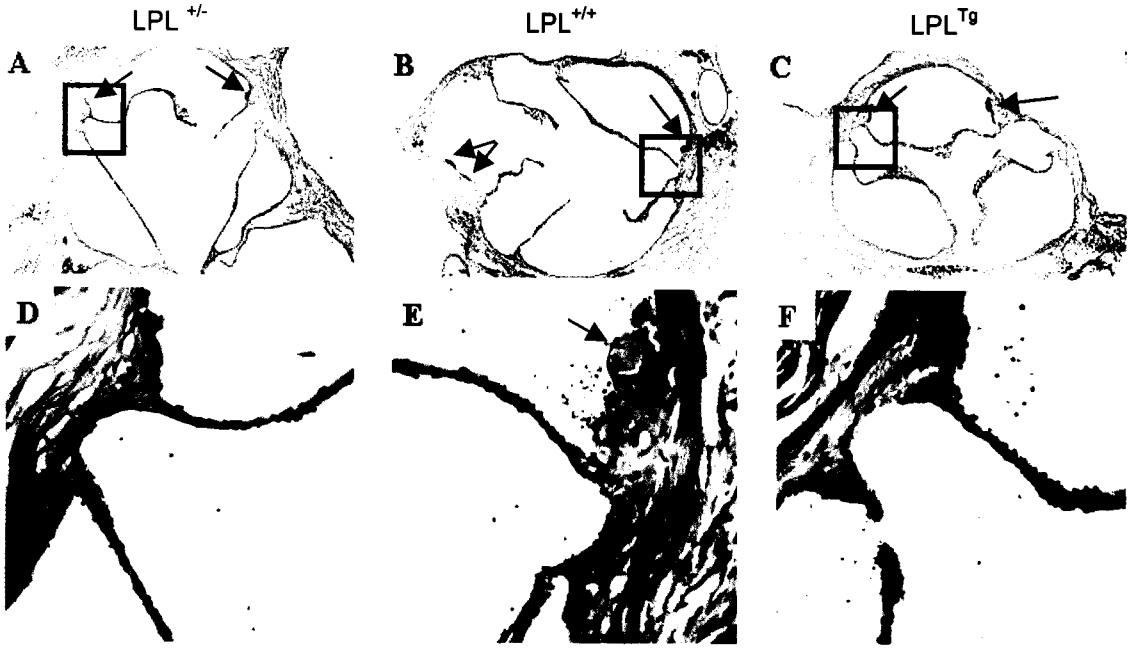

Figure 3.3. Atherosclerotic lesions in C57BL/6 mice.
Panels A-C display representative aortic cross-sections, 15x magnification. Lipid deposits are stained with oil red O (red, marked with arrows), and sections were counterstained with hematoxylin (blue). Panels D-F depict magnified (50x) images from adjacent serial sections, stained with Movat's pentachrome (as described in Figure 3.1). Lesion areas included small collections of foam cells (purple granular cytoplasm colocalized with lipid staining), especially evident in panel E (e.g. arrow). As evident in panels B and E, the lesions in the $LPL^{+/+}$ mice tended to be larger than in either the $LPL^{+/-}$ or LPL^{Tg} mice.

3.5 The cholesterol fed LDL receptor deficient model

3.5.1 Lipids and lipoproteins

On the LDLr deficient background, heterozygosity for *LPL* was associated with a similar pattern of lipid changes as in the other two models, both prior to, and during, atherogenic diet

feeding (Table 3.5). $LPL^{+/-}LDLr^{-/-}$ mice had increased TG both pre- (147±49 vs. 74±26 mg/dL, $p<0.001$) and post atherogenic diet feeding (92±54 vs. 48±26, $p=0.002$) compared with $LPL^{+/+}LDLr^{-/-}$ mice. Total cholesterol was also higher (170±33 vs. 144±24 mg/dL, $p=0.04$ pre-diet; 1628±354 vs. 1433±249 mg/dL, $p=0.04$ post-diet) in the $LPL^{+/-}LDLr^{-/-}$ mice. This increased total cholesterol can be attributed to increased nonHDL cholesterol, as there were no significant differences between the mice in HDL-C (Table 3.5). Total and nonHDL cholesterol were increased in the $LDLr^{Tg}$ mice (Table 3.5). No significant differences in TG were observed, while HDL-C was significantly decreased in the $LPL^{Tg}LDLr^{-/-}$ compared to the $LPL^{+/+}LDLr^{-/-}$ mice.

Table 3.5. Lipid levels in $LDLr^{-/-}$ mice, by *LPL* genotype

	LPL +/- (n=24)	LPL +/+ (n=28)	LPL Tg (n=20)	P-Values +/- vs. +/+	P-Values Tg vs. +/+
On Chow (Pre-Diet)					
TG (mg/dL)	147±49	74±26	75±30	**<0.001**	NS
TC (mg/dL)	170±33	144±24	185±53	**0.04**	**0.001**
HDL-C (mg/dL)	63±18	55±15	66±25	NS	*0.12*
Non-HDL chol. (mg/dL)	107±29	89±21	119±36	0.06	**0.001**
Post Atherogenic Diet feeding					
	(n=21)	**(n=25)**	**(n=17)**		
TG (mg/dL)	92±54	48±26	69±45	**0.002**	*0.10*
TC (mg/dL)	1628±354	1433±249	1679±311	**0.04**	**0.01**
HDL-C (mg/dL)	19±11	23±15	13±7	NS	**0.008**
Non-HDL chol. (mg/dL)	1609±354	1410±248	1666±313	**0.03**	**0.009**
	(n=20)				
Lesion area (μm^2)	387,888±147,647	412,350±110,190	412,427±152,928	NS	NS

Lipid levels at 10 weeks of age, consuming a standard chow diet, and at 22 weeks of age following 10 weeks consumption of an atherogenic diet. Lesion areas were measured following 12 weeks consumption of the atherogenic diet.

The nonHDL cholesterol particles of the cholesterol-fed $LDLr^{-/-}$ mice were primarily of the VLDL size, and LDL was not detected in any of the animals (Table 3.3). $LPL^{+/+}LDLr^{-/-}$ mice had a mixture of $VLDL_1$ and $VLDL_2$ sized species. $LPL^{+/-}$ $LDLr^{-/-}$ mice also had a mixture of the

two species, but tended to have more $VLDL_1$ than $VLDL_2$. The $LPL^{Tg}LDLr^{-/-}$ mice had larger particles, predominantly $VLDL_1$ and $VLDL_0$ sized (no $VLDL_2$), but also IDL.

Analysis of the post-diet lipoproteins by FPLC revealed differences in the sizes of the main cholesterol carrying particles between the *LPL* genotypes (Figure 3.2). In all three groups, the predominant particle was a VLDL species eluted in fraction 15. In the $LPL^{+/-}LDLr^{-/-}$ animals, this was accompanied by an almost similar size peak of larger particles. In the $LPL^{Tg}LDLr^{-/-}$ animals, it was accompanied by a second major peak containing smaller particles.

3.5.2 Atherosclerosis

Interestingly, in contrast to the other two models, there were no differences in atherosclerotic lesion areas (Table 3.5, Figure 3.4). Large, complex lesions were observed in all *LPL* genotypes.

Lesions in the $LPL^{+/-}LDLr^{-/-}$ mice included a foam cell and smooth muscle cell rich interior, with deep areas of necrotic core and prominent extracellular cholesterol. A fibroelastic cap has formed. It appears these lesions have an intact endothelial layer. Adventitial inflammation with numerous lymphocytes was noted at the origin of the right coronary artery where a foam cell lesion has disrupted the media, "spilling" into the adventitial space. Small foam cell lesions were even present in the origin of the coronary arteries in some mice.

The lesions in the $LPL^{+/+}LDLr^{-/-}$ mice appear to cover the aortic root surface more comprehensively than in the $LPL^{+/-}LDLr^{-/-}$. The lesions in these mice have a somewhat altered complexity compared to those of the $LPL^{+/-}LDLr^{-/-}$ mice. Foam cells are more predominant, with a smaller deep necrotic core and less early fibrous cap formation. Where extracellular matrix was present, it tended to be deep within lesions up against the internal elastic laminae of the aorta, and was more clearly glycosaminoglycan rich. Some smooth muscle cell proliferation is evident at the bases of the necrotic cores.

Extensive lesions similar in size to the $LPL^{+/-}LDLr^{-/-}$ and $LPL^{+/+}LDLr^{-/-}$ mice were found in the $LPL^{Tg}LDLr^{-/-}$ mice. Lesions were somewhat intermediate in complexity compared with the $LPL^{+/-}LDLr^{-/-}$ and $LPL^{+/+}LDLr^{-/-}$ mice. The lesions were characterized by very prominent deep pools of glycosaminoglycan, extracellular cholesterol, and a focal necrotic core. There were areas of smooth muscle cell and matrix accumulation within the lesion. Overall they had a morphology more similar to the $LPL^{+/+}LDLr^{-/-}$ mice.

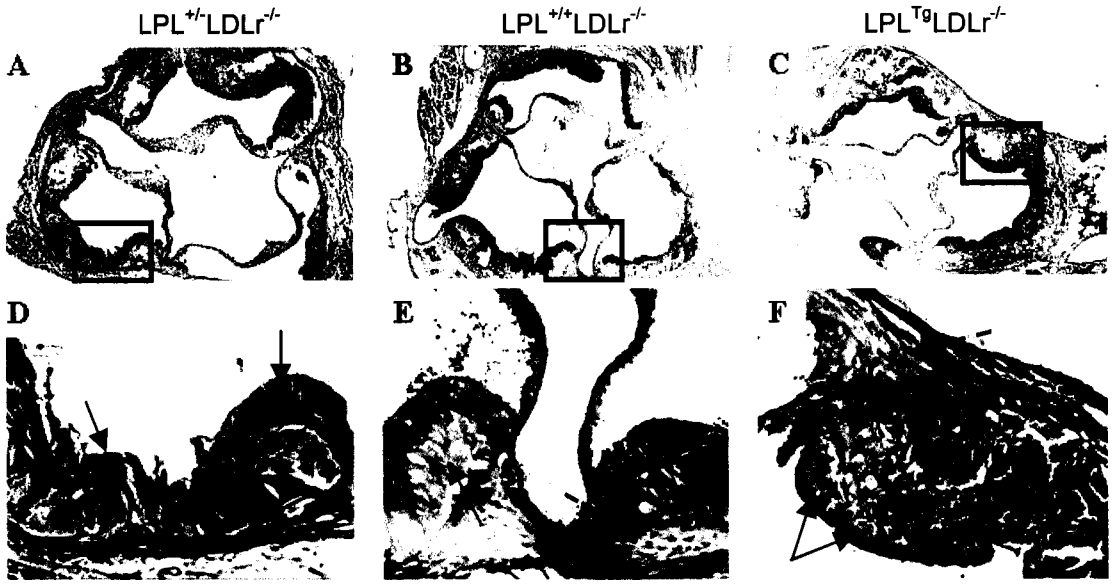

Figure 3.4. Atherosclerotic lesions in $LDLr^{-/-}$ mice.
Complex lesions are visible in the $LDLr^{-/-}$ mice. Panels A-C are stained with ORO (neutral lipid, red) and counterstained with hematoxylin (nuclei, blue; 15x magnification), while panels D-F are adjacent serial sections stained with Movat's pentachrome (muscle red, nuclei and elastin black, proteoglycans aqua, collagen yellow; 50X magnification; as in Figures 3.1, 3.3). Large proteoglycan-rich (aqua), acellular areas are visible at the core of the lesions (e.g. solid arrow in E), while the edges contain predominantly foam cells (purple granular cytoplasm, filled with lipid, e.g. solid arrows in F and D). Some evidence of smooth muscle cell proliferation into the intima is visible, particlularly in the $LPL^{+/+}LDLr^{-/-}$ and $LPL^{Tg}LDLr^{-/-}$ mice (dashed arrows in E,F).

3.6 Discussion

This chapter describes experiments allowing us to directly assess the relative atherogenicity of vessel wall and plasma LPL. In two different models of atherosclerosis, we have provided *in vivo* evidence for the differing influence of LPL in the development of atherosclerosis depending on its site of expression. Decreased LPL in the vessel wall due to heterozygosity for a null allele at the *LPL* locus was associated with decreased atherosclerotic lesion formation, despite the dyslipidemia caused by low plasma LPL activity. In work published while this study was in progress, it has been shown that atherosclerotic lesions of $LPL^{+/-}$ mice have decreased LPL protein mass as compared with $LPL^{+/+}$ mice[259]. Furthermore, using a complementary approach, it has also since been shown that abolition of macrophage LPL was associated with reduced lesion formation in both apoE deficient and LDLr deficient mice[260], and in cholesterol fed C57BL/6 mice[261,262]. Mice with only partially diminished macrophage LPL activity also displayed reduced lesion formation in the proximal aorta, suggesting a dose-response relationship between macrophage LPL levels and atherosclerosis susceptibility[260,262]. Cumulatively, these findings suggest that macrophage-derived vessel wall LPL plays a crucial

pro-atherogenic role in determining susceptibility to atherosclerotic lesion formation (Figure 1.5 (B)). Indeed, it has since been shown that addition of LPL to macrophage cultures increases their uptake of VLDL, independent of apoE and the LDLr[155].

These studies also showed that increased LPL activity specifically in the plasma (and not in macrophages, and thus the vessel wall) is protective, as might be predicted by the anti-atherogenic lipid profile changes associated with increased plasma LPL activity (Figure 1.5 (A)). These results confirm the early findings of Shimada and colleagues in low density lipoprotein receptor deficient mice[245], and subsequent to this study, in apoE deficient mice[263]. We have previously shown that liver-directed LPL overexpression was associated with increased plasma catalytic activity and improved lipoprotein profiles[264]. The data presented herein provides *in vivo* evidence that therapies designed to increase LPL activity should be targeted specifically to increasing plasma (and not vessel wall) LPL, where LPL may be of significant therapeutic potential in reducing susceptibility to atherosclerosis.

It is noteworthy that we were able to demonstrate similar trends to the $E^{-/-}$ mice, and an even larger percentage decrease in lesion area, in the C57BL/6 model of early lesion formation. The finding that there was no difference in lesion formation between the *LPL* genotypes in the LDLr deficient model is interesting, and has several possible interpretations. The lack of a difference in lesion areas in the absence of the LDLr could imply that the LDLr is a key component in the mechanism by which LPL affects lesion formation. However, Shimada et al[245] have been able to detect differences in lesion formation with mice overexpressing LPL on the $LDLr^{-/-}$ background, and it has been shown in humans that LPL may moderate the phenotype of individuals lacking functional LDL receptors[265-267]. Thus, a more likely interpretation of the data is that the role LPL plays in lesion formation is indeed in the early stage, by aiding lipoprotein retention within the vessel wall, and/or altering plasma lipid concentrations, and thus the likelihood of entry into the vessel wall.

At later stages of lesion development, changes of lesion complexity such as fibrous cap formation may predominate, or additional lipid accumulation may be masked by the already large lipid-rich core. Interestingly, Shimada et al[245] examined lesions after only 8 weeks of atherogenic diet feeding and observed differences, whereas we and Semenkovich et al[259] used 12 weeks of atherogenic diet feeding, and were unable to detect any differences in lesion areas. This may also be due, in part, to the method of lesion assessment used. In a very recent study by

Babaev and colleagues, examining the effects of macrophage LPL depletion on lesion formation in $LDLr^{-/-}$ mice, when complex lesions were induced by extended cholesterol feeding, no differences in cross-sectional lesion areas were observed, although differences were observed in the total percentage of the aorta covered by lesions[262]. This suggests that once the lesion has become complex, further growth may occur by a broadening of the lesion along the length of the vessel, not further outward growth into the lumen.

The predominant lipoprotein determining atherosclerotic susceptibility in each model was VLDL, the primary nonHDL-C carrying particle of the $apoE^{-/-}$, cholesterol-fed C57BL/6 and $LDLr^{-/-}$ mice. As the LPL^{Tg} mice had reduced lesion formation despite lower HDL-C levels, this suggests that the HDL did not have a strong protective role in these animals, as may be expected in CETP deficiency, where HDL can not function as effectively in reverse cholesterol transport. Instead, the decreased atherogenesis in the LPL^{Tg} mice seems to be more related to decreased TG and/or nonHDL cholesterol, providing additional evidence that TGRL are atherogenic.

The finding of differing roles for vessel wall and plasma LPL in atherogenesis has additional clinical relevance. *LPL* mutations may be present at cumulative frequencies approaching 20% in Caucasian populations. Some patients have *LPL* mutations associated with a catalytic defect and stable LPL immunoreactive mass, for example the I194T, R243H, and G188E mutations (class II[268]), while others have mutations resulting in decreased LPL protein mass in addition to defective catalytic activity, such as the P207L mutation[269] and several insertions, deletions and premature truncations (class I[268]). We would thus predict that mutations such as the former may confer a significant atherosclerotic risk, as they are associated with decreased catalysis and dyslipidemia, but normal vessel wall LPL protein mass, which may still function normally in the retention of lipoproteins. In contrast, other mutations that are associated with loss of LPL activity and less stable LPL protein mass, and consequently lower levels of LPL protein in the vessel wall available to retain lipoproteins, might be predicted to be less atherogenic.

Recently there have been several publications examining the relationship between LPL and atherogenesis in humans[141,266,270-280]. The only functional *LPL* variants frequent enough to allow investigators to study the relationship between *LPL* genetic variation and disease at the population level are three common polymorphisms: N291S, D9N, and S447X. However, the effects of these variants on LPL catalytic function are mild[281], making it very difficult to discern

differences in the absence of large sample sizes. Some studies have suggested that these variants in the *LPL* gene may be associated with an altered risk of developing or an increased progression or severity of atherosclerosis[141,266,270-274], while others have found no association[275-280]. The data presented here provide support for a pro-atherogenic effect of the N291S and D9N variants, as these variants retain near normal protein levels (Chapter 5)[281] but have catalytic defects associated with pro-atherogenic changes in lipids, and would thus be predicted to be associated with increased risk for atherosclerosis.

An intriguing finding in this study was that HDL-C levels increased with decreasing LPL activity, particularly in the $E^{-/-}$ model. Of note, we have also shown increased HDL-C levels in our heterozygous LPL deficient feline model, another CETP deficient system (Chapter 4). Animals deficient in plasma CETP activity rely on other mechanisms of cholesterol delivery to the liver for efficient functioning of the reverse cholesterol transport pathway. Thus, selective uptake of CE may be even more important in mice and other CETP deficient animals. Recently, LPL has been shown to aid in the selective uptake of HDL-CE by macrophages and hepatic cells[282,283]. Thus, in the absence of CETP and apoE, which may also aid in the bridging of lipoproteins to the cell surface[258], a reduction in the amount of LPL protein may compromise the ability of HDL to deliver its CE's to the liver. This, in turn, would result in an increased plasma HDL-C concentration. The lipid data presented in the $E^{-/-}$ model is thus consistent with LPL having a role in selective uptake of HDL-CE. Furthermore, this may explain why VLDL, the primary nonHDL particle in each model, was the predominant lipoprotein determining atherosclerotic susceptibility, and changes in HDL-C levels did not appear to reflect changes in atherosclerosis susceptibility, as increasing HDL was not indicative of increased reverse cholesterol transport in these animals, and thus was not associated with protection against atherosclerosis.

In summary, we have demonstrated important roles for LPL in the initial stages of lesion formation, in two separate model systems. These roles in atherosclerosis are related to both the amount of vessel wall LPL protein available for functions such as trapping lipoproteins and to the level of plasma LPL activity influencing plasma lipid concentrations, as we have shown by comparing the effects of decreased plasma and vessel wall LPL with increased LPL in tissues contributing only to LPL in the plasma. Specifically, we provide *in vivo* evidence that increasing plasma LPL activity without altering macrophage, and hence vessel wall, LPL levels is

associated with decreased lesion formation, while increased vessel wall LPL protein is pro-atherogenic. Whether these pro-atherogenic roles of vessel wall LPL are due solely to non-catalytic bridging functions, or whether they are due at least in part to LPL catalytic activity resulting in the localized generation of smaller particles which are more easily taken up cannot be determined from this study. Although these differences in lesion formation were small in relative terms, when extrapolated throughout an entire organism over a lifetime, such differences may have a significant impact on overall disease status. The lack of observed difference in the LDLr deficient model suggests that LPL plays an especially important role in the early stages of lesion formation. Our findings also provide further evidence as to the atherogenic nature of TGRL, and are consistent with a role of LPL in the selective uptake of HDL-CE. In conclusion, these findings suggest that therapies designed to increase LPL activity in the plasma, without increasing expression within the vessel wall, such as with targeted gene delivery, are likely to be of significant therapeutic potential in reducing the risk for atherosclerosis.

Chapter 4: Lipoprotein analysis and atherosclerosis susceptibility in LPL deficient cats

The work presented in this chapter has been published in part in

Ginzinger D. G., **Clee S. M.**, Dallongeville J., Lewis M. E. S., Henderson H. E., Bauje E., Rogers Q. R., Jansen D., Eckel R., Dyer R., Innis S., Jones B., Fruchart J.-C., and Hayden M. R. Lipid and Lipoprotein Analysis of Cats with Lipoprotein Lipase Deficiency. *Eur. J. Clin. Invest.* 1999 **29**:17-26.

The atherosclerosis data is unpublished.

Preface

The results described herein represent studies originally designed by a former Ph.D. student in our lab, David Ginzinger, with key contributions from Dr. Suzanne Lewis. The colony of cats was originally identified by Dr. Boyd Jones, and some cats were provided from the colony maintained by Dr. Quinton Rogers. The detailed lipoprotein analysis was performed in the laboratories of Drs. Jean Dallongeville and Jean-Charles Fruchart. The plasma lipid and LPL activity measurements were performed by Ms. Li Miao. I participated in the monthly blood-sampling, and the necropsies of the cats. All of the data in this chapter was analyzed, and the manuscript written, by me. Drs. Bauje, Jansen, Eckel, Dyer and Innis provided additional data which was part of the manuscript but which is not included here. All lesion assessments were performed by Darlene Redenbach and Wengao Lu under the expert supervision of Janet and Dr. Bruce McManus, in the Department of Pathology at the University of British Columbia.

4.1 Introduction

A number of animal models have been used to study the consequences of abnormalities in lipoprotein metabolism[7,207,284]. Both LPL transgenic and gene targeted mice have been engineered to assess the role of LPL in lipid metabolism[211,212,214-217,234]. Although the transgenic mouse model has proven to be useful in studying the effects of increased LPL activity on lipid and lipoprotein metabolism, mice homozygous for targeted disruption of LPL do not survive beyond the first day of life[214,215], thereby preventing studies in this model to assess the consequences of complete LPL deficiency. Furthermore, because the mouse is a small animal, and as such necessarily has small organs and vessels, detailed pathological analysis is difficult in these animals.

We have previously described the underlying genetic defect in a colony of domestic cats with chylomicronemia originating from New Zealand[285]. This colony of cats was the first viable animal model of complete LPL deficiency available for study. Cats are carnivorous animals, and consume a diet rich in protein and fat, not too different from a typical North American diet. The cat is a relatively large animal, which facilitates detailed pathological assessment, and as it has a larger blood volume than the mouse, more detailed lipid and lipoprotein assessments can be performed. Thus the cat is a useful animal model in which to study lipid metabolism and atherosclerosis.

Two prior studies of lipid metabolism in cats have been performed[286,287]. These have shown that the cat has many similarities to humans with respect to lipid metabolism. Like mice and many other animals, cats are "HDL animals" in that the majority of their cholesterol is carried in these particles[286,288]. Unlike most other animals, however, cats have separable HDL_2 and HDL_3 subfractions, similar to humans[286,287]. Although the absolute levels of LDL-C are much lower in cats than humans, LDL generally has a similar composition to humans[287]. Furthermore, in contrast to mice, $apoB_{48}$ production appears restricted to the intestine in cats, as in humnas[287].

Another larger animal model of LPL deficiency has recently been described[289]. A line of mink have been shown to have complete LPL deficiency due to a missense mutation near the catalytic domain. The mink is another "HDL animal", carrying most of its cholesterol in HDL particles, and lacking CETP activity[289,290]. However, mink are difficult to work with, and even less is known about lipid metabolism and atherosclerosis in this animal model than in cats. In

addition, the mink is a smaller animal than a cat. Thus, the cat still seems a preferential animal model of complete and partial LPL deficiency.

The role of LPL within feline lipid metabolism has not been specifically assessed. Prior to the use of the LPL deficient cat as a model for studies on the pathogenesis of human LPL deficiency and the role of LPL in atherosclerosis, a detailed knowledge of both the similarities and differences in the phenotypic effects of LPL deficiency in these cats as compared with humans is required. Data presented in this chapter includes an expanded and more detailed lipid and lipoprotein characterization of our feline model of LPL deficiency. In addition, we have examined the consequences of LPL deficiency on oral fat tolerance. This has provided a basis for direct comparison to human LPL deficiency.

Cats, like mice, are naturally resistant to atherosclerosis, and one needs to feed supplemental cholesterol in the diet to induce atherosclerotic lesion formation in these animals[291]. The initial reporting that atherosclerosis can be induced in these animals, described in 1970, reported general atherosclerosis susceptibility in a number of animal models[291]. We have subsequently replicated and extended these findings, elaborating on the conditions necessary for inducing atherosclerosis in this animal model[292]. This formed the baseline on which we have now been able to assess the atherosclerotic susceptibility of $LPL^{+/-}$ cats compared to $LPL^{+/+}$.

4.2 Methods

4.2.1 Animals

All cats used in this study were derived from a colony of LPL deficient cats originally discovered by Jones et al., in New Zealand[293]. The exact level of consanguinity between individual cats used in this study cannot be determined. However, for at least 4 to 5 generations this colony has been interbreeding with primarily three male studs in sib-sib and parent-offspring type matings. Animals are group housed in a facility maintained at UBC that greatly exceeds space recommendations set forth by the U.S. National Institutes of Health guide for care and use of laboratory animals. Cats have both indoor and outdoor access, and as such, light cycles vary according to natural daily patterns. Animals included in the study were adult cats, all less than 4 years of age. The average age of the cats, for each sex within each genotype in the lipoprotein

studies was between one and two years old. Average body weights of female cats were between 2-3 kg and of males were 3.5-4 kg over the duration of the study.

Throughout the course of the study, except where noted in the oral fat load studies and for the atherosclerosis study, cats were fed *ad libitum* a standard commercial dry-type expanded diet, containing approximately 7% fat (58% protein, 24% fat and negligible carbohydrate on a dry weight basis), primarily from fish and poultry sources. Each cat likely consumes approximately 6 to 8 g fat on a daily basis. It should be noted that the diet followed in this study contained a lower fat content than that used in the initial study describing these cats[285].

For all LPL, lipid and lipoprotein measurements, cats were fasted overnight, approximately 18 hours, and provided with free access to water. Prior to withdrawing blood samples from the jugular vein, animals were anaesthetized with Ketamine and Versed. All experimental procedures conducted on these animals were approved by the University of British Columbia Committee on Animal Care.

4.2.2 Lipoprotein and LPL analysis

Blood was collected in tubes containing EDTA (1 mg/mL). Plasma was separated by centrifugation (2500 rpm) for 20 min at 4°C. Samples were stored at 4°C until analysis, which was performed within one week. Basic lipoprotein analysis was performed as described in Chapter 2.

For detailed lipoprotein profile analysis, lipoproteins were separated using a combination of ultracentrifugation and phosphotungstate precipitation. TGRL were separated by a single ultracentrifugation at density 1.006 g/mL according to a previously described procedure[37] but with increased run times and with modifications due to the smaller volumes of plasma obtainable from cats as compared with humans. Briefly, plasma (500 μL) was diluted with an equal volume of NaCl (0.9%) and centrifuged in a polycarbonate tube (3 hr at 440, 000 x g at 20°C) in a Beckman Ti 100.2 rotor. The tube was sliced and the remaining 0.5 mL infranate ($d>1.006$ g/mL, "bottom") fraction was analyzed for lipids. The TGRL ($d<1.006$ g/mL) fraction was measured by subtracting lipid values of this bottom fraction from plasma values. HDL lipoprotein measures were determined after precipitation of apolipoprotein B containing lipoproteins in whole plasma with phosphotungstate, using commercially available reagents (Cholesterol HDL, CHOD/PAP, Boehringer Mannheim, Mannheim Germany). HDL_2 and HDL_3

fractions were separated by precipitation with polyethylene glycol, using a commercially available kit (Quantolip HDL2/HDL3, ImmunoFrance, Rungis, 94577, France). LDL lipid measures were quantified by subtracting precipitated HDL values from lipid values in this bottom fraction. All lipid levels are presented in mg/dL.

For LPL activity, blood was withdrawn 10 minutes after injection of 100 U/kg sodium heparin, separated by centrifugation at 4°C for 10 minutes, and immediately frozen at -80°C. LPL activity was measured as described in Chapter 2, and is presented in mU/mL.

4.2.3 FPLC lipoprotein analysis

As these analyses were performed in France, the procedure is a slight modification of the one described in Chapter 3. Gel filtration chromatography was performed on pooled plasma samples using a Superose™ 6HR 10/30 column (Pharmacia, Pharmacia LKB Biotechnology, S-751 82 Uppsala Sweden). The column was allowed to equilibrate with phosphate buffered saline (10 mM) containing 1 mM EDTA. Plasma (130 μL) was eluted with this buffer at room temperature at a flow rate of 0.2 mL/min. The effluents were collected in 260 μL fractions, and the elution profile was monitored at 280 nm. Calibration was carried out with human VLDL ($d<1.006$ g/mL), LDL ($1.019<d<1.063$ g/mL), HDL ($1.063<d<1.21$ g/mL) and Bovine albumin Fraction V (Sigma, Sigma Chemical Co. St. Louis, MO).

4.2.4 Oral fat tolerance

The oral fat load study was performed in male cats of each genotype. Individuals were deprived of food for 48 hours, at which time baseline blood samples were taken for TG analysis. Each cat was then given a small meal (0.94 g fat/kg body weight, approximately 4-5g fat per cat) of a high-fat canned cat food, consisting of 2.9% carbohydrate, 51% protein and 38.5% fat on a dry weight basis. The meals were consumed within 10 minutes. Blood samples were withdrawn for TG analysis at intervals of 1, 2, 3, 5, 7, 12, 24 and 48 hours postprandially. Water was available *ad libitum* throughout the course of the experiment.

4.2.5 Atherosclerosis study

Six male $LPL^{+/-}$ and 6 male $LPL^{+/+}$ cats were used in this study. Their ages ranged from approximately 2-4 years at the start of the study. Cats were placed on a diet of 30% fat, 3%

cholesterol for a period of 6 months, and their plasma lipid levels were measured monthly. At this time, many of the cats were experiencing severe liver dysfunction, as noted on routine clinical chemistry screening performed at the same time as lipid measurements. So, after the 7th month (no plasma samples for lipid analysis were taken) the cats were taken off the diet for a period of one month. During this time their liver function was restored, and the cats were weaned back on to a diet consisting of 30% fat, 1% cholesterol. Lipids were measured after 1 and 2 months back on this diet. Cats were sacrificed after a total of 3 months on this diet, although lipids were not measured immediately prior to sacrifice. The plasma lipid levels observed on this diet were similar to those on the higher cholesterol diet (data not shown). Thus, the cats consumed a cholesterol-enriched diet for a period of 10 months.

Monthly lipid and bimonthly LPL assessment was performed as described in Chapter 2. Detailed lipoprotein analysis was performed at months 2 and 5 on the diet, as described above.

Blood vessels were removed immediately after sacrifice in a detailed necropsy. Vessels were divided into three pieces, and preserved either in formalin and paraffin embedded, flash-frozen, or flash-frozen and embedded in OCT. Serial cross-sections were made from the OCT-embedded samples, and stained with ORO. Lesion areas were assessed in both a semi-quantitative and quantitative manner, as described[292]. Semiquantitative analysis was based on a score of 0-5+ for the presence of foam cells and lipid rich lesions, and was performed by trained pathologists. Quantitative analysis was performed on a specific cross-section from each vessel segment as described in Chapter 3 for the mice. Lesion areas are expressed in μm^2.

4.2.6 Statistical analysis

For lipoprotein analysis, between genotype comparisons were performed using an ANOVA, with a Tukey adjustment for multiple comparisons. Comparisons between males and females were made using Student's T-tests, assuming separate variances.

For the atherosclerosis study, P-values comparing the two groups were calculated using the non-parametric Kruskal-Wallis test. A non-parametric test was used because of the small numbers which resulted in a non-normal distribution of the data, and due to the fact that some of the cats were related. This approach yielded values very similar to those obtained with a *t*-test.

All statistical analysis was performed using Systat (Systat version 7.0, SPSS Inc., Chicago IL).

4.3 Lipid and lipoprotein analysis of cats with lipoprotein lipase deficiency

Plasma lipid concentrations within each *LPL* genotype are presented in Table 4.1. Cats with complete LPL deficiency ($LPL^{-/-}$, hereafter referred to as homozygotes) manifested with profoundly higher concentrations of plasma TG ($p<0.001$ in males and $p<0.01$ in females), due mainly to increased TGRL-TG concentrations ($p<0.001$, $p<0.01$ for males and females, respectively) when compared with their $LPL^{+/+}$ (hereafter referred to as normal) counterparts. This was accompanied by higher levels of TGRL-C ($p<0.05$) in males. In contrast, LDL-C was generally lower in homozygotes of both genders, consistent with what is seen in humans with complete LPL deficiency[42]. HDL-C was higher in male ($p<0.05$) but not female homozygotes, and was accounted for predominantly by elevated levels of HDL_2 ($p<0.05$).

Heterozygous cats did not show any significant change in lipoprotein profile, compared to their normal counterparts. However, the power to detect smaller differences is quite low with the relatively small numbers studied. Male heterozygotes did show a trend towards increased plasma and TGRL-TG, and total and HDL cholesterol.

These findings are consistent with the cholesterol and TG FPLC profiles of pooled cat plasma from each cohort, shown in Figure 4.1. Similar to a previous report on single animals[285], the principal difference among the groups was an evidently higher concentration of TG and cholesterol in the largest FPLC fractions, and a lower concentration of cholesterol in the fractions corresponding to LDL in the homozygous cats, as compared with normal and heterozygous cat profiles, consistent with the differences in lipid levels. In addition, there was evidence for a slight enlargement of particles in the HDL fraction in homozygous as compared with control cats, as the HDL peak in these animals appeared to the left of fraction number 37, while in the heterozygous and normal cats it appears between fractions 37 and 40. This might reflect the increased HDL_2-C in these animals.

4.3.1 Lipoprotein composition

The TGRL, LDL and HDL lipid composition is summarized in Table 4.2 according to *LPL* genotype. There were no statistically significant changes in the relative composition of TGRL with respect to TG, cholesterol and PL among the different genotypes. Although in male homozygotes TGRL were PL enriched at the expense of TG, in female homozygotes TGRL were

Table 4.1. Feline lipid profiles by *LPL* genotype

	Male			Female		
	LPL +/+ (n=7)	**LPL +/-** (n=8)	**LPL -/-** (n=8)	**LPL +/+** (n=6)	**LPL +/-** (n=8)	**LPL -/-** (n=4)
Triglycerides						
Total Plasma (mg/dL)	24.8±4.4[c]	33.7±8.0[c,d]	210.9±120.5	25.7±19.5[b]	24.8±8.0[b,d]	202.0±145.3
TGRL (mg/dL)	13.3±3.5[c]	18.6±5.3[c,d]	185.2±101.0	17.7±17.7[b]	12.4±5.3[b,d]	185.2±149.7
LDL (mg/dL)	5.3±6.2	7.1±5.3	20.4±26.6	2.7±3.5	6.2±7.1	9.7±18.6
HDL (mg/dL)	7.1±4.4	7.1±4.4	6.2±3.5	7.1±6.2	6.2±4.4	8.0±3.5
Cholesterol						
Total Plasma (mg/dL)	77.8±15.1	87.5±11.2	97.5±13.0	87.8±32.9	98.7±34.1	84.4±17.8
TGRL (mg/dL)	4.3±6.2[a]	4.3±3.9[a]	16.3±11.6	4.3±2.7	6.6±6.6	8.5±4.3
LDL (mg/dL)	19.4±10.8	22.1±8.5	13.5±8.1	22.1±16.6	23.2±13.2	12.0±5.4
HDL (mg/dL)	54.6±5.4[a]	61.9±10.4	67.7±9.3	61.5±18.2	72.0±25.2	63.9±12.8
HDL_2 (mg/dL)	12.8±5.0[a]	16.3±7.0	22.1±5.4	16.6±8.9	20.1±12.8	14.3±6.2
HDL_3 (mg/dL)	43.3±5.0	47.6±9.7	50.7±10.1	44.9±7.7	49.9±10.8	51.9±10.8

P-values: -/- vs. +/+ or +/-: [a] ≤0.05, [b] <0.01, [c] <0.001; male vs. female: [d] <0.05
All comparisons between LPL +/+ and LPL +/- were non-significant.

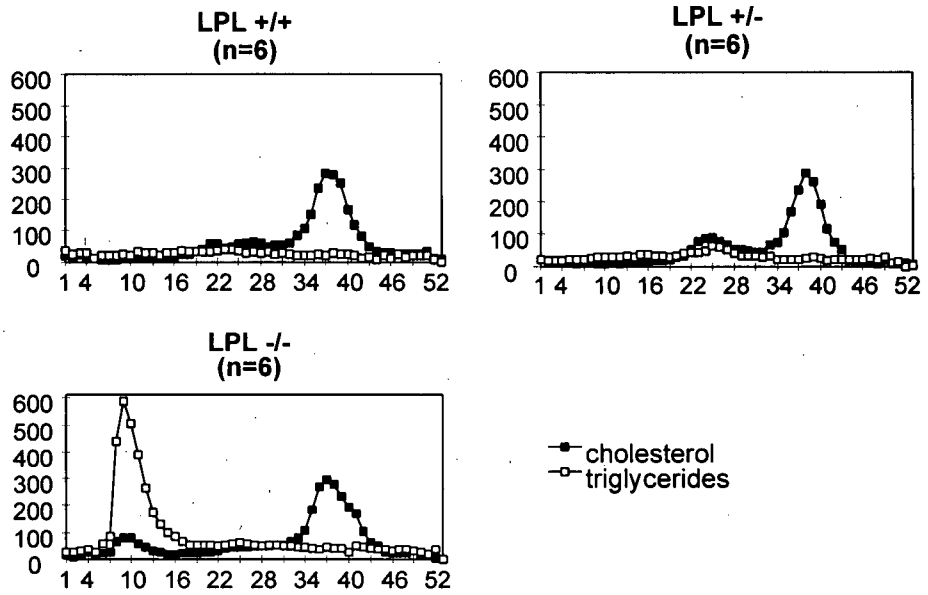

Figure 4.1. FPLC profile of pooled fasting cat plasma.
Fraction numbers are given on the X-axis, while the Y-axis depicts cholesterol (black squares) and triglycerides (white squares) in arbitrary units. Fractions 7-14 correspond to VLDL, 22-28 to LDL and 34-42 to HDL. A marked increase in VLDL is evident in the LPL-/- (homozygous) cats.

Table 4.2. Particle composition of feline lipoproteins by *LPL* genotype

	Males			Females		
	LPL +/+ (n=7)	**LPL +/-** (n=8)	**LPL -/-** (n=8)	**LPL +/+** (n=6)	**LPL +/-** (n=8)	**LPL -/-** (n=4)
TGRL						
Chol.	11±7	13±6	13±8	10±4	20±14	21±15
TG	74±10	74±5	68±8	81±11	74±17	70±17
PL	14±9	13±10	19±7	10±8	6±8	9±8
LDL						
Chol.	45±14	44±12	36±26	47±16	54±19	39±30
TG	16±6[b]	20±5[a]	36±17	16±8	16±9	31±18
PL	39±10	36±11	29±14	38±9	29±16	30±16
HDL						
Chol.	26±5[a,c]	29±3[d]	41±18	40±7[c]	44±11[d]	51±16
TG	ND	ND	1±2	1±1	1±1	1±1
PL	74±5[a,d]	71±3[d]	58±18	57±5d	52±10d	49±15

Values expressed as a percentage of total lipid in that particle.
ND = not detected
P-values: -/- vs. +/+ or +/-: [a]<0.05, [b]<0.01; males vs. females [c]<0.05, [d]<0.01

cholesterol-enriched at the expense of TG. The LDL particle composition differed among *LPL* genotypes, such that LDL particles from male homozygotes contained proportionately more TG (36±17%) than those of heterozygotes (20±5%, $p<0.05$) or normal cats (16±6%, $p<0.01$). A similar pattern was observed in females, but was not significant, likely because of the smaller number of females available for study. The relative lipid composition of HDL differed according to *LPL* genotype. HDL from homozygous males was enriched in cholesterol and depleted in PL when compared to HDL from normal male cats (41±18% versus 26±5% cholesterol, $p<0.05$; and 58±18% versus 74±5%, PL, $p<0.05$). Similar results were observed in females, although they did not reach significance.

4.3.2 Fat tolerance test

To examine the functioning of LPL in this model under an environmental challenge, we have examined the oral fat tolerance in these cats. The oral fat tolerance profiles of the various genotypes are given in Figure 4.2. Following a fat rich meal, mean plasma TG of normal cats increased only moderately from a fasting baseline value of 0.49 mmol/L (43 mg/dL) to a peak value of 1.1 mmol/L (97 mg/dL), 3 hours after the oral fat load. Triglyceride concentrations then decreased progressively, returning to baseline after 7 hours, and remained stable up to 48 hours. All heterozygotes demonstrated a more gradual increase in TG concentrations after the fat load, and a significantly different postprandial TG profile from that of normals. The heterozygous cats (n=4) had mean TG concentrations, which increased from 0.31 mmol/L (27 mg/dL) at baseline, to a peak of 2.35 mmol/L (208 mg/dL) after 5 hours. The mean peak concentration was nearly eight times the fasting value, compared with a two-fold increase in normals, and the peak time was delayed to 5 hours, with TG concentrations only returning to baseline after 12 hours. The area under the mean TG clearance curve (AUC) was approximately 6 fold higher than that of normals (13.1 vs. 2.2 hr x mmol/L).

The alteration in fat tolerance was even more profound in cats with complete LPL deficiency. Postprandial concentrations increased approximately 10-fold (from 0.97 mmol/L (86 mg/dL) to 9.36 mmol/L (829 mg/dL)) and peak time was delayed to almost 7 hours after fat ingestion, the time by which normals had cleared their TG load. Twelve hours after the fat load the TG concentrations were still more than twice the fasting baseline value. Moreover, even after 48 hours, homozygous cats still had not reached a plasma TG concentration comparable to

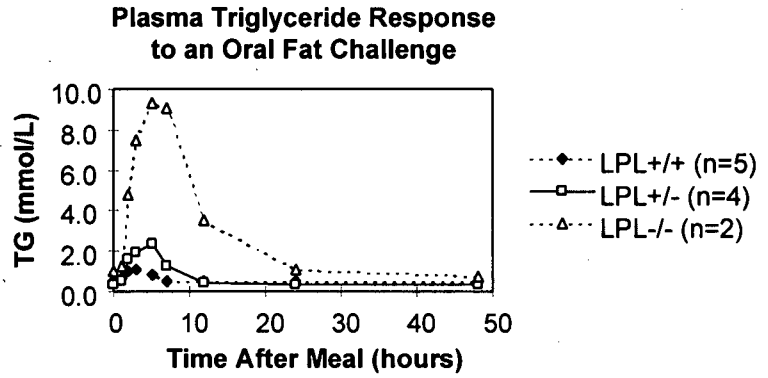

Figure 4.2. Oral fat load studies of LPL deficient cats.
Plasma triglyceride concentrations of normal, heterozygous, and homozygous LPL deficient cats prior to (t=0), and as measured over the indicated time intervals following an oral fat load. Normal cats (black diamonds) have a small rise in plasma TG which rapidly return to baseline. Heterozygotes (grey squares, solid line) have an increased TG peak and a delayed return to baseline following the oral fat load. Homozygotes have a markedly increased postprandial lipoprotein peak and severely delayed clearance of TG.

those of normals. The AUC was thus significantly greater in cats with complete LPL deficiency (280.3 hr x mmol/L) than in heterozygotes and normals.

4.4 Development of atherosclerosis in $LPL^{+/-}$ and $LPL^{+/+}$ cats

4.4.1 Lipoproteins

Cats, like mice, only develop atherosclerosis following high fat, high cholesterol feeding. Baseline comparisons of lipid levels and LPL activities (mean $\pm$ SD) between the groups used in the atherosclerosis study are presented in Table 4.3. The results were very similar to those already shown in this chapter for $LPL^{+/+}$ and $LPL^{+/-}$ cats. In general, TG, and specifically TGRL-TG were increased in heterozygotes compared to controls. Other lipoprotein differences did not reach significance.

During the cholesterol feeding, lipids were measured monthly. From the monthly lipid measurements while the animals were consuming the cholesterol enriched diet, average lipid levels over the time on the diet were calculated, to give an estimate of the lipid levels the cats were exposed to through the duration of the diet. Differences in lipids between the groups are presented in Table 4.4. Again, the main difference between heterozygotes and normals was increased TG in the heterozygotes. There was also a trend to increased HDL in the heterozygotes. This was not, however, significant, likely due to the large standard deviations.

Cholesterol feeding had a dramatic impact on lipid levels. To obtain a measure of the response to the diet, for each lipid measurement at each time point, the levels were expressed as

Table 4.3. Baseline LPL, lipid and lipoprotein comparisons between groups used in the atherosclerosis study

	LPL +/+ (n=6)	LPL +/- (n=6)	P-value
LPL activity (mU/mL)	258±84	91±43	**0.006**
TC	82.7±12.1	90.5±10.2	0.52
TG	26.0±4.1	35.8±7.4	**0.03**
HDL-C	54.8±5.9	62.3±11.9	0.30
HDL-TG	6.5±4.8	7.5±4.9	0.69
HDL_3-C	43.2±5.6	49.0±11.2	0.42
HDL_2-C	13.0±5.6	16.3±8.3	0.47
TGRL-C	6.2±5.5	3.5±4.2	0.37
TGRL-TG	13.5±3.4	20.2±5.3	**0.05**
LDL-C	22.0±9.0	25.8±5.6	0.62
LDL-TG	6.3±6.3	8.2±6.3	0.57
Particle Compositions (%)			
TGRL- chol	11.7±7.2	14.3±6.0	0.38
TG	74.3±11.1	76.7±2.1	0.52
PL	14.0±10.3	9.2±7.9	0.37
LDL- chol	46.5±13.3	46.8±13.1	1.00
TG	15.7±6.1	20.2±5.9	0.23
PL	37.8±9.9	32.7±13.4	0.42
HDL- chol	26.2±5.3	28.0±3.7	0.42
TG	ND	ND	-
PL	73.8±5.3	72.0±3.7	0.42

a percentage of the baseline value. These values were averaged over the 8 points on the diet, resulting in average percentage change from baseline values (Table 4.4). The response to the diet was generally very similar in both genotypes. High cholesterol feeding resulted in large (approximately 6-fold) increases in plasma total cholesterol levels, and approximately 2-fold increases in plasma TG. Interestingly, LPL activity also showed an increase on high-fat, high-cholesterol feeding in both genotypes.

The detailed particle compositions are an average of two measurements, at 2 and 5 months on the diet, respectively (Table 4.4). All particles became cholesterol-enriched (TGRL>LDL>HDL) compared to pre-diet values. TGRL were cholesterol enriched at the

Table 4.4. Average feline lipid values on the cholesterol-enriched diet

	LPL +/+ (n=6)	LPL +/- (n=6)	p-value
Monthly average lipid and LPL levels			
TC	441.9±211.4	507.6±255.3	0.63
HDL	72.9±24.0	96.3±27.2	0.20
TG	33.3±13.9	52.1±38.6	0.42
TC/HDL	8.27±5.64	8.3±6.2	1.00
LPL (mU/mL)	394±87	184±90	**0.02**
Detailed Lipid and Lipoprotein Profiles			
TC	501.9±227	601.2±404.5	0.87
TG	27.3±10.0	75.7±90.5	*0.08*
HDL-C	75.8±31.0	83.4±19.9	0.63
HDL-TG	11.7±2.6	12.6±3.5	0.52
HDL_3-C	32.0±10.4	42.7±14.7	0.13
HDL_2-C	39.2±22.3	36.2±12.6	0.94
TGRL-C	294.4±203.9	297.8±243.3	1.00
TGRL-TG	20.6±9.9	66.3±89.1	*0.11*
LDL-C	131.7±53.8	220.0±198.4	0.75
LDL-TG	0±0	0.5±1.2	0.32
Particle Compositions (%)			
TGRL- chol.	65.2±9.8	64.6±9.3	0.63
TG	8.7±9.9	11.8±8.8	*0.08*
PL	26.1±1.4	23.6±6.0	0.42
LDL- chol.	52.3±1.6	56.5±6.2	0.42
TG	2.1±1.6	3.8±2.0	0.11
PL	45.3±2.5	39.7±5.3	**0.04**
HDL- chol.	49.5±5.1	47.6±5.2	0.38
TG	0.2±0.3	0.7±0.8	0.16
PL	50.2±5.0	51.7±5.5	0.52
Average Percent Change from baseline (%)			
TC	503±163	522±303	0.63
HDL	19±51	23±23	0.63
TG	91±49	108±143	0.63
TC/HDL	649±503	655±508	0.87
LPL	66±55	168±271	1.00

expense of TG. LDL had a slight increase in cholesterol and decrease in TG. HDL were cholesterol enriched and PL depleted. Plasma TG and TGRL-TG were increased in

heterozygotes compared to normals. Heterozygotes also had a trend towards increased HDL_3-C compared to controls, similar to the trends seen in both the apoE deficient and C57BL/6 mouse models. Compared to normals, heterozygotes had a trend towards increased TGRL-TG, decreased LDL-PL and LDL-TG, and a trend to an increase in HDL-TG levels.

4.4.2 Atherosclerosis severity

Atherosclerotic lesion formation was measured in different ways. Lesions were scored on a semiquantitative basis from 0-5+. The scores from all sections within a given vessel segment (e.g. descending aorta, carotid, etc.) were averaged for each cat. Comparisons of these values between groups are presented in Table 4.5. Results are given for each segment, as well as for the average over the whole aorta, and as a sum of scores in all vessels. Information was not available for all cats for all segments due to difficulties obtaining/sectioning some segments. Where the number of cats was less than 6, the number is given to the right of that mean. Although no comparisons reached significance, lesion scores were generally decreased in heterozygotes compared to controls.

Lesion data was also calculated quantitatively as the absolute ORO staining area. A single defined section of each vessel was used (largest, smallest, etc.), so each group is the mean of the 6 measurements for the 6 cats. In the thoracic aorta, all available sections were measured (number of segments varies from cat to cat), and values were averaged over this segment to produce a single score, similar to the above vessels where only one segment was measured. It is these average values, which were then compared between the groups (Table 4.5). Also, the maximal staining within this segment was compared between groups (Table 4.5). Again, except in a small number of segments, lesion areas were generally smaller in heterozygotes compared to controls for all measures.

As individual quantitative lesion measures did not show any significant differences between the genotypes, the presence of general trends among all the vessels were examined, separating the aorta from the peripheral vessels. The average ORO staining areas over the aorta (average of the arch measurement, the mean thoracic measurement, and the two abdominal sections), and the sum of the absolute staining areas for the above aortic segments were calculated. Similar results were compiled for the peripheral segments (the two brachiocephalic/subclavian sections, the left and right carotid sections, and the two femoral

sections). A comparison of these measurements between the genotypes is presented in Table 4.6. While the differences still did not reach significance, the same trends were maintained, whereby lesions were decreased in the heterozygous cats compared to controls.

Table 4.5. Comparison of lesion measures between *LPL* genotypes

	LPL +/+ (n=6)	n	LPL +/- (n=6)	n	P-value
Semiquantitative scores					
Carotid (1)	1.64±2.40	5	1.80±2.77	3	1.00
Carotid (2)	2.28±2.37		1.33±1.93		0.68
Heart	1.10±1.13		1.28±1.21		0.81
Brachio/Sub[a]	3.33±1.97		1.86±2.11	5	0.19
Iliac	2.54±2.29	5	2.03±2.31		0.71
Aortic Arch	2.10±1.25	5	1.62±1.36		0.35
Descending Aorta	2.00±1.23		1.53±1.31		0.42
Thoracic Aorta	1.68±1.05		1.50±1.45		0.63
Abdominal Aorta	2.02±1.49		1.83±2.11		0.87
Lower Abd. Aorta[b]	1.92±1.52		1.85±2.14		0.68
Whole Aorta	2.01±1.26		1.68±1.62		0.75
Sum of all vessels	21.56±14.52		17.12±18.04		0.52
Quantitative ORO+ areas					
Left Coronary	11,274±15,781	5	1711±1340		0.20
Brach/Sub[a] (large)	966,988±1,187,769	5	456,585±806,736		0.27
Brach/Sub[a] (small)	356,549±656,680	5	186,742±417,482		0.36
Left Carotid	314,752±472,903		221,207±456,586		0.52
Right Carotid	149,832±317,047		140,914±339,225		*0.06*
Femoral (large)	141,492±221,100	5	475,765±934,556		0.72
Femoral (small)	19,195±30,820	4	122,553±244,344	4	0.25
Aortic Arch (large)	569,750±766,258		30,850±30,948	4	*0.06*
Abd. Aorta[b] (large)	809,644±1,486,271	4	757,397±1,102,649	5	0.81
Abd. Aorta[b] (small)	461,541±903,960	5	165,680±311,019	4	0.22
Thoracic Aorta	277,240±539,430		410,889±846,211		1.00
Maximum ORO+ area					
Thoracic Aorta	493,072±917,231		492,207±876,781		0.75

[a] brachiocephalic/subclavian
[b] Abd. = abdominal

As there were no significant differences between the genotypes, we next asked whether there were general trends between increasing LPL activity and the various measures of atherosclerosis. Correlations between baseline LPL and lesions, average LPL on the diet and

lesions, and % change in LPL on the diet and lesions were examined. No significant correlations between any LPL measurement and any lesion measurement were evident (data not shown).

As there did not appear to be any correlations between any measure of LPL and lesion formation, we examined whether there were correlations between lipid parameters and lesions.

Table 4.6. Comparison of summary lesion measures between genotypes

μm^2	LPL +/+ (n=6)	LPL +/- (n=6)	P-value
Aortic segments			
Average absolute ORO area	495,174±815,307	190,374±359,880	0.20
Sum of absolute ORO areas	1,771,370±3,279,215	1,173,073±2,037,064	0.34
Peripheral segments			
Average absolute ORO area	328,683±540,009	267,056±521,211	0.34
Sum of absolute ORO areas	1,698,239±2,687,878	1,562,914±3,135,162	0.42

Table 4.7. Correlations between lipid measures and lesions

	TC		HDL		TG		TC/HDL	
	r	p	r	p	r	p	r	p
% change in lipids								
Aortic segments								
Avg. absolute area	0.593	*0.042*	0.324	0.304	0.49	0.106	0.316	0.318
Sum absolute areas	0.688	*0.013*	0.304	0.337	0.637	*0.026*	0.392	0.208
Peripheral segments								
Avg. absolute area	0.747	**0.005**	-0.296	0.35	0.755	**0.005**	0.417	0.177
Sum absolute areas	0.767	**0.004**	-0.275	0.387	0.793	**0.002**	0.414	0.181
Average lipids on diet								
Aortic segments								
Avg. absolute area	0.739	**0.006**	-0.222	0.488	0.496	0.101	0.401	0.196
Sum absolute areas	0.827	**0.001**	-0.182	0.571	0.646	*0.023*	0.471	0.123
Peripheral segments								
Avg. absolute area	0.861	**<0.001**	-0.151	0.64	0.774	**0.003**	0.469	0.124
Sum absolute areas	0.867	**<0.001**	-0.124	0.702	0.824	**0.001**	0.455	0.137

These results are shown in Table 4.7. Due to the number of comparisons being made, p-values above 0.01 (or even likely 0.005) should not be considered significant. Plasma TC and TG levels were highly correlated with lesion areas. These correlations were primarily for VLDL-C and LDL-C, mainly VLDL-C. Individual particle-TG levels were not as strongly correlated as plasma TG.

As there were significant relationships between lipids and lesions, we next looked to see if there were any correlations between LPL and any of these lipid measurements, to look for an indirect influence of LPL activity on lesions. While there were correlations between LPL and TG at baseline ($r=-0.73$, $p=0.007$), following the diet, neither average LPL on the diet, nor the percent change in LPL on the diet were correlated with any lipid measures (either average or percent change, data not shown).

4.5 Discussion

LPL is the principal enzyme involved in TG hydrolysis of the TGRL. Although complete LPL deficiency in humans is generally not very common (about 1 in a million worldwide), individuals with partial reduction in LPL lipolytic activity are common, representing between 5-7 % of populations of European descent[141,144,276,278,280]. Characterization of this enzyme's role in lipoprotein metabolism and in the progression of atherosclerosis has yet to be fully elucidated. Studies performed in mouse models are described in Chapter 3, however such studies would be enhanced by complementary investigations in a well characterized larger animal model.

In cats, as in humans, complete LPL deficiency is associated with increased plasma TG concentrations. Chylomicrons were detected in the fasting plasma of the homozygous cats, as in humans with complete LPL deficiency[42]. TGRL-TG and TGRL-C concentrations were higher in homozygotes than in normal cats, however, the relative lipid composition remained unchanged, suggesting that there is an increase in total particle number, in all likelihood due to defective clearance of these particles. LDL-C levels are lower in homozygous cats, as is seen in humans with complete LPL deficiency[42,294]. This supports the concept that LDL production from VLDL is subsequently reduced in LPL deficiency, and that lipolysis is a key step in the production of LDL[294].

The cat has been considered an "HDL-animal" due to studies showing that the cat possesses more HDL-C than LDL-C[287], a finding confirmed in this investigation. It is likely that

the higher concentrations of HDL in cats, as compared to humans, is a consequence of the absence of any measurable plasma CETP activity in cats[295]. Significantly higher HDL concentrations are also found in human genetic CETP deficiency[296], and in other species lacking plasma CETP activity[297], or where CETP activity has been inhibited by an immunological blockade[298,299]. In the absence of CETP activity, HDL-C cannot be transferred to TGRL, while TG cannot be transferred in the reverse direction, resulting in cholesterol enrichment of HDL at the expense of TG. Of interest, we have observed almost no measurable TG in cat HDL, suggesting that a large portion of this component arises through CETP mediated transfer from TG rich particles, although it should be noted that lower HDL-TG levels may also in part arise from increased hydrolysis by HL.

Human hypertriglyceridemia is associated with several changes in lipoprotein composition, many of which are attributable to CETP activity. These include cholesterol enrichment and TG depletion of VLDL, and TG enrichment and cholesterol depletion of HDL[300]. In the present study we have characterized the relative effect of hypertriglyceridemia, due to complete and partial LPL deficiency, on lipoprotein composition in a model lacking plasma CETP activity. Despite a 10-fold increase in plasma TG concentrations, there were no significant changes in HDL lipid composition observed among genotypes. As the changes in HDL normally observed in human hypertriglyceridemia were not observed in this CETP deficient model, this suggests that CETP plays a critical role in HDL compositional changes in hypertriglyceridemia[300].

In LPL deficient cats ($LPL^{-/-}$), it is probable that lipoproteins (TGRL, LDL and HDL) compete for the remaining available lipolytic pathways. Hepatic lipase (HL) has been shown to function in the lipolysis of TGRL lipids, as well as those of the smaller apoB containing lipoproteins[244]. In cats HL activity is approximately two-fold greater than those of humans (D. Ginzinger, unpublished observations). This suggests that HL may play a role in the hydrolysis of VLDL-TG and PL in homozygous cats. However, as TGRL-TG are not the usual substrate of HL, TGRL would likely be inefficiently lipolyzed, which may contribute to the subsequent TG enrichment of LDL shown here. Also, as cats do not possess CETP activity, this increase in LDL-TG may reflect the lack of TG transfer from apoB containing lipoproteins to HDL.

If there is increased competition with HDL for HL by apoB containing lipoproteins in LPL deficiency, this may lead to increased HDL concentrations, as seen here with decreasing

LPL activity. However, HDL has been shown to be the preferential substrate of HL when competing with VLDL and LDL[33,301]. Thus, this explanation is unlikely. Alternatively, these findings are consistent with those observed in the mouse models (Chapter 3), and might again suggest a role for LPL in the selective uptake of HDL-CE.

Oral fat tolerance studies of complete human LPL deficiency have illustrated a dramatically reduced post-prandial TG clearance due to decreased LPL hydrolytic activity[302,303]. In this report we have demonstrated that homozygous cats similarly possess a markedly lower clearance rate of plasma TG after an oral fat load (Figure 4.2). Furthermore, they had increased fasting TG at baseline compared to normal cats, and demonstrated a profound increase in TG concentration, peaking at about 7 hours after the meal. After 12 hours these cats still had over a 3-fold elevation in TG concentration versus baseline. However, after a further 12 hours the majority of the circulating TG had been cleared, indicating that alternate pathways are able to metabolize ingested fat within 24 hours.

Heterozygosity for mutations in the *LPL* gene in humans has been associated with increased post-prandial lipemia[302,303], and with higher fasting plasma TG, due to elevations in the TG content of apoB-containing lipoproteins[145]. Here, while no significant differences in lipoprotein concentrations and compositions were found between fasting heterozygous and normal cats we show that, similar to humans[303], differences between heterozygotes and normals can be unmasked in response to an oral fat challenge (Figure 4.2). Heterozygous cats demonstrated an increased peak concentration and AUC, and delayed clearance of TG following a fat load.

Atherosclerosis was examined in $LPL^{+/-}$ compared to $LPL^{+/+}$ cats. Homozygous LPL deficient cats were not included in these studies, as in order to avoid the clinical manifestations of LPL deficiency (such as peripheral neuropathy, splenomegaly, etc.), they must be maintained on a low fat diet. Thus, feeding of a high-fat diet high-cholesterol diet needed to induce atherosclerosis would likely trigger the clinical sequelae of the disease. The inflammatory nature of some of these symptoms may influence the development of atherosclerosis, and thus might confound our analysis if not equivalent in all animals. In addition, as complete deficiency of LPL activity is relatively rare in the general population, examination of the effects of partial enzymatic deficiency is much more relevant to the general population.

As discussed in Chapter 1, there has been less support for the atherogenicity of TG and TGRL. The predominant particle in the cats during high cholesterol, high fat feeding was TGRL. The strong correlations between TC and TG and lesion areas thus provides additional validation of the atherogenicity of these predominantly TGRL particles, illustrating that increased plasma concentrations of TG-rich particles are associated with atherosclerosis. These findings have been obtained in the absence of CETP. Thus the effects of TG are not due to the CETP-mediated decrease in HDL-C that often accompanies hypertriglyceridemia in humans. These data specifically suggest that increased TG are associated with increased atherosclerosis.

However, as shown in Chapter 3 for the $LDLr^{-/-}$ mice, we were not able to detect any significant differences in lesion formation between the *LPL* genotypes. It is likely the atherogenic insult was too large in these animals, and perhaps overwhelmed the difference in LPL between heterozygotes and normals. Indeed, the cats were unable to tolerate the initial high cholesterol diet and had to be placed on a diet containing a lower percentage of cholesterol. However, it is also possible that the lack of measured difference may be in part technical. The lesions in these animals were obviously diffuse, and had progressed well beyond any initial lesion-prone sites. Thus, without measuring lesions extensively throughout the vascular tree, and examining the percentage of the total vasculature covered by lesions, it may be difficult to detect differences. Furthermore, with the large variability in lesion areas that have been observed in these large, complex lesions, it is quite likely much larger numbers would be needed to achieve a reasonable statistical power to detect small differences.

Although none of the differences reached statistical significance, lesion areas were generally smaller in the heterozygous cats, similar to what was observed in the mice (Chapter 3). This occurred despite the increased TG levels in these animals, and despite the increased postprandial TG response of the $LPL^{+/-}$ cats. While the initial study describing these cats was able to detect normal levels of LPL protein in the deficient cats[304], we have subsequently been unable to replicate these findings, using multiple different antibodies[285,305]. Thus, it is likely the mutant cat allele does not produce a stable protein product, and that heterozygotes have decreased LPL in the vessel wall as well as in the plasma. These findings are therefore consistent with what was observed in the mice, whereby decreased LPL protein was associated with decreased lesion formation. This further strengthens the idea that any increase in vessel wall is bad, despite the beneficial effects of increasing LPL in the plasma. These findings are

further strengthened by our inability to detect correlations between plasma LPL activity and any lesion measurement, despite correlations between lipid levels and lesions. The beneficial effects of increased LPL in the plasma are counteracted by the negative effects of increased LPL in the vessel wall.

This chapter describes a detailed analysis of plasma lipoproteins in normal and LPL deficient cats. In general, feline lipoprotein particles demonstrate similar properties to those of humans. LPL deficiency in cats, as in humans, is associated with higher concentrations of plasma TG, TGRL-TG and TGRL-C, and lower LDL-C concentrations. Homozygous cats demonstrated a markedly enhanced postprandial lipemia. Similar to what has been described in humans[303] (and Chapter 5), the effects of LPL deficiency in heterozygous cats also became more pronounced when stressed by an oral fat load, highlighting the impaired clearance of TG in these animals. Hence, these results are consistent with what is predicted for LPL deficiency in humans. Similarity of phenotypes is fundamental to the use of these animals in studies to further assess the role of LPL in lipoprotein metabolism and atherogenesis, providing additional validation of this colony of cats as a model of human LPL deficiency and in which to test potential therapeutics.

This chapter also describes an initial study of the atherosclerosis risk in these animals. Although significant differences in lesion formation were not detected, these studies need to be extended and replicated, likely at various stages of lesion formation, before firm conclusions can be drawn. The data do provide additional suggestive evidence that increased LPL within the vessel wall is an atherogenic risk factor.

Chapter 5: The role of LPL variants in atherosclerosis in human populations

The work presented in this chapter has been published in part in the following manuscripts

Zhang H., Henderson H., Gagne S. E., **Clee S. M.**, Miao L., Liu G., and Hayden M. R. Common Sequence Variants of Lipoprotein Lipase: Standardized Studies of In Vitro Expression and Catalytic Function. *Biochimica et Biophysica Acta* 1996 **1302**:159-166.

Pimstone S. N., **Clee S. M.**, Gagne S. E., Miao L., Zhang H., Stein E. A., and Hayden M. R. A Frequently Occurring Mutation in the Lipoprotein Lipase Gene (Asn291Ser) Results in Altered Postprandial Chylomicron Triglyceride and Retinyl Palmitate Response in Normolipidemic Carriers. *Journal of Lipid Research* 1996 **37**:1675-1684.

Ehrenborg E., **Clee S. M.**, Pimstone S. N., Reymer P. W. A., Benlian P., Hoogendijk C. F., Davis H. J., Bissada N., Miao L., Gagne S. E., Greenberg L. J., Henry R., Henderson H., Ordovas J. M., Schaefer E. J., Kastelein J. J. P., Kotze M. J., and Hayden M. R. Ethnic Variation and In Vivo Effects of the -93t→g Promoter Variant in the Lipoprotein Lipase Gene. *Arteriosclerosis, Thrombosis and Vascular Biology* 1997 **17**:2672-2678.

Clee S.M., Loubser O., Collins J. A., Kastelein J. J. P., and Hayden M. R. The LPL S447X cSNP is associated with decreased blood pressure, plasma triglycerides and risk of coronary artery disease. (submitted to *Arteriosclerosis, Thrombosis and Vascular Biology*, Mar. 2001)

The sections on the N291S and S447X variants have also been published in abstract form

Pimstone S. N., et al. *Oral presentation*, 68th Scientific Sessions of the American Heart Association, Anaheim CA Nov 13-16, 1995. Published in *Circulation* 92 (8, Suppl.) I493. Oct. 15, 1995.

Clee S. M., et al. *Poster presentation*, 72nd Scientific Sessions of the American Heart Association, Atlanta GA Nov. 7-10, 1999. Published in *Circulation* 1999 100(18):I822.

Preface

While not solely my own work, the data in the chapter is presented to illustrate the studies I have been involved in, in collaboration with others in the lab, which extend the findings I have obtained in the animal models to human populations. In the section on *in vitro* work, I have assisted in the transfections, and performed the LPL protein mass measurements. The study described in the section on the N219S variant was designed and carried out by Dr. Simon Pimstone. I performed the statistical analysis of the data and participated in the writing of the manuscript. In the section on the D9N variant, designed by Dr. Ewa Ehrenborg, I again performed the statistical analysis of the data and contributed largely in the writing of the manuscript. The section on the S447X was a study performed by Mrs. Odell Loubser, a technician in our lab, under my guidance. I have been responsible for all data interpretation and writing of that section, and this represents a manuscript recently submitted for publication.

5.1 Introduction

Assessment of the role of *LPL* genetic variation in the development of atherosclerosis in human populations is difficult. Heterozygosity for *LPL* mutations occurs in about 1 individual in 500 in most populations[42], which is too rare to identify sufficient individuals for studies of CAD. Thus, the relationship between LPL and atherosclerosis can only be examined in relation to more common variation in human populations.

A large degree of genetic variation is common in most genes, with differences between individuals occurring, on average, approximately every 200-1000 base pairs[306-309]. These variants are most commonly single nucleotide polymorphisms (SNPs) resulting from the substitution of one nucleotide with another, although they can often include other types of polymorphism such as the insertion or deletion of nucleotides or the repetition of one or more nucleotides. Three common polymorphisms have been identified in the coding region of the *LPL* gene (cSNPs)[310], which are associated with small changes in enzyme function and have effects on plasma HDL-C, TG and severity of coronary artery disease (CAD).

An A to G transition of the second base at codon 291 (nucleotide 1127) in exon 6 of the *LPL* gene results in a substitution of serine for asparagine (N291S)[144,311,312]. The carrier frequency of this variant has been reported to vary between approximately 2-5% in the general population[274,276,278,311-315]. Several studies, in different population groups, have reported an association of this variant with hyperlipidemia, specifically increased TG[266,274,276,312,314,316,317] and decreased HDL-C[144,266,274,276,278,312,316,318,319]. Other studies have failed to show these associations[271,315,320]. Carriers of this variant have more recently been shown to have increased CAD[266,271,274], an increased risk of cerebrovascular disease in women[321]. However, as homozygotes for this cSNP do not appear to have a more severe phenotype than heterozygotes, and as not all early studies showed associations of the N291S with plasma lipid levels, questions were raised about the functional nature of this cSNP[313].

The second variant arises from a point mutation in exon 2 at codon 9 (G280A) that results in a substitution of asparagine for aspartic acid. This D9N variant has been reported at a mean carrier frequency of approximately 1-4% in individuals from European countries[278-280,314,322,323]. It has been found at 2-3 fold increased frequencies in patients from the same communities with familial combined hyperlipidemia[280]. Carriers of this variant have been shown to have increased

TG[267,278-280,314,322-324] and decreased HDL-C[141,267,272,323] and apoAI[277]. Furthermore carriers have an increased risk of atherosclerotic coronary or cerebrovascular disease[141,267,272,322]. Although, again, not all studies have found differences in lipid levels[320] or CAD[277].

Factors which are associated with hypertriglyceridemia have been shown to exacerbate the phenotype of *LPL* mutations. The first such factor described was pregnancy[325]. Excess alcohol intake[326,327] and diabetes[328-330] have also been shown to exacerbate the phenotype of *LPL* mutations. The phenotype of both the D9N and N291S cSNPs may become exaggerated in the presence similar environmental factors. Increased body mass index (BMI) has been shown to exaggerate the phenotype of the N291S[276,278,312] and D9N[267,279], as have other factors predisposing to hyperlipidemia such as the E2 isoform of apoE[311], hyperinsulinemia[317], smoking[314], alcohol intake[327] and reduced physical activity[323]. These variants have also been seen at a higher frequency in individuals with diabetes and/or obesity[331]. Gender-specific factors may also influence the phenotype of variation in *LPL*, as some effects have been reported only in males from some cohorts, while others have only been seen in females. Other cohorts have shown effects in the opposite genders, however, so the role for sex-dependent effects remains unclear.

The third common variant of *LPL*, S447X (C1595G), results in the generation of a premature stop codon, truncating the last two amino acids of the mature LPL protein, and has been reported at carrier frequencies of 10-30%[315,332-335]. This cSNP was first reported as the molecular basis for a patient with familial chylomicronemia[336]. However, recent studies have found that this cSNP occurs at a lower frequency in hyperlipidemic patients than in normolipidemic subjects[333,335] and at a reduced frequency in individuals who have had a myocardial infarction (MI)[273]. This variant has been associated with decreased plasma TG[271,273,277,334,337,338], and increased HDL-C[271,315,334,337,339,340] in most, but not all[275,320] studies. The effects of this cSNP on CAD have been less clear. It has been associated with a decreased family history of MI[273] and a decreased risk of CAD events[333,334]; with no difference in atherosclerosis or coronary events[271,275,277,341]; or with a trend to increased CAD[315]. It has also been suggested that the effects of this variant on CAD may occur independent of its effects on plasma lipid levels[334]. Thus, there is still some question as to the functional nature of this variant.

The data presented in this chapter describe studies I have been involved in that have contributed to our understanding of these cSNPs and how they might contribute to the population risk of CAD.

5.2 Characterization of the three common *LPL* cSNPs *in vitro*

Early *in vitro* studies of these SNPs yielded conflicting data on whether these variants were truly associated with altered LPL levels or activity. Decreased activity[144,317,331] has been reported for the N291S cSNP, but this was not found in all studies[342]. Both reports of normal and reduced catalytic activity for the D9N variant have appeared in the literature[280,328,343]. The S447X has been shown to have increased LPL protein mass *in vitro*[336], decreased activity *in vitro*[336], increased post-heparin activity *in vivo*[337], an increased kinetic activity[344], and to be found at an increased frequency in individuals selected for high LPL activity[151]. Thus, the effects of these variants on LPL production and catalytic activity were uncertain.

Potentially confounding variables in the *in vitro* assessment of cSNP function included poorly standardized cell transfection procedures. Studies performed by Dr. Hanfang Zhang in our lab were therefore designed to re-examine these variants using optimized experimental conditions for the in vitro expression of their cDNA constructs and to determine their catalytic activities, stabilities, and cell surface binding affinities in rigorously controlled and comparable experiments. These properties of the variants were compared to those of normal LPL expressed under identical conditions.

5.2.1 Methods

5.2.1.1 Transient expression of LPL constructs

Transfection of CMV-driven wildtype LPL cDNA or constructs containing the D9N, N291S or S447X cSNPs in the pcDNA3 vector was performed by lipofection. Transfections were performed using Lipofectamine, according to the manufacturer's protocol (Gibco-BRL). Briefly, plasmid DNA (0.6 μg) was mixed with OPTI-MEM reduced serum medium (Gibco-BRL) to a final volume of 400 μL. This was gently added to an equal volume of Lipofectamine diluted in OPTI-MEM (20 μL Lipofectamine + 380 μL OPTI-MEM), and incubated at room temperature for 15 minutes. A further 3.2 mL of OPTI-MEM was then added. This mix was added in 1.2 mL aliquots to triplicate wells of COS-1 cells (80% confluent) in 6-well plates, after

they had been washed with PBS and OPTI-MEM. Cells were incubated at 37°C for 5 hours, and 0.3 mL of OPTI-MEM with 25% fetal bovine serum (FBS) was added to the cells to give a final concentration of 5% FBS. The incubation continued for another 8-10 hours (overnight). Antibiotics were not present in the OPTI-MEM medium during the total transfection period. The medium was replaced with culture media (Dulbecco's modified eagle medium (DMEM) with high glucose, 5% FBS, 1 mM glutamine, 2 mM pyruvate and penicillin/streptomycin supplemented with 7 mU/mL heparin) the following morning, and changed on the subsequent three days, at 24, 48, 72 hours post-transfection, respectively. These media collections were cleared of dead cells by centrifugation, aliquoted, snap frozen and kept at -70 °C until assayed.

5.2.1.2 Measurements of LPL protein mass and catalytic activity

LPL immunoreactive mass levels in the media were measured by an ELISA using the 5F9 and 5D2 LPL monoclonal antibodies as capture and detection antibodies respectively[142,345]. The epitope of the 5F9 monoclonal is undetermined, but known to be exposed only on denatured or monomeric LPL, while the 5D2 epitope is exposed in both the dimer and monomer forms of LPL, and is located between residues 396-405[345,346]. Total LPL protein mass was determined after dissociation of dimeric enzyme into monomers by denaturation with 1M guanidinium hydrochloride (GuHCl)[345]. LPL dimeric mass was indirectly determined by subtracting the monomer mass determined in the absence of denaturation with GuHCl from the total monomer mass after denaturation[345]. Microtitre plates were coated with the 5F9 monoclonal in PBS by incubation for 4 hours at 37°C. Media samples or purified bovine LPL (used as standard controls) were added to each well and incubated for 18 hours at 4°C. Wells were then washed to remove the unbound LPL and incubated with the 5D2 monoclonal, conjugated to horseradish peroxidase, for 4 hours at room temperature. Wells were washed 5 times in PBS/Triton X-100 and substrate (3,3',5,5' tetramethyl benzidine) was added for colour development. The reaction was quenched after 10 minutes with 4 M H_2SO_4, and the optical densities read at 490 nm.

LPL lipolytic activities were measured as described in Chapter 2.

5.2.2 Results

5.2.2.1 Measurement of LPL protein mass and catalytic activities of the variants in COS media

The mean LPL activity and protein mass data from multiple transfections is shown in Table 5.1. The data are expressed as the percentage of the average of the wildtype LPL replicates within that transfection. Each construct was tested in 3 to 5 independent experiments. The LPL activities of wildtype ranged from 76 to 161 mU/mL and the total mass levels from 450 to 1290 ng/mL within these experiments.

Two of the variants showed decreased catalytic activities when compared to normal LPL, with the N291S manifesting the lowest activity at 57% of normal ($p< 0.0005$). The D9N variant had slightly reduced LPL activity that was 85% of control ($p<0.0005$). The S447X truncated variant gave 94% of normal activity, which was not significantly different from control.

Table 5.1. LPL protein mass and activity from wildtype and LPL variants in transfected COS-1 medium

	LPL Mass		LPL Activity	Ratio of Activity to Total Mass
	Total	Dimer/Monomer		
Wildtype (n=15)	100.03±11.09	1.09±0.19	100.00±7.04	0.139±0.033
N291S (n=8)	76.99±7.61[a]	0.66±0.14[a]	56.70±4.05[a]	0.104±0.009[b]
D9N (n=9)	88.62±14.33[c]	1.00±0.32	84.57±7.35[a]	0.147±0.014
S447X (n=12)	131.11±22.34[a]	0.75±0.31[b]	93.78±15.53	0.100±0.026[b]

n=total number of wells in which that construct was expressed
[a] $p < 0.0005$ compared with wildtype
[b] $p < 0.003$ compared with wildtype
[c] $p < 0.05$ compared with wildtype

We measured LPL protein mass for both the monomeric and dimeric forms of LPL and could therefore express specific activities using either protein mass level as the denominator. Specific activities derived from the dimeric mass (homodimer specific activity) are determined largely by the kinetic properties of the enzyme, while specific activities derived from the total mass (total mass specific activity) are additionally determined by the rates of dissociation of active dimer into inactive monomer. A decrease in total mass specific activities can therefore, indicate changes in either of these parameters, although the latter is likely to predominate.

The N291S enzyme gave the lowest protein mass level, but this decrease was less than the reduction in lipolytic activity (Table 5.1). This variant also had the lowest dimer to monomer ratio and therefore yielded a low total mass specific activity. The low ratio of dimer to monomer could have arisen from either an increased rate of dissociation of dimer into inactive monomers, or excess secretion of monomeric LPL. Although the D9N variant gave a significant reduction in activity, this decrease was paralleled by a reduction in protein mass, giving a normal dimer to monomer ratio and therefore a normal total mass specific activity. While the catalytic activity of S447X did not differ from normal, it showed substantially increased monomeric mass, thereby significantly lowering the dimer to monomer ratio and the total mass specific activity. Again, this raised the possibility of increased dimer dissociation or excess secretion of the monomeric enzyme.

5.2.3 Discussion

Initially, I participated in these studies examining the phenotypic expression of the LPL polymorphisms *in vitro*, using carefully controlled and standardized conditions established by Dr. Zhang. The data clearly indicate that the N291S sequence variant of LPL manifests with a partial reduction of catalytic activity (57% of normal) and a reduction of LPL protein mass (76% of normal) in the COS-1 cell expression system. This was in keeping with the previous studies from this laboratory, reporting a 40% reduction in catalytic activity[144,311]. Dr. Zhang subsequently demonstrated that the N291S homodimer is less stable than the normal counterpart, which might, in part, account for its decreased lipolytic activity[281]. Loss of activity due to an accelerated rate of dimer dissociation is supported by the higher LPL monomer concentrations measured in the harvesting medium. An inherently unstable N291S homodimer and reduced secretion of the protein may therefore explain the lower *in vivo* LPL activities seen in N291S carriers[144].

The D9N sequence variant showed a marginal but significant reduction in overall catalytic activity despite a normal homodimer specific activity and dimer stability. This *in vitro* demonstration of some impairment of lipolytic function is in accord with the reported tendency of D9N carriers toward lowered post heparin LPL levels[280].

Catalytic activity and dimeric mass levels for the S447X truncation variant were similar to those of control LPL. However, total mass levels (after denaturation) were significantly

elevated, giving a 28% reduction in the total mass specific activity of the variant (Table 5.1). Dr. Zhang subsequently showed that this excess monomer is not derived from enhanced denaturation of the dimer form, and most likely arises from a higher constitutive secretion rate[281]. We found the S447X variant to manifest normal homodimer specific activity (data not shown), suggesting the activity of the enzyme is normal. The functional importance of the terminal residues of LPL is unclear, but it is possible that they are involved in the post-translational modification processes that determine the dimerization of LPL monomers prior to secretion. This hypothesis is supported by the demonstration that the S447X truncation abolishes the residual 10% activity of the Glu410Val homodimer[347] possibly through inhibition of dimerization. These findings are more recently supported by studies that overexpress this variant *in vivo* through an adenovirus delivered construct, yielding dramatically increased LPL protein mass in plasma (K. Ashbourne Excoffon, unpublished observations). Interestingly, truncation of the enzyme ten amino acids earlier (at 437) results in decreased production or secretion of the LPL protein, suggesting a significant reduction in stability[348]. This suggests the C-terminal part of LPL is a critical determinant of dimer stability.

5.3 The N291S cSNP in the lipoprotein lipase gene results in altered postprandial chylomicron triglyceride and retinyl palmitate response in normolipidemic carriers

Early studies variably suggested that the N291S cSNP was associated with low HDL-C and elevated TG levels in the fasting state. Some carriers, however, are normolipidemic and may have LPL activity in the normal range in the fasting state[144]. The high frequency of this cSNP in Western populations and the finding of normolipidemia in some persons with this cSNP, have raised questions as to the functional effects of this common variant[313].

Postprandial metabolic studies have been performed previously on individuals heterozygous for mutations in the *LPL* gene[302,303]. Carriers of true null alleles, had exaggeration of lipid abnormalities after a fat challenge despite the fact that overlap existed between carriers and non-carriers for both LPL lipolytic and specific activity measurements in the fasting state[302]. Humans spend approximately 75% of their time in the postprandial state[349], therefore postprandial assessment of lipoprotein metabolism may provide a more physiological perspective of disturbances in lipoprotein homeostasis compared to assessment in the fasting state. As much controversy existed regarding the functional nature of the N291S cSNP, and due

to its potential importance as a result of its high frequency in the general population, we studied postprandial metabolism in three normolipidemic N291S heterozygotes and compared the response to five healthy BMI-matched controls and one subject with a catalytically defective LPL protein.

5.3.1 Methods

5.3.1.1 Subjects

The studies were performed on five normal subjects, three N291S heterozygotes (subjects 1, 2 and 3) and one individual with a catalytically defective LPL protein, as defined by 50% of normal LPL activity (subject 4) identified through screening a population of healthy, unrelated volunteers. The ages of all subjects ranged from 24 to 49 years. All subjects had normal fasting lipids (TC < 5.2 mmol/L; LDL-C < 3.4 mmol/L; TG < 2.3 mmol/L; and HDL-C > 0.9 mmol/L) and normal body mass index (BMI < 26 kg/m^2). No subject had any disease, and none were taking medications known to affect lipoprotein metabolism. None of the subjects smoked, took alcohol in excess (> 2 glasses of alcohol/day), or were pregnant. One of the male N291S carriers (subject 1) was a marathon runner, but all other subjects exercised moderately (< 2 times weekly). The protocol was approved by the Clinical Screening Committee of the University of British Columbia and informed, written consent from all subjects was obtained prior to the study.

5.3.1.2 Study design

Three day dietary records completed by all subjects in the week prior to the study were analyzed by St. Paul's Hospital Lipid Clinic dietitians. All subjects were following diets in accordance with American Heart Association step 1 guidelines and were instructed to remain on their current diets. Study participants were asked to refrain from alcohol for 72 hours prior to the fat load study.

After a 12 hour overnight fast, subjects were given a standard fat-rich meal consisting of cream (65g), skim milk powder (51g), sugar (24.7g), and 2.5 mL corn oil. The composition of the meal was 67% fat, 22% carbohydrate and 11% as protein, containing 238 mg cholesterol. Vitamin A (retinyl palmitate (RP) at a dose of 60 000 IU/m^2 body surface area in the form of Vitamin A Palmitate Type P1MO/BH, a kind gift from Hoffman-La Roche Ltd., Mississauga, Ontario) was added to each meal to biosynthetically label intestinal lipoproteins. After fasting

blood samples were drawn, meals were consumed by all subjects within 10 minutes. Meals were well tolerated. No evidence of malabsorption, bloating or nausea were recorded. Subjects were permitted to be ambulatory and were allowed unlimited access to water and sugar-free gum throughout the duration of the study.

5.3.1.3 Analysis of samples

Following the meal, blood samples were drawn at 2 hour intervals for 12 hours. Blood samples were immediately put onto ice and were protected from light after removal and during transport and processing. All processing of samples was performed in a dark room. Plasma was separated by centrifugation (2000 rpm for 10 minutes at 4°C) and aliquoted into multiple tubes and into 20 mL transfer vials containing aprotinin and sodium azide preservatives. The tubes were stored at -70°C and the transfer vials were wrapped in aluminum foil and kept at 4°C until analysis, which was performed within one week of sampling. Plasma from the transfer vials was used for sequential ultracentrifugation and for RP assays. Frozen plasma was used for lipid analysis and apolipoprotein assessment. Samples were transported overnight at 4°C and -20°C respectively to the analytical laboratory (Medical Research Laboratories, Highland Heights, KY).

5.3.1.4 Lipid, lipoprotein and retinyl palmitate analysis

Total cholesterol, TG and various lipoprotein cholesterol and TG fractions were measured on a Hitachi 737 analyzer (Boehringer Mannheim Diagnostics, Indianapolis, IN) using a microenzymatic procedure.

HDL was isolated from total plasma following precipitation of apoB containing lipoproteins with heparin and 2 M manganese chloride[350]. Various lipoprotein fractions were isolated by sequential preparative ultracentrifugation, using the following parameters. Large TG-rich particles (predominantly chylomicrons) were isolated following centrifugation of plasma at 33,000 rpm for 27 minutes at 20°C. The 'chylomicron free' (bottom) fraction was subjected to 40,000 rpm at 4°C for 20 hours at d=1.006 g/mL. The 'VLDL' fraction was defined as this chylomicron free fraction minus the d > 1.006 g/mL fraction. This fraction would include predominantly $apoB_{100}$ but also smaller $apoB_{48}$ containing lipoprotein particles. IDL was isolated by ultracentrifugation of the d> 1.006 g/mL fraction using the same parameters except at

d = 1.019 g/mL. LDL was defined as the difference between the d > 1.019 g/mL and the isolated HDL fraction described above. HDL_3 was isolated by ultracentrifugation at d = 1.125 g/mL and HDL_2 by the difference between HDL by precipitation and the HDL_3 isolate.

Plasma RP and the RP content of chylomicron and non-chylomicron fractions were measured by HPLC as previously described[302].

ApoAI and apoB were analyzed by immunonephelometry using monoclonal antibodies and standardized to World Health Organization referenced calibrators (Behring, Germany)[351]. ApoCIII and LpAI (lipoprotein particles containing only apoAI) were quantified using hydrogel electroimmunodiffusion as previously described[352]. Glucose was measured on the Hitachi 747 using a hexokinase based reagent. Insulin was quantified by a radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA).

5.3.1.5 Postheparin plasma lipolytic activities and protein mass

One week prior to the fat load study, all subjects received an intravenous dose of heparin (60 U/kg body weight, Hepalean, Organon Teknika, Toronto, Canada). Subjects were fasted for 12 - 14 hours and were asked to refrain from alcohol for 72 hours prior to the heparin challenge test. Fasting plasma samples were withdrawn before and 15 minutes post-heparin challenge for assessment of plasma lipase activities. Plasma was separated by centrifugation at 2000 rpm for 10 minutes at 4°C and thereafter frozen at -70°C.

Lipolytic activities (LPL and HL) were measured as described in Chapter 2. Total and dimeric LPL protein mass in post-heparin plasma samples were measured as described in Section 5.2.

5.3.1.6 DNA analysis of *LPL* variants

DNA was extracted from leukocytes by standard procedures[353]. The N291S cSNP was assayed as previously described[144]. Exon 6 was amplified with the following two primers, the second of which incorporates a mismatch (in bold) creating an *Rsa*I restriction site on the 291S allele: 5'GCCGAGATACAATCTTGGTG and 5'GAGAACGAGTCTTCAGGTGCATTTTGCT GCTTCTTTTGGCTCTGACT**G**TA. Reactions, using 10 pmol of each primer, were carried out in the presence of 2.5 mM $MgCl_2$ and 1.6 μM dNTPs. Cycle conditions constituted: 94°C 5 minutes; 35 cycles 94°C 1 minute, 52°C 1 minute, 72°C 2 minutes; 72°C 10 minutes. The 264

bp PCR product was digested with *Rsa*I and resolved on a 2.5% agarose gel, yielding bands of 215 and 49 bp for the variant allele, while the wildtype allele is not cut.

The other two *LPL* cSNPs associated with functional effects in the general population, the D9N and S447X, were excluded in all subjects by PCR techniques previously described[275,354]. For detection of the D9N, exon 2 was amplified with 10 pmol each of the following primers: 5'CTCTTCCCCAAAGAGCCTCC and 5'CTCATATCCAATTTTTCCTTT CCAGAAAGAAGAGATTT**G**ATC. The longer of the two primers incorporates a mismatch, creating a *Bcl*I restriction site in the presence of the 9N. The S447X was detected by amplifying exon 9 with 10 pmol each of the two primers shown: 5'GGATGCCCAGTCAGCTTTAGCCCA GAATGCTCACCAGA**C**T and 5'TATTCACATCCATTTTCTTC. As with the D9N and N291S assays, the long primer incorporates a mismatch to generate a *Hinf*I site on the 447X allele. Both reactions were performed in the presence of 1.5 mM $MgCl_2$ and 1.6 μM dNTPs. Cycle conditions were as follows for the D9N: 94°C 5 minutes; 35 cycles of 94°C 1 minutes, 52°C 1 minutes, 72°C 2 minutes; 72°C 10 minutes, and with an annealing temperature of 53°C and cycle times of 45 seconds at each temperature for the S447X. PCR products were digested with *Bcl*I and *Hinf*I for the D9N and S447X, respectively, and resolved on 2.5% agarose gels. The D9N amplifies a fragment of 210 bp which is cut to fragments of 172 and 38 bp on the variant allele, while the 164 bp S447X product results in fragments of 124, 40 for the variant allele. Neither wildtype allele is digested by the respective enzymes.

Subject 4, with 50% normal LPL activity, was assessed for mutations in the *LPL* gene by SSCP analysis as previously described[343]. No bandshifts were noted in exons 1-9 and the molecular cause for the underlying functional defect remains unknown.

5.3.1.7 Apo E genotyping

Apo E genotyping was performed using PCR amplification of a 244 bp fragment with the F4 and F6 primers (5'ACAGAATTCGCCCCGGCCTGGTACAC and 5'TAAGCTTGGCA CGGCTGTCCAAGGA), followed by digestion with the restriction enzyme *Cfo*I, an isoschizomer of *Hha*I. This method has been described in detail[355]. Restriction fragments were then separated on polyacrylamide gel electrophoresis and isoforms read against a PUC 18 marker digested with *Msp*I. In addition to invariant bands of 38,18, and 16 bp, the E2 allele produces

bands of 91 and 81 bp, the E3 bands of 91, 48 and 33 bp, and the E4 allele results in bands of 72, 48, 33, and 19 bp, following digestion. This allows unambiguous genotyping of all three alleles.

5.3.1.8 Statistical methods

Means and standard deviations (SDs) were calculated using conventional methods. Within group comparisons of means at various time points during the fat load study were made using the Student's paired t-test (Microsoft excel, version 5.0). Between group comparisons were tested using the one-way ANOVA after variance was shown to be equal by the Bartlett test for homogeneity of group variance. If the variances of the two groups were unequal, a non-parametric Mann-Whitney U test was employed to detect between group significance. Where the coefficient for age as a covariate was significant, an analysis of covariance was performed to take into account age effects on the parameter measured.

Where indicated, values in our study were corrected for baseline by subtracting t=0 (baseline) values from values at each subsequent timepoint. The TG and retinyl palmitate (RP) response curves were drawn by plotting the corrected for baseline values versus time and fitting a smooth curve through the data points, using the cubic spline fit (KaleidaGraph, version 2.0.2). The area between this curve and the x-axis (area under curve, AUC) was integrated using the trapezoidal rule (KaleidaGraph, version 2.0.2).

TG had returned to baseline in all subjects by 10 hours and therefore plasma and CM TG AUC were calculated between t = 0 and t = 10. RP, on the other hand, had not returned to baseline by 10 hours in all subjects and therefore RP AUCs were calculated as the area between t = 0 and t = 12. To determine the correlations between various measured parameters, data for both groups was pooled and the Spearman (non-parametric) correlation coefficients were determined. To account for the effect of multiple comparisons, the accepted level of significance was adjusted using the Bonferroni correction (adjusted level of significance, p = 0.01). Unless otherwise stated, statistical analyses were performed on the Systat statistical package (Version 5.2 for the Macintosh).

5.3.2 Results

5.3.2.1 Subject characteristics

The baseline characteristics of the subjects are shown in Table 5.2. N291S carriers, controls and subject 4, with a catalytically defective LPL, were matched for BMI and were between 24 and 49 years of age. No significant difference was noted between carriers and controls for age or BMI. All subjects were normotensive and non-smokers.

Table 5.2. Baseline characteristics and metabolic parameters of individuals in the oral fat load study

	Subject 4	N291S Heterozygotes (n=3)	Controls (n=5)	P-Value[a]
Age	49	37.7±10.5	29.2±5.2	NS
Sex	M	2M, 1F	2M,3F	
BMI	25.8	24.1±1.3	22.5±1.3	NS
Apo E Genotypes	E2/3	2 E2/3, 1 E3/3	2 E3/4, 3 E3/3	
Plasma TG	2.18	1.72±0.27	0.85±0.29	0.005
LDL-TG	<0.01	0.05±0.08	0.02±0.03	NS
HDL-TG	0.52	0.54±0.05	0.42±0.06	NS
HDL_2 -TG	0.29	0.35±0.01	0.24±0.03	0.02
HDL_3-TG	0.23	0.18±0.07	0.18±0.03	NS
VLDL-TG	1.12	0.66±0.21	0.23±0.12	0.02
IDL-TG	0.11	0.15±0.07	0.09±0.03	NS
Total Cholesterol	5.1	5.2±1.0	4.5±0.6	NS
LDL-C	2.8	3.2±1.1	2.7±0.5	NS
HDL-C	1.14	1.24±0.16	1.37±0.27	NS
HDL_2-C	0.41	0.53±0.15	0.55±0.11	NS
HDL_3-C	0.72	0.71±0.02	0.81±0.16	NS
VLDL-C	1.19	0.81±0.02	0.43±0.14	0.02
IDL-C	0.23	0.29±0.13	0.25±0.09	NS
Apo CIII (mg/L)	35	35.0±2.0	21.0±5.2	0.004

[a]P-values are for comparisons between N291S heterozygotes and controls. NS= not significant. All lipid levels are in mmol/L. To convert mmol/L to mg/dL, multiply cholesterol by 38.7, TG by 88.6 and PL by 75.0.

Fasting plasma TG were below the 80th percentile for age and sex in all N291S subjects, a criterion for inclusion in the study. Despite fasting plasma TG levels in the normal range, the N291S heterozygote group had significantly higher values compared with controls ($p = 0.005$).

Table 5.3. Total, hepatic and lipoprotein lipase activities and protein mass in post heparin plasma

	Total Lipase Activity	LPL Activity (mU/mL)	HL Activity (mU/mL)	Total LPL Mass (ng/mL)	Dimeric Mass (ng/mL)	Monomeric Mass (ng/mL)	Specific Activity (mU/ng)
Mean±SD							
Controls (n=5)	330±87	209±57	121±43	1137±211	705±90	432±226	0.26±0.02
N291S Heterozygotes (n=3)	364±67	201±54	164±43	950±364	538±201	412±211	0.39±0.11[a]
Subject 4	401	106	295	821	507	314	0.21
Individual N291S heterozygote values							
Subject 1	401	258	142	1313	657	655	0.39
Subject 2	405	192	213	953	650	303	0.29
Subject 3	287	152	136	585	306	279	0.5

[a] $p < 0.004$ for comparisons between N291S Heterozygotes and Controls.

VLDL-TG and VLDL-C were both significantly higher in N291S carriers compared with controls. In addition, HDL_2-TG was increased in N291S carriers compared with controls ($p = 0.02$). No significant difference was seen in fasting HDL-C levels between N291S carriers, controls and subject 4. Mean plasma apoCIII was significantly higher in carriers compared with controls at baseline ($p = 0.004$). No significant difference was seen in mean apoAI and apoB levels between the groups (data not shown). Fasting glucose and insulin levels were normal in all subjects and no significant difference was observed between the groups (data not shown).

Mean LPL activity between carriers of the N291S cSNP and controls were similar (Table 5.3). This was primarily due to the high LPL activity of subject 1, an N291S carrier and a marathon runner whose higher LPL activity is consistent with what has previously reported in endurance trained athletes[356,357]. Subject 4 had an LPL activity approximately 50% of controls. Total and dimeric LPL protein mass were not significantly different between N291S carriers and controls (Table 5.3). Overlap existed in specific activity measurements among subjects in both N291S carrier and control groups. Subject 4 had the lowest specific activity of all study participants (Table 5.3).

5.3.2.2 Total plasma, chylomicron and non-chylomicron retinyl palmitate response to the vitamin A fat load

RP is absorbed through the intestine and carried on chylomicrons and their derivatives. Once picked up by the liver, resecretion is minimal, as is transfer by CETP to other particles. Thus it is used as a marker of intestinally-derived lipoproteins. The mean plasma RP response over time is shown in Figure 5.1 (A). The mean plasma RP peak was reached at 4 hours in controls and was delayed to 6 hours in the N291S carriers and 8 hours in subject 4. Variation in the time to reach peak RP levels was seen in individuals within both N291S carrier and control groups, with one of the five controls peaking at 6 hours and the female N291S carrier (subject 3), having a marginally higher level at t=4 hours than at t=6 (data not shown). Mean plasma RP peak levels were higher in N291S carriers compared with controls ($p=0.03$) (Table 5.4). All N291S carriers had higher RP peak levels than the mean RP peak for the control population. Significance was reached between N291S carriers and controls in plasma RP levels at t=4 ($p=0.008$) and t=6 ($p=0.03$) (Figure 5.1 (A)). Peak plasma RP was higher in subject 4 than in all N291S carriers (Table 5.4).

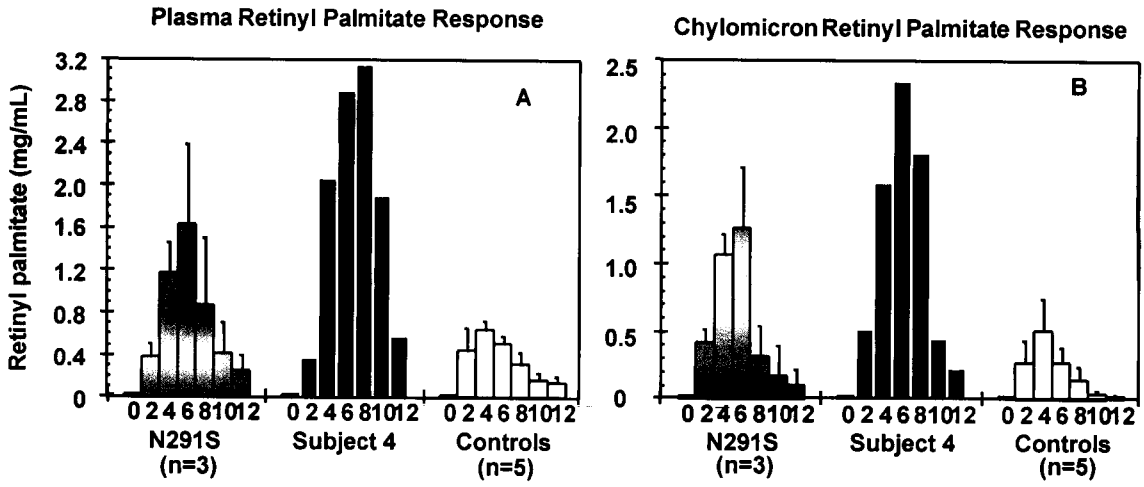

Figure 5.1. Retinyl palmitate responses of N291S carriers and controls.
Mean plasma **(A)** and chylomicron **(B)** retinyl palmitate levels at baseline (t=0) and up to 12 hours following the oral fat load for N291S carriers (grey), subject 4 (black) and controls (white) are shown. Error bars represent the SD of the mean at each timepoint for the N291S carriers and controls.

Table 5.4. Peak retinyl palmitate and triglyceride levels following the oral fat load

	Retinyl Palmitate			Triglycerides	
	Plasma (mg/mL)	Chylomicron (mg/mL)	Non-Chylomicron (mg/mL)	Plasma (mmol/L)	Chylomicron (mmol/L)
Mean±SD (range)					
Controls (N=5)	0.68±0.10 (0.55-0.82)	0.52±0.24 (0.25-0.89)	0.26±0.15 (0.08-0.45)	0.38±0.12 (0.22-0.50)	0.27±0.10 (0.10-0.35)
N291S Heterozygotes (N=3)	1.67±0.69[a]	1.30±0.38[b]	0.55±0.43	1.25±0.69	0.86±0.40[a]
Subject 4	3.15	2.36	0.95	1.28	1.30
Individual N291S heterozygote values					
Subject 1	1.97	1.38	1.05	0.47	0.59
Subject 2	2.16	1.65	0.32	1.77	1.32
Subject 3	0.88	0.90	0.28	1.51	0.67

P-values compared to controls: [a]p=0.03, [b]p=0.02

The mean plasma RP area under curve (AUC) was significantly higher in N291S carriers compared with controls ($p = 0.05$), but lower than the plasma RP AUC in subject 4 (Table 5.5).

Plasma RP encompasses that in CM and their lipolyzed remnants, and thus also reflects rates of remnant clearance. Therefore we examined RP clearance specifically from the CM fraction. N291S carriers had significantly increased chylomicron RP (CRP) peak heights compared with controls ($p = 0.02$) (Table 5.4). The difference between the two groups was particularly evident at t=6 ($p = 0.001$). No overlap existed between any N291S carrier or control in CRP peak height (data not shown). As with peak plasma RP, peak CRP was delayed in the male N291S carriers (t=6) compared with controls (t=4, Figure 5.1(B)). Subject 3 had her CRP peak at t=4, earlier than the male N291S carriers. All controls had peaked at 4 hours (data not shown). The CRP peak level of subject 4 was higher than in any N291S carrier (Table 5.4). The mean CRP AUC was significantly higher in the N291S carriers compared with controls ($p = 0.01$) (Table 5.5) and no overlap existed between the two groups. Subject 4 had the greatest CRP AUC (Table 5.5).

Table 5.5. Retinyl palmitate and triglyceride areas under the curve

	Retinyl Palmitate			Triglycerides	
	Plasma (mg/mL x hr)	Chylomicron (mg/mL x hr)	Non-Chylomicron (mg/mL x hr)	Plasma (mmol/L x hr)	Chylomicron (mmol/L x hr)
Mean±SD (range)					
Controls	4.43±0.53 (3.90-5.32)	2.59±1.12 (1.16-4.01)	1.93±1.09 (0.54-3.28)	0.91±0.63 (0.08-1.55)	1.11±0.53 (0.23-1.55)
N291S Heterozygotes	9.28±4.05[a]	6.66±2.06[b]	3.37±2.33	4.32±3.15	2.87±1.12[c]
Subject 4	21.6	13.8	6.62	4.92	5.63
Individual N291S heterozygote values					
Subject 1	12.6	6.78	6.03	0.75	1.96
Subject 2	10.5	8.66	2.37	6.72	4.11
Subject 3	4.77	4.55	1.72	5.48	2.53

P-values compared to controls: [a]p=0.05, [b]p=0.01, [c]p=0.02

The non-chylomicron (NC) fraction reflects remnant lipoproteins. The mean NCRP peak was higher in the N291S heterozygotes compared with controls, but this did not reach significance (Table 5.4). Within the N291S group, one male subject (subject 1), displayed a greater NCRP compared with the other two carriers whose peak NCRP levels were more similar to those of the control group (Table 5.4). An elevated NCRP curve was also observed in subject 4. Both subjects 1 and 4 with greater NCRP curves are carriers of the apo E2 isoform (E2/E3 genotypes) and these results are in keeping with the delayed remnant clearance that is often seen in carriers of the E2 isoform[358]. This suggests the N291S defect is in chylomicron processing to remnants, not in remnant clearance.

5.3.2.3 Plasma and lipoprotein TG response to the vitamin A fat load

As RP is only a marker of intestinal lipoproteins and may be influenced by differences in absorption, we have also directly examined plasma and CM TG responses. It should be noted that plasma TG levels reflect those in all particles, including VLDL that may be secreted by the liver in response to incoming remnants. The mean plasma TG clearance is shown in Figure 5.2(A). All subjects had a mean peak level at t=4 and had returned to baseline by t=10. Significantly increased plasma TG levels were noted in the N291S group at t=4 ($p = 0.02$) and t=6 ($p = 0.03$) when compared with controls. Variation existed within N291S carriers and controls in the peak plasma TG level. Within the N291S heterozygote group, subject 1, a long distance runner, had a lower peak level compared with the other two N291S carriers in the group. This is probably a reflection of the substantially reduced peak VLDL-TG in this individual (data not shown). His peak plasma TG level however, was still marginally higher than the mean peak plasma TG level for the control group (Table 5.4). The mean plasma TG AUC was increased nearly 5-fold in N291S carriers compared with controls, however this did not reach statistical significance. The highest peak plasma TG level was observed in subject 4.

Mean peak chylomicron TG (CTG) levels were significantly higher in the N291S carriers compared with controls ($p = 0.03$, Table 5.4). Levels were higher at t=4 ($p = 0.001$) and t=6 ($p = 0.05$, Figure 5.2 B). CTG had returned to baseline by 10 hours in all subjects. As with CRP, all carriers had higher CTG peaks than any control. The peak CTG level was highest in subject 4. The mean CTG AUC was significantly higher in N291S carriers compared with controls ($p = 0.02$, Table 5.5), and was highest in subject 4.

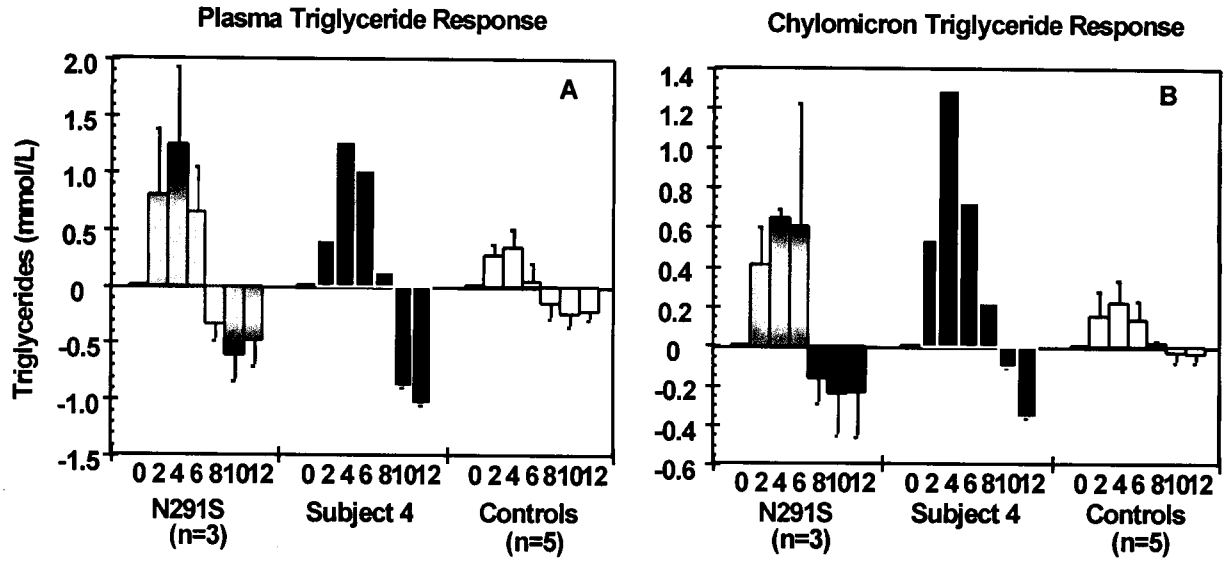

Figure 5.2. Plasma triglyceride responses in N291S carriers and controls.
Mean plasma **(A)** and chylomicron **(B)** TG levels at baseline (t=0) and up to 12 hours following the oral fat load for N291S carriers (grey), subject 4 (black) and controls (white) are shown. Error bars represent the SD of the mean at each timepoint for the N291S carriers and controls.

5.3.2.4 Correlation between fasting TG, LPL activity and TG AUC

Fasting TG levels were positively correlated with plasma TG AUC (p= 0.04), but did not reach the Bonferroni adjusted significance level of p= 0.01. However, baseline TG were positively associated with CTG AUC (p= 0.001, Figure 5.3 A). Baseline TG were also positively correlated with CRP AUC (p= 0.001, Figure 5.3 B). No significant correlations were observed between fasting LPL activity and either CTG or CRP AUC (data not shown).

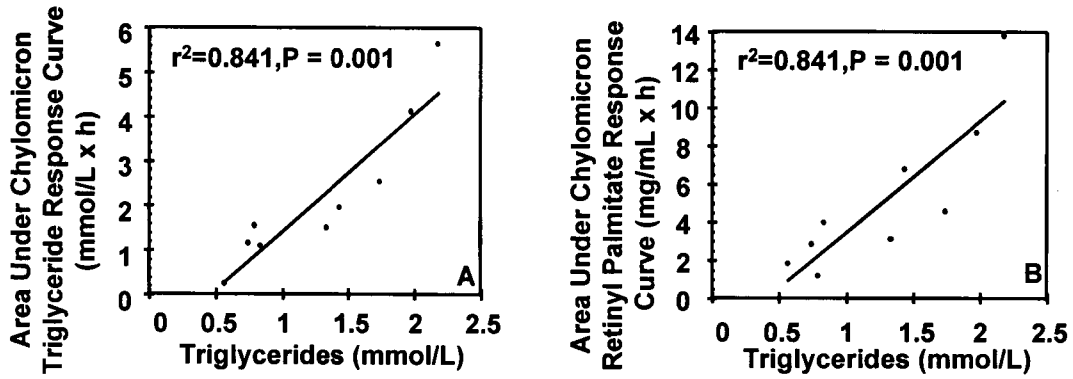

Figure 5.3. Correlations between TG response and baseline TG levels.
Area under chylomicron TG **(A)** and RP **(B)** response curves versus baseline TG levels. A significant positive correlation (Spearman correlation) is shown between these variables.

5.3.3 Discussion

The present study was undertaken to determine the influence of an environmental stress in the form of an oral fat load on the phenotype of carriers of the common N219S cSNP in the *LPL* gene. We find that despite normolipidemia amongst carriers in the fasting state, carriers of the N291S variant had a significantly impaired response to an oral fat challenge. The response in these N291S carriers was intermediate between that of controls and a subject with 50% LPL catalytic activity, suggesting a partial lipolytic defect associated with the N291S cSNP, even for subjects with normal lipid levels in the fasting state. Although fasting TG levels fell within the normal range in N291S carriers, these levels were approximately twice those of the control group. As fasting TG levels have been shown previously to be a predictor of postprandial response[359,360], all relevant analyses were performed correcting for baseline lipid values similar to other investigations[361,362].

Carriers for mutations in the *LPL* gene have previously been shown to have exaggeration of disturbances in lipoprotein values following a dietary fat challenge[303]. We have now extended these findings to a common cSNP in the *LPL* gene. The lipolytic defect induced by this common DNA alteration is partial in nature, resulting in a loss of approximately 30% of LPL activity when measured after a heparin challenge[144]. Furthermore, these moderate reductions in lipolytic activity are not evident in all N291S carriers, many of whom are clearly normolipidemic in the fasting state[144,313].

Despite a small sample size, significantly different results were observed when markers used for determining postprandial response were compared between the N291S carriers and controls. Peak TG and RP levels in the CM fraction were significantly higher in carriers of the N291S cSNP compared with controls ($p = 0.03$ and $p = 0.02$ respectively). This was true even in subject 1, a marathon runner. One may have expected that this individual, with elevated LPL activity and reduced VLDL-TG, would not have an abnormal postprandial chylomicron response. However, despite this, an increased postprandial response was observed. Delayed plasma and CM RP peaks at 6 hours were observed in both male carriers compared with 4 hours in controls. This was not however the case in the female N291S carrier (subject 3), who peaked at 4 hours. This finding is in keeping with prior reports suggesting earlier peak RP levels and lower postprandial RP responses in women compared with men matched for age and BMI[363]. Delayed CRP peaks in both male N291S carriers compared with male controls, suggests a delay

in clearance of large, TG-rich (predominantly CM) particles. While the apo E genotype may influence the metabolism of the non-chylomicron (predominantly remnant) fraction, postprandial CTG and CRP response has not been shown to be influenced by the apo E genotype[358,364-366]. As NCRP clearance was not delayed, except in the two individuals with apoE2 alleles, this suggests the defect in the N291S individuals is in the conversion of CM to remnants.

Another assessment of CM particle clearance was made by determining the magnitude of the area under the CM curve postprandially. The mean CTG AUC was significantly higher in N291S carriers compared with controls ($p = 0.02$), as was the mean CRP AUC ($p = 0.01$), suggesting delayed clearance of large, TG-rich particles. The AUC is a measure of the total postprandial stress, as it reflects not only increased levels of postprandial lipoproteins, but also a prolonged postprandial lipemic phase. Thus, increased postprandial AUC would reflect a dramatically increased insult to the vessel wall.

An abnormal postprandial response in N291S carriers in our study would not have been predicted by measurements of (fasting) post-heparin plasma LPL activity and protein mass. Subject 3 had the lowest postprandial response of the N291S carriers despite the lowest LPL activity. Subject 1 on the other hand had a marked postprandial response despite the second highest LPL activity of all subjects studied. In addition, fasting LPL activity measurements did not correlate significantly with CTG or CRP AUC. Fasting LPL activity measurements therefore may not always predict postprandial response, particularly in individuals with a functional defect in the *LPL* gene. It is possible that mechanisms regulating LPL activity *in vivo*, either transcriptional or translational, may be different in the fasting state compared to when challenged by a high fat meal. In fact, LPL activity in both animal and normal human subjects have shown significant increases after fat feeding (Chapter 4 and references[133,234]). Carriers of defective alleles may upregulate *LPL* in an attempt to compensate for their defect, and thus not be able to respond to a challenge, such as fat feeding, with further upregulation. Carriers of the N291S cSNP in the *LPL* gene may therefore be unable to respond to a high fat diet by an increasing their LPL activity in contrast to normal subjects. Thus a high fat diet may unmask a previously hidden defect in lipolysis in these subjects.

5.4 *LPL* D9N is in linkage disequilibrium with the g(-93)t promoter variant: effects of the combined –93g/9N haplotype on lipoprotein profiles

In addition to the three common cSNPs of the *LPL* gene, three promoter SNPs at positions -93, -53 and -39 have been identified in patients with familial combined hyperlipidemia[367,368]. This disorder is characterized by elevated plasma levels of total cholesterol, TG, or both, in multiple individuals of the affected family[369]. While the substitutions at -53 and -39 are rare, the change at -93 (t→g) was reported to occur in 3/183 (1.6%) control individuals and at an increased carrier frequency of 5.2% (6/115) in a cohort of CAD patients[368]. This suggested that this particular SNP may contribute to dyslipidemia and atherosclerosis[368]. Both decreased and increased *LPL* transcriptional activity *in vitro* have been suggested[367,368] as the functional effects of the –93g allele *in vitro*.

Thus far, the frequency of the –93g allele in the *LPL* gene has not been examined in populations other than Caucasians. In this study we sought initially to investigate the ethnic variation in frequency of this mutation. We studied the frequency of this DNA change in three ethnically diverse populations (Caucasian, South African Black, and Chinese) and show marked differences in the frequency of this DNA change in different populations. Haplotype analysis revealed that the -93g allele was almost always seen in association with the D9N cSNP in Caucasians but not in Blacks. As the D9N variant has been shown to be associated with the disturbances in lipids and increased progression of atherosclerosis, the question therefore remained as to whether the D9N cSNP alone caused the phenotypic effects previously reported, or whether the promoter SNP in almost complete non-random association might be contributing to this finding. The high frequency of -93gg homozygotes and -93tt homozygotes in the absence of the D9N cSNP in the South African Black population allowed us to directly address the phenotypic effects of this DNA transition.

5.4.1 Methods

5.4.1.1 Subjects

5.4.1.1.1 Caucasians

To assess the frequency of the –93g allele in a Caucasian population, 232 unrelated male subjects < 60 years of age, ascertained from a large Dutch population-based risk-factor study (RIFOH) were studied[370]. All subjects were normolipidemic, with no history of CAD. No

subject had any disease known to affect lipid metabolism including diabetes mellitus, hypertension, thyroid, renal or liver disease and none were taking medications known to affect lipoprotein metabolism (diuretics, beta-blockers, calcium channel blockers and steroids including hormone replacement therapy and oral contraceptives).

To assess the chromosomal relationship between the –93g allele and the D9N cSNP a larger cohort of Caucasian carriers of these SNPs was obtained for analysis of flanking microsatellite markers. Seventy six subjects from the Framingham Offspring Study cohort consisting of individuals with mixed European descent[334,371] were ascertained. This cohort comprised 39 D9N and –93g carriers and 37 subjects who did not carry the –93g allele, -53, -39, D9N, or the N291S SNPs. The D9N and –93g carriers did not carry the -53, -39 or N291S SNPs.

5.4.1.1.2 South African Blacks

One hundred sixty-one unrelated Black South African subjects were ascertained for these frequency studies, including volunteers from the hospital staff and patients from the Medical Outpatient Department at the Red Cross Children's Hospital, Cape Town[372]. All 161 individuals were analyzed for the presence of the –93, -53, -39, D9N and N291S SNPs.

The influence of the –93g allele on lipid levels independent of the D9N cSNP was assessed in 92 male subjects for whom fasting lipid profiles were available. All were from the Venda tribe, in rural areas of South Africa and were between 18 and 70 years of age. None of these subjects were taking medications known to affect lipid metabolism, consumed alcohol in excess (i.e. >3 drinks/day), or were carriers of the -53, -39 or N291S SNPs.

5.4.1.1.3 Chinese

One hundred thirty unrelated individuals of Chinese (Cantonese) ancestry recruited from six Chinese family physician practices in Vancouver were screened for the –93 and the D9N cSNPs. Subjects were selected as consecutive unrelated patients being assessed for a routine physical examination, and were part of a more detailed study identifying coronary risk factors in the Cantonese speaking Chinese population (S. McGladdery, Atherosclerosis, In Press). Only individuals 20-70 years of age were included in the study. Thirty eight of these individuals were

ascertained for analysis of flanking microsatellite allele frequencies, described in section 5.4.2.2. In addition, the frequency of the –93, -53, -39, D9N and N291S SNPs were analyzed.

5.4.1.2 DNA analysis

The D9N and N291S cSNPs were detected as described in the previous section. PCR amplifications of genomic DNA for promoter SNP analysis and allelic variation in the 5' region and intron 6, respectively, were performed in 25 μL reactions in the presence of 0.3 mM specific primer, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.4 at 70°C, 0.1% Triton X-100, 0.2 mM each dNTP, and 1.25 units Taq DNA polymerase (BRL). The promoter variants at -93 and –53 were analyzed by amplification with primer prLPL-8 (5'-GTGTTTGGTGCTTAGACAGG) and primer prLPL-1 (5'-GCTAGAAGTGGGCAGCTTTC). The analysis of the –39 mutation was performed using a mismatch primer, -39prLPL (5'-AATAGGTGATGAGGTTTATTTGTA) and primer prLPL-1. The reactions were incubated for 5 minutes at 96°C, followed by 40 cycles at 96°C for 45 seconds, 57°C for 30 seconds, and 72°C for 45 seconds. The PCR products were digested with 10 U *Hae*III for detection of the –93 substitution, with 10 U *Bcl*I for -53 detection and with 10 U of *Rsa*I for detection of the -39 SNP. The digested fragments were separated on 3.5% agarose gels.

Allelic variation in the 5' region was obtained by PCR amplifications with oligonucleotides previously described[373]. The reactions specific for the CA repeat were incubated for 5 minutes at 96°C, followed by 20 cycles at 96°C for 1 minute, 60°C for 30 seconds, and 72°C for 1 minute. PCR products were run on a 6% denaturing polyacrylamide gel. The sizes of alleles were determined by comparison with an M13 sequencing reaction as a standard.

5.4.1.3 Biochemical analysis

Plasma lipid levels (TC, TG, HDL-C, nonHDL-C) were measured as described in Chapter 2. LDL-C was calculated according to the Friedewald formula[374].

5.4.1.4 Statistical analysis

The significance of frequency distributions of SNPs/alleles between and within populations was determined using Chi Square analysis and Fisher's Exact two-tail probability,

where appropriate. The SNPs were all in Hardy-Weinberg equilibrium. Allele sizes of the simple sequence repeat were grouped until a minimum of one allele in each group was obtained. Linkage disequilibrium is presented as a D value[375], and the corresponding significance was assessed by Fisher's exact two-tail probability test using maximum likelihood estimated haplotype frequencies. In the South African Black population, group differences in biochemical parameters were determined using an analysis of variance between the following pairs: tt/DD vs. gg/DD; gg/DD vs. gg/DN; and gt/DD vs. gt/DN. All pairs were matched for age, systolic and diastolic blood pressure, mean alcohol consumption and smoking behavior. The first two of these pairs were also matched for BMI but small differences in BMI were seen between the -93gt/DD vs. -93gt/DN group (p=0.03). Statistics for TG levels were performed on log transformed data. Statistical analysis of the data was carried out using All Stats (UBC) and Systat (SPSS Inc.).

5.4.2 Results

5.4.2.1 Frequency of the –93g allele in different populations

The frequency of the sequence substitution in the promoter region at -93, described as a t→g substitution[367] was investigated in three ethnically distinct populations: Caucasian (n=232), South African Black (n=161) and Chinese (n=130). Significant differences in the frequency of the –93g allele were observed between the populations (Table 5.6). The g allele at -93 was identified in 5/232 Caucasians (carrier frequency of 2.2%). In the South African Black population, however, this allele was identified in 76.4% of the individuals (123/161). In contrast, this SNP was not identified in the Chinese population.

Furthermore, when the genotypic information obtained for the Caucasian population was compared with that of previous studies regarding the D9N and N291S cSNPs in this cohort[141,144], the -93g allele was in observed to be in almost complete linkage disequilibrium with the D9N cSNP (D=0.0085[375], p=8 x 10^{-9}). The D9N cSNP was identified in four individuals from this Dutch cohort, representing a carrier frequency of 1.7% (4/232), all of whom were heterozygous for the -93g. One individual homozygous for the -93g allele without the D9N cSNP was also identified in the Dutch population. The D9N cSNP was not found in the absence of the -93g allele in the Caucasian population. In contrast, of the 123 Black subjects carrying the -93g

Table 5.6. Carrier frequencies of the -93g allele and the D9N in the *LPL* gene in three different populations

	CAUCASIAN N=232	BLACK N=161	CHINESE N=130
-93t/D[a] (%)	227 (97.8)	38 (23.6)	130 (100.0)
(95% CI)	(95.9-99.7)	(16.9-30.3)	(100.0)
-93g/D[b] (%)	1 (0.4)	103 (64.0)	0 (0.0)
(95% CI)	(-0.5-1.3)	(56.4-71.6)	(0.0)
-93g/N[c] (%)	4 (1.7)	20 (12.4)	0 (0.0)
(95% CI)	(0.0-3.4)	(7.2-17.6)	(0.0)

CI= confidence interval
[a]-93t/D includes individuals with genotype-93tt/DD.
[b]-93g/D includes individuals with genotypes -93gt/DD or -93gg/DD.
[c]-93g/N includes individuals with genotypes -93gt/DN or -93gg/DN.

$p<10^{-6}$ Caucasian vs. Black vs. Chinese
$p<10^{-6}$ Caucasian vs. Black
$p<10^{-5}$ Black vs. Chinese
$p<10^{-4}$ Caucasian -93g/N vs. Black -93g/N

allele, only 20 were carrying the D9N cSNP. Similar to the –93g allele, the D9N cSNP was not identified in the 130 Chinese subjects (Table 5.6).

5.4.2.2 Allele distribution of a highly polymorphic marker upstream of the *LPL* coding region

To further investigate the chromosomal origins of both the -93g allele and D9N cSNP in the *LPL* gene, the CA repeat polymorphism located approximately 5 kb upstream of the transcription start site[373] was analyzed. In order to assess whether the N9 and the -93g alleles were in complete linkage disequilibrium, and to obtain larger numbers of Caucasian D9N carriers for dinucleotide repeat analysis, 39 individuals identified as heterozygous D9N carriers in the Framingham Offspring study[376] were also screened for the presence of the -93g allele. All 39 were found also to be heterozygous carriers of the -93g allele. These 39 subjects were typed for the CA repeat, as were Black subjects with genotype gg/DD (n=17), gt/DN (n=2) and gg/DN

(n=3). In addition, 5' CA repeat lengths in 37 Caucasians, 17 South African Blacks and 38 Chinese subjects with the genotype tt/DD were assessed (Table 5.7).

Repeat lengths varied from 14 to 28 repeats[373]. Due to the small numbers of each repeat size identified, allele sizes were grouped until the minimum number of alleles in all categories was at least one. Significant differences in allele distributions between all three populations were observed (Table 5.7). Thus, further analysis of the alleles was performed in each population separately.

Table 5.7. Frequencies of the grouped allele sizes of the CA repeat located upstream of the *LPL* gene in individuals with different genotypes in three different populations

	CAUCASIAN		BLACK				CHINESE
CA repeat sizes	tt/DD n (%)	gt/DN n (%)	tt/DD n (%)	gg/DD n (%)	gt/DN n (%)	gg/DN n (%)	tt/DD n (%)
14-16	59 (79.7)	38 (48.7)	20 (58.8)	6 (17.7)	1 (25.0)	1 (16.7)	57 (75.0)
17-19	11 (14.9)	1 (1.3)	12 (35.3)	8 (23.5)	1 (25.0)	1 (16.7)	5 (6.6)
20-28	4 (5.4)	39 (50.0)	2 (5.9)	20 (58.8)	2 (50.0)	4 (66.6)	14 (18.4)
Total number of alleles	74	78	34	34	4	6	76

p=0.05 Caucasian tt/DD vs. Black tt/DD
p=0.02 Caucasian tt/DD vs. Chinese tt/DD
$p<10^{-4}$ Black tt/DD vs. Chinese tt/DD
$p<10^{-6}$ Caucasian tt/DD vs. Caucasian gt/DN
$p=10^{-5}$ Black tt/DD vs. Black gg/DD
All other pairwise comparisons were non-significant

In Black subjects with the gg/DD genotype the -93g allele is seen on chromosomes with many different CA repeat alleles (Table 5.7, Figure 5.4 (B)), while the -93t allele in subjects with the tt/DD genotype was primarily associated with CA alleles containing 16 or 17 repeats (Table 5.7, Figure 5.4 (A)). Furthermore, repeat lengths of 16 and 17 are the predominant alleles in both Caucasian (Table 5.7, Figure 5.4 (E)) and Chinese (Figure 5.4 (C)) populations where the -93t allele is predominant. Thus, as the -93t allele is primarily associated with the CA allele of 16 repeats in all 3 populations (Figure 5.4 (A, C, E)), while no such associations are seen for the -

93g allele, this suggests the t allele arose on a specific haplotype containing 16 or 17 repeats and is compatible with a common origin of the carriers of the -93t allele.

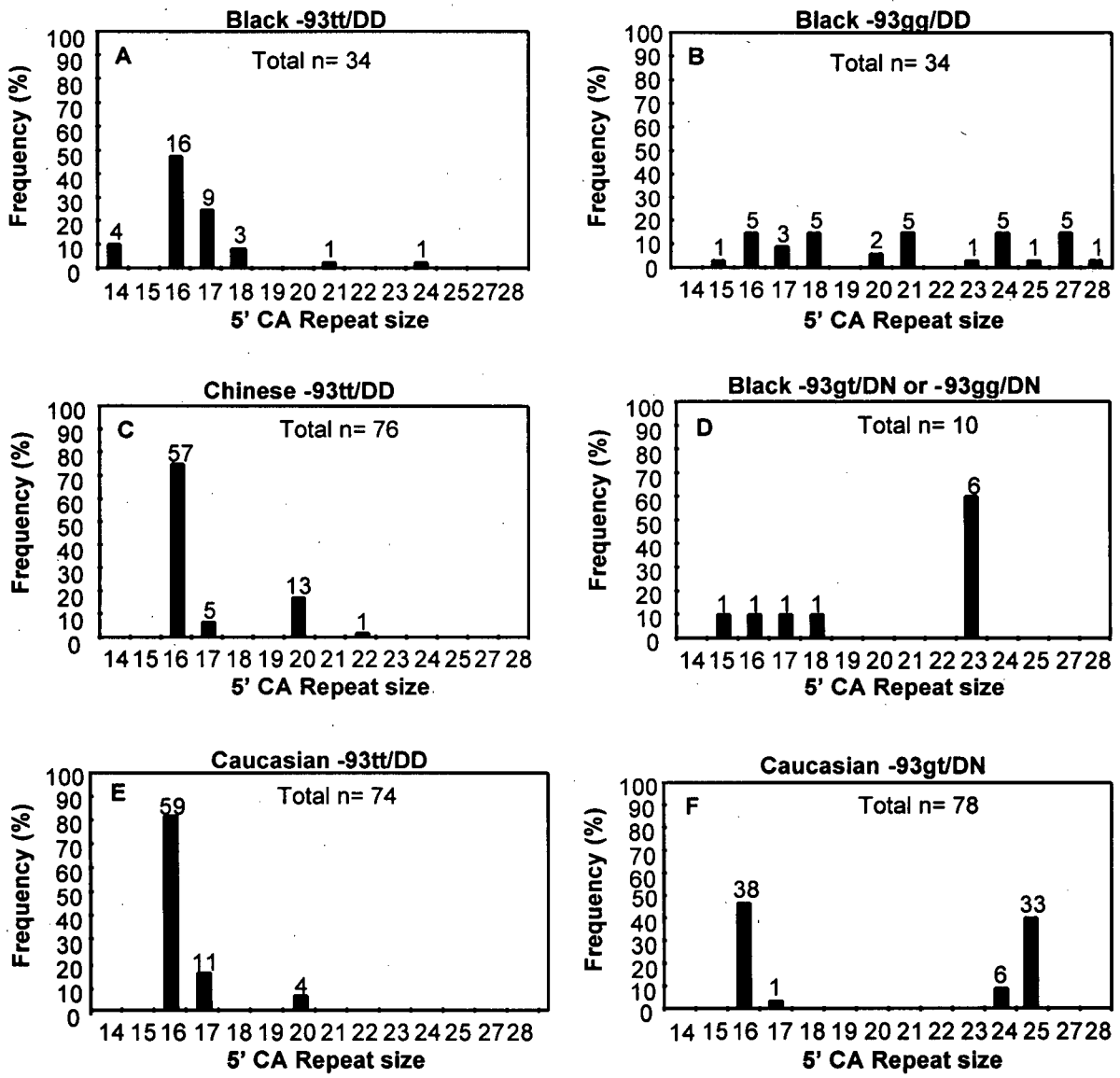

Figure 5.4. Allelic distributions by LPL –93 and D9N genotypes within different ethnic populations. **(A-F)** Allele distribution of the 5' dinucleotide repeat in separate populations with different genotypes at position –93, with and without the D9N cSNP. The number of alleles is illustrated above each bar. Allele sizes are indicated by the number of repeats on the x-axis. The –93 g allele haplotype is seen on many different CA repeat alleles in the Black population (B), while in Caucasians it is associated with the 9N and repeats of 24-25 (F). The –93 t/D9 allele is associated with repeats of primarily 16-17 in all populations (A,C,E).

The D9N variant is associated with the 23 repeat CA allele in the Black population (Figure 5.4 (D)). Similarly, in the Caucasian population a significant shift ($p<10^{-6}$, Table 5.7) towards CA repeats of particularly 24-25 repeats in size are seen in subjects of the -93gt/DN

genotype compared with individuals carrying the -93tt/DD genotype (Figure 5.4 E, F). This suggests that the D9N cSNP is associated with longer CA repeat lengths like those associated with chromosomes carrying the -93g allele (Figure 5.4 (D, F)) as opposed to the highly prevalent -93t allele seen in Caucasians. Again, this suggests an independent and common origin of the D9N. Taken together these data would suggest that the -93t allele and the D9N cSNP each occurred independently on distinct chromosomal backgrounds. As the –93g allele is present on many haplotypes, it seems more likely that this is the ancestral allele, on which the other two variants arose.

Table 5.8. Lipid levels in Black South African carriers and non carriers of the -93g allele and the D9N

	gg	gt	tt
DD			
n	20	36	21
Age (years)	36.5± 11.6	35.2± 9.3	36± 11.4
BMI (kg/m^2)	21.48± 4.00	20.09± 2.12	21.22± 2.66
TC (mmol/L)	3.00± 0.66	2.82± 0.71	2.97± 0.77
HDL-C (mmol/L)	1.28± 0.36	1.22± 0.29	1.22± 0.57
TG (mmol/L)	0.82± 0.30*	1.00± 0.54[a]	1.14± 0.66
LDL-C (mmol/L)	1.33± 0.68	1.19± 0.50	1.22± 0.66
TC/HDL	2.49± 0.90	2.38± 0.60	2.68± 0.97
DN			
n	8	7	
Age (years)	33.1± 12.8	39.4± 12.6	
BMI (kg/m^2)	21.04± 3.12	22.28± 3.49	
TC (mmol/L)	3.24± 0.60	3.19± 0.88	
HDL-C (mmol/L)	1.17± 0.38	1.28± 0.34	
TG (mmol/L)	0.97± 0.33	1.05± 0.41	
LDL-C (mmol/L)	1.64± 0.41	1.42± 0.73	
TC/HDL	2.94± 0.73	2.49± 0.38	

[a]p=0.04 gg/DD vs. tt/DD
Values are presented as mean±SD. No significant differences were observed for any other comparisons.

5.4.2.3 Phenotypic effects of the -93g allele

In Caucasians, as the D9N cSNP is in linkage disequilibrium with the -93g allele, the effects of the D9N variant and -93g allele cannot be distinguished. Furthermore it is unclear

whether either one or both of these changes may contribute to the phenotypic effects previously described in Caucasian D9N carriers[141,280]. The presence of the -93g allele at a high frequency and independent of the N9 allele in the South African Black population represents an ideal opportunity to study the phenotypic effects of this DNA substitution.

Lipid profiles were assessed in 92 Black rural South African males originating from Venda (Table 5.8). Individuals with the gg/DD genotype (n=20) showed significantly lower TG levels when compared to subjects (n=21) carrying the tt/DD genotype (0.82 ± 0.30 mmol/L vs. 1.14 ± 0.66; p=0.04). No significant differences were found for total, HDL- or LDL-cholesterol levels (Table 5.8).

Carriers of the gg/DN (n=8) had a trend to higher TG levels compared to persons without the D9N cSNP (gg/DD) but this did not reach significance, likely due to the variability of the results and the small sample size of the 9N carriers (n=8, Table 5.8).

5.4.3 Discussion

The variant at nucleotide -93 in the *LPL* promoter was originally described in a Caucasian population as a t to g substitution, with a carrier frequency of 1.6%[367]. In this study we sought to determine its frequency in populations of different ethnic origins.

The frequency of this SNP varied widely between different ethnic groups. The carrier frequency of the -93g allele was 1.7% in the Dutch Caucasian population studied, similar to prior reports. In contrast, in the South African Black population, the carrier frequency was 76.4%. The –93g allele was not found in 130 Chinese individuals screened. Of further interest, was the finding of near complete linkage disequilibrium between the –93g allele and the D9N cSNP in Caucasians (D=0.0085[375], p= 8 x 10^{-9}). In contrast, within the Black population, most carriers of the g allele at –93 did not carry the D9N cSNP.

To further study the origin of the -93g allele and its association with the D9N variant, analysis of a highly polymorphic CA repeat 5' to the *LPL* gene was performed. Interestingly, the -93g allele was seen across many different CA alleles in the South African Blacks (Figure 5.4 (B)). In contrast, the -93t allele was seen predominantly in association with CA alleles of 16 repeats in size, in all three populations (Fig 5.4 (A, C, E)). This raises the question as to whether the -93g or -93t allele is the original ancestral allele. The data presented here suggest that the -93t allele, reported as the wild-type allele in Caucasians[367], may be derived from the -93g allele,

which is most frequent in Blacks and spread across numerous repeat alleles, and that the -93g allele might in fact be the ancestral allele. In further support of this hypothesis is the fact that the -93g and not the -93t allele is conserved among other species including mouse[99,377], chicken[378] and cat (K.A. Excoffon, unpublished observation), again suggesting the -93g allele is the more ancient allele. Thus the data suggest that a mutation arose early at position -93, resulting in a g→t substitution on a single or few chromosomes carrying the CA allele with the size of 16 repeats. The European and Asian populations derived later from Africa[379] and due to a possible founder effect would therefore be expected to carry predominantly the CA allele of 16 repeats in size and the -93t allele, which is evident.

The fact that the D9N variant is seen associated with CA alleles with 23 to 25 repeats in size suggests it arose on a specific -93g allele carrying larger CA repeat sizes (Table 5.7, Figure 5.4 (D, F)). Furthermore, the absence of the D9N cSNP in the Chinese population suggests this mutation occurred after branching off of the Asian population approximately 50 000 years ago[379]. This could then explain the linkage disequilibrium between the –93g allele and the D9N cSNP in the Caucasian population, and the apparent absence of both from the Chinese population. However, due to the relatively small number of Chinese individuals screened for the –93g allele and the D9N cSNP these DNA changes may still be present at reduced frequencies.

To further explore the frequency and possible genetic relationships between both the promoter SNPs and cSNPs, all subjects were also screened for the SNPs at -39 and -53 in the promoter[367], and the N291S cSNP[144] (data not shown). In this study, the nucleotide substitution at -39 was not detected in either Caucasian (control n=232), Black South African (n=161) or Chinese (n=130) individuals. The SNP at -53 was found in 2/232 controls of Dutch origin but not in either the Black (n=161) or Chinese populations (n=130). Thus, in contrast to the –93 substitution, the –39 and -53 promoter SNPs appear to be rare in all populations. The N291S variant was present in only the Caucasian population, with a carrier frequency of 4.3% (10/232). None of these SNPs appeared to be in linkage disequilibrium.

It has previously been suggested that the -93g allele reflects a functional variant[368,380]. However, the reports concerning the transcriptional activity *in vitro* for the –93g allele are conflicting[367,368,380]. This allele has been shown to have a reduced (40-50% of wildtype) transcriptional activity *in vitro* using the human monocytic leukemic cell line THP-1 and the mouse myoblast cell line C2C12[367,368] while this variant was associated with an approximate

24% increase in promoter activity using a smooth muscle cell line[380]. On the other hand, the D9N cSNP has been reported to have decreased catalytic activity *in vitro*[281] (and Section 5.2), and to be associated with hypertriglyceridemia *in vivo*[280].

The relative *in vivo* contribution of each of these mutations to the phenotype of hyperlipidemia cannot easily be distinguished in studies of Caucasian patients since there is almost complete linkage disequilibrium between the -93g allele and the D9N cSNP in Caucasians. However, the presence of the -93g allele alone on more than 53% of alleles in South African Blacks afforded us an ideal population in which to study the independent phenotypic effects of the -93g allele.

Within the South African Black population we have shown that the -93g allele is associated with lower TG levels compared to the -93t allele. These results support what might be expected if the –93g allele increased transcriptional activity *in vivo*[380]. An interesting question is thus raised in carriers of both the –93g allele and the D9N cSNP as to the relative effects of both DNA changes. From the data obtained in the Black population, the –93g allele would be expected to lead to decreased TG, while the D9N variant has been shown to have decreased activity *in vitro* and *in vivo*, and would thus be expected to result in increased TG (Section 5.2 and references[280,281]). We have previously reported the phenotypic effects of the D9N cSNP in carriers who are also carriers of the –93g allele[141]. In this situation these SNPs are associated with elevated TG *in vivo*, suggesting the D9N cSNP, which results in decreased catalytic activity is dominant in its effect over the –93g allele. Similar trends were observed in the small number of Black individuals carrying the 9N allele compared to individuals without the 9N, matched for alleles at position –93.

Some caveats however should be noted. The TG and cholesterol levels in all Black individuals were generally low, compared with typical Caucasian levels. This may be due in part to the consumption of a rural (low-fat) diet by these individuals in contrast to the typical diet of Western populations, and may relate to a significantly lower incidence of CAD reported in South African Black versus Caucasian populations[381]. Similar low cholesterol and TG concentrations have been observed in rural Chinese populations and are suggested to be in part dietary related, as increased urbanization results in significantly elevated cholesterol and TG levels[382]. However, when comparing lipid profiles of Black and Caucasian South Africans on the same Western diet for two years, Black South Africans were still found to have significantly lower cholesterol and

TG concentrations[383]. This suggests that genetic differences may contribute to these findings. The increased frequency of the –93g allele in the Black population in the absence of the D9N cSNP could contribute to the findings of lower TG levels in Blacks compared to Caucasians.

The –93g allele has been shown to be associated with increased transcriptional activity[380]. Increased production of catalytically normal LPL would be predicted to lower TG levels, as was observed in gg vs. tt individuals. However, in the company of the D9N cSNP on the same allele, increased production of a catalytically defective protein might now be expected to be associated with higher TG levels as is seen in studies of Caucasians with both these DNA changes[280], and similar to trends observed in the Black individuals described here[141].

5.5 The *LPL* S447X variant is associated with decreased plasma triglyceride levels and risk of CAD, and with decreased systolic and diastolic blood pressure

Familial hypercholesterolemia (FH) is a disorder caused by mutations in the LDLr gene[45]. Heterozygotes occur with a frequency of approximately 1/500 in Caucasian populations[45,140]. Individuals with impaired LDLr activity have a marked accumulation of LDL in the plasma, associated with a dramatically elevated risk of coronary disease[45]. Heterozygotes manifest with an approximate 2-fold elevation in LDL-C from birth, and typically have premature CAD (often in their 30's-40's)[36,45], with an increased CAD mortality being evident even in their 20's[140]. Individuals homozygous for LDLr mutations (1/1 000 000 in Caucasian populations[140]) have 6-10 fold elevations in LDL-C, often have coronary events beginning in childhood[36], and succumb to CAD by their 20's[45]. Heart attacks have been noted in children as young as 4 years of age[140]. Although it is usually associated with premature coronary disease, the phenotypic expression of heterozygosity for LDLr mutations in FH can be variable[69,384]. It has thus been suggested that interactions with other gene products, such as LPL, may influence the phenotypic expression of FH[69,265,385].

SNP studies examining the risk of CAD events are limited by the frequency of events within the population studied (typically low). Having a small percentage of the total subjects with events makes it difficult to detect small differences in risk when this group is subdivided by genotypes. We have previously shown that the effects of variants having small effects on risk of CAD events may be more easily observed on a background of increased CAD events, such as a

population with FH[267]. Specifically, we have shown that both the D9N and N291S cSNPs are associated with a significantly increased risk of CAD in FH patients[266,267].

The S447X variant, in contrast to the N291S and D9N variants, has been associated with decreased TG[271,273,277,334,337,338], increased HDL-C[337,339], and a significantly decreased risk of CAD[273,333,334,339]. However, others have failed to find such associations[275,320,333,334,341]. Furthermore, the suggested protective effects of the S447X cSNP on risk for CAD may occur, at least in part, independent of its anti-atherogenic lipid changes[270,334]. Thus the effects of this variant, particularly on CAD, are unclear. We have examined the effects of the S447X on lipids and CAD in a population of FH heterozygotes.

Linkage of a quantitative trait locus for systolic blood pressure to genetic markers within the *LPL* gene has previously been identified[386]. Individuals heterozygous for *LPL* mutations have been shown to have increased systolic blood pressure[387]. Furthermore, a recent study has suggested that the LPL protein may have direct effects on the vessel wall, perhaps influencing the production or release of nitric oxide and thus vascular tone[388]. The phenotype of the S447X variant is opposite to that observed in *LPL* heterozygotes. Thus, we hypothesized that one mechanism whereby the S447X variant may have beneficial effects is by lowering blood pressure. To assess other potential beneficial effects of this cSNP in addition to its effects on plasma lipid levels and risk of CAD, we therefore also examined blood pressure in this cohort.

5.5.1 Methods

5.5.1.1 Subjects

We identified a total cohort of 650 heterozygous FH patients from the St. Paul's Hospital lipid clinic in Vancouver. All individuals with either kidney or liver disease, who were pregnant, who were homozygous for the apoE2 allele, who were diabetic, or who had impaired glucose tolerance were excluded. All N291S and D9N carriers were also excluded, as were individuals of French Canadian ancestry who carried either the G188E or the P207L mutations which are common to that population and are associated with significantly decreased LPL activity[102,140]. This left a primary cohort of 534 individuals.

For analysis of lipid levels, we excluded those individuals on medications known to affect lipids (lipid lowering medications, diuretics, β-blockers, hormone replacement therapy, steroids, testosterone or anti-epileptics) for whom pre-treatment lipid values were not available,

and those who consumed excess alcohol (> 2 drinks/day). All individuals were adult (≥18 years). This left a cohort of 407 individuals.

As individuals who had vascular disease were often those who were currently taking lipid-lowering medication, the prevalence of vascular disease and its mean age of onset was assessed in the baseline cohort of 534 individuals.

The prevalence of hypertension was also assessed in the basic cohort of 534 subjects. For blood pressure analysis, only individuals from the cohort of 534 not on blood pressure lowering medications or for whom pre-treatment blood pressure measurements were available were included (n=461). Mean blood pressures were also examined in age-defined subgroups of this main group.

5.5.1.2 Genotyping

Genotyping of the S447X variant was performed by PCR and restriction fragment length analysis as previously described[337]. This is an alternate method to the one described earlier in this chapter. Briefly, exon 9 of the *LPL* gene was amplified with the following 2 primers: 5'TACACTAGCAATGTCTAGGTGA and 5'TCAGCTTTAGCCCAGAATGC. The resulting PCR product was digested with 10 U *Mnl* I. The 488 bp PCR product is digested to produce fragments of approximately 203 and 285 bp on the wildtype allele. The S447X variant introduces an additional restriction site, which splits the 285 bp fragment to 247 bp and 38 bp. Thus, heterozygotes for the S447X have three visible bands (247, 203, and 38 bp), while individuals homozygous for the S447X display 2 bands (285, 203 bp).

5.5.1.3 Patient assessment

All individuals were assessed by physicians at the St. Paul's Hospital lipid clinic. Plasma lipid levels were measured at the lipid laboratory of St. Paul's hospital. Blood pressures were measured from individuals at rest. For those already on medication when first seen at the lipid clinic, pre-treatment blood pressures were obtained from family physicians.

Vascular disease was classified as either coronary heart disease (CHD), peripheral vascular disease (PVD) or cerebrovascular accident (CVA). CHD was defined as those who had had an MI, coronary artery bypass graft surgery (CABG), percutaneous transluminal coronary angioplasty (PTCA), angina treated with medication, or angiographic evidence of CHD. PVD

was classified as those who had claudication and surgery on carotid or abdominal arteries due to atherosclerosis. This did not include individuals with bruits only, aneurysms, or evidence from ultrasound only. The diagnosis of CVA included individuals who had had a stroke or transient ischemic attack (TIA). The diagnosis of hypertension was made either by physicians at the lipid clinic or by their referring family physicians.

5.5.1.4 Statistical Analysis

Differences in frequency were compared using a Chi-square analysis (demographics) or Fisher's exact test (vascular disease), where appropriate. Differences in mean lipid levels and blood pressure were assessed by a two-tailed Student's *t*-test, assuming independent variances. As TG are not normally distributed, statistics were performed on log-transformed values, although untransformed values are given in the table so that they may be interpreted. Pearson correlation coefficients are presented. The effects of TG on blood pressure were accounted for using an analysis of covariance (ANCOVA). All values are reported as mean $\pm$ standard deviation.

5.5.2 Results

We identified an initial cohort of 650 individuals heterozygous for FH. All individuals with liver or kidney disease, who were homozygous for apoE2 allele, who were diabetic or had impaired glucose tolerance, plus those with other *LPL* variants were excluded, which left a cohort of 534 individuals for analysis. We then examined differences in lipid levels, CAD and blood pressure between those who were carriers of S447X compared to the non-carriers.

Table 5.9. Baseline demographics of S447X carriers and non-carriers

	S447X carriers	Non-carriers	P value
n	91	316	
Age (years)	41.5±14	43±13.4	0.39
BMI (kg/m^2)	24.39±3.64	24.69±3.82	0.52
M/F	45/46	141/175	0.47
Glucose (mmol/L)	5.12±0.41	5.17±0.42	0.42

We examined mean plasma lipid levels in those not taking medications known to alter lipid levels (n=407). There were no significant differences in age, BMI plasma glucose levels, or the relative proportions of males and females in carrier vs. non-carrier groups (Table 5.9).

Mean plasma lipid levels are shown in Table 5.10. Carriers of the S447X had significantly decreased TG compared to non-carriers (1.21±0.47 vs. 1.52±0.67, p<0.001). There were no significant differences in total, LDL or HDL cholesterol levels. Similar results were obtained if males and females are examined separately (data not shown).

Table 5.10. Lipid values in S447X carriers and non-carriers

	S447X carriers	Non-carriers	P value
TG (mmol/L)	1.21±0.47	1.52±0.67	**<0.001**
TC (mmol/L)	8.89±1.89	8.86±1.62	0.90
HDL-C (mmol/L)	1.26±0.32	1.26±0.34	0.90
LDL-C (mmol/L)	7.06±1.86	6.84±1.56	0.30
TC/HDL	7.49±2.77	7.5±2.64	0.97

As individuals who had documented vascular disease were more likely to have been placed on lipid-lowering medication, we examined the prevalence of vascular disease in the cohort of 534 individuals. The prevalence of vascular disease was reduced in S447X carriers compared to non-carriers (12.7% vs. 19.5%, p=0.10, Table 5.11). However, likely due to the still small number of vascular disease cases this did not quite reach significance. If we examine specific forms of vascular disease (CHD, PVD or CVA), in each subgroup the prevalence of disease was lower in carriers compared to non-carriers. None of these comparisons reached significance (Table 5.11). There was no significant difference in mean age of onset of any form of vascular disease between the carriers and non-carriers (Table 5.11).

We also examined the subgroup of individuals who were adults (≥18 years of age), as they would be more liekly to have developed vascular disease. Although the overall frequencies of disease were higher, the relative difference between carriers and non-carriers remained similar. The prevalence of all vascular disease was 14.7% in the carriers (total n=102) compared to 21.3% in the non-carriers (total n=380, p=0.16). The corresponding frequencies for CVD,

PVD and CVA were: 14.7 vs. 18.4%, p=0.46; 1.0 vs. 4.2%, p=0.14; and 0% vs. 2.6%, p=0.13, respectively.

Table 5.11. Vascular disease in S447X carriers and non-carriers

	S447X carriers (n=118)	Non-carriers (n=416)	P-value
Prevalence of vascular disease			
Vascular disease (all)	15 (12.7%)	81 (19.5%)	0.10
CHD	15 (12.7%)	70 (16.8%)	0.32
PVD	1 (0.8%)	16 (3.8%)	0.14
CVA	0 (0%)	10 (2.4%)	0.13
Age of onset			
Vascular disease (all)	46.7±12.7	49.5±11.9	0.45
CHD	47.3±11.8	48.3±11.8	0.77
PVD	57	58.2±10.3	NA[a]
CVA	-	53.1±12.1	NA

[a]NA= not assessed

As we have previously shown that the effects of the S447X may be independent of lipids, and as changes in blood pressure have been linked to the *LPL* locus, we examined the prevalence of hypertension within our cohort of 534 individuals. A much lower frequency of hypertension was observed in the S447X carriers (8.5% (10/118)) compared to the non-carriers (17.3% (72/416), p=0.02, Figure 5.5).

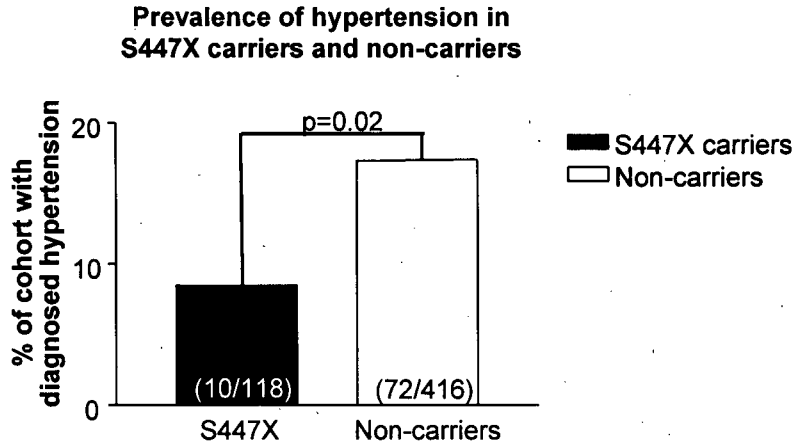

Figure 5.5. Frequency of hypertension in S447X carriers and non-carriers.
The percentage of S447X carriers (black) and non-carriers (white) with diagnosed hypertension is plotted. S447X carriers have approximately half the frequency of hypertension compared to non-carriers. The number of individuals with hypertension out of the total number in the cohort is shown in parentheses.

Table 5.12. Mean systolic and diastolic blood pressures in S447X carriers and non-carriers

	S447X Carriers	Non-carriers	P value
n	101	360	
SBP (mm Hg)	125±18	130±19	**0.01**
DBP (mm Hg)	78±10	82±11	**<0.001**

To further examine the relationship between this variant and blood pressure, we identified the subgroup of individuals who were not currently taking blood pressure lowering medication, or for whom pre-medication blood pressure measurements were available (n=461). Both mean untreated systolic (SBP) and diastolic (DBP) blood pressures were decreased in S447X carriers compared to non-carriers (125±18 (n=101) vs. 130±19 mm Hg (n=360), p=0.01; 78±10 vs. 82±11 mm Hg, p<0.001, respectively, Table 5.12). Similar results were also observed when males (p=0.06 SBP and p=0.05 DBP) and females (p=0.07 SBP and p=0.001 DBP) were analyzed separately (Table 5.13).

Table 5.13. Mean systolic and diastolic blood pressures in men and women

	S447X Carriers	Non-carriers	P value
Males			
n	44	164	
SBP (mm Hg)	126±14	131±18	0.06
DBP (mm Hg)	80±9	84±11	0.05
Females			
n	57	196	
SBP (mm Hg)	124±20	130±20	0.07
DBP (mm Hg)	76±11	81±11	0.001

To address whether these changes were evident in younger individuals who may have fewer factors such as increased salt intake, smoking or excess alcohol consumption influencing blood pressure, we assessed blood pressures in age-defined groups. When the cohort was split

by age, similar results for mean systolic and diastolic blood pressures in youths and adults were observed (Table 5.14). In those <20 years systolic (p=0.04) and diastolic (p=0.004) blood pressures were reduced by approximately 10 mm Hg. This difference was even larger than what was observed in those ≥20 years (p=0.05, p<0.001 for SBP and DBP respectively).

Table 5.14. Mean systolic and diastolic blood pressures in youths and adults

	S447X Carriers	Non-carriers	P value
Age <20 years			
n	10	31	
SBP (mm Hg)	102±17	116±21	0.04
DBP (mm Hg)	64±8	74±8	0.004
Age ≥ 20 years			
n	91	336	
SBP (mm Hg)	128±16	131±18	0.05
DBP (mm Hg)	79±9	83±11	0.001

Plasma lipid levels, especially TG, have been shown to influence endothelial function and vascular tone, and thus may directly influence blood pressure[389-393]. Here we have shown that the S447X variant was associated with lower plasma TG. Thus, we sought to examine whether the differences in blood pressure observed for this variant are due solely to the differences in plasma TG between the two groups. First, we examined whether TG levels were related to blood pressure in this cohort. Indeed, TG were significantly correlated with both SBP (r=0.26, p<0.0001) and DBP (r=0.29, p<0.0001). Similar correlations were observed in carriers and non-carriers separately. Thus, we re-examined the differences in blood pressure between the groups including TG as a covariate. The differences in blood pressure between carriers and non-carriers were still significant when the effects of TG were accounted for (p=0.02 for DBP, although p=0.26 for SBP). This suggests, that the effects of the *LPL* S447X on blood pressure may be at least in part independent of its effects on plasma lipid levels.

5.5.3 Discussion

Here we have shown that in a cohort at an increased risk of CAD, the S447X variant was associated with reduced plasma TG levels. We were unable, however, to detect any differences in HDL-C between carriers and non-carriers in this cohort. We also observed trends to less CAD in carriers of the S447X compared to non-carriers. Despite the increased prevalence of CAD, it is possible there is still not enough power to detect more minor effects. Interestingly, we also observed decreased blood pressures in carriers of the S447X compared to non-carriers, at least partly independent of plasma TG levels.

Data from the first section of this chapter suggests that the S447X variant is associated with an increased secretion of (inactive) LPL monomer, with no change in catalytic activity. It is uncertain at this stage exactly what effect this would have. Monomeric LPL has been shown to be carried on lipoprotein (TGRL/remnant) particles[132], so it is possible the lipoproteins in S447X individuals contain excess monomeric LPL. If the LPL monomer is capable of binding to proteoglycans, this may facilitate uptake of TGRL and their remnants in the liver, providing an additional mechanism whereby plasma TG are decreased. These findings may be more important when LDLr activity is compromised. As lipolysis may not be significantly increased in carriers of this variant (Section 5.2), this may not generate sufficiently increased surface remnants to detect differences in HDL-C levels, similar to what we have suggested in normal mice (Chapter 2). It is also possible that the beneficial effects of this variant in comparison with wildtype may be brought out more in stressed states. Carriers of the S447X have a decreased postprandial response compared to non-carriers[273], suggesting that the beneficial effects of this variant may be more evident when the system is challenged.

This was the first description of reduced blood pressure levels in carriers of the S447X, findings which have very recently been confirmed in women[394]. Triglycerides are known to influence endothelial function through a number of different mechanisms[393]. Endothelial dysfunction has been documented in individuals with increased levels of remnant lipoproteins[391]. Individuals with hypertriglyceridemia have also been shown to have higher levels of circulating adhesion molecules (sVCAM-1 and sICAM-1), markers of endothelial dysfunction[390]. Endothelium-dependent vasodilation in response to acetylcholine or shear-stress has been shown to be impaired in hypertriglyceridemic individuals[389,390]. In rats, hypertriglyceridemia has been associated with endothelial dysfunction due to an increased superoxide production and thus

decreased availability of nitric oxide (endothelial-derived relaxing factor)[392]. Thus, one mechanism whereby alterations in LPL may influence blood pressure is indirectly through modulation of plasma TG levels.

Plasma TG were significantly correlated with blood pressure in our cohort, and had significant effects on mean blood pressures. However, the effects of the S447X were still evident, even after correction for TG levels, which suggests that there may be a direct effect of the S447X on the vessel wall.

We have previously shown that the severity of angina is decreased in individuals with increased LPL activity[388]. This was related to both LPL activity and protein levels, and it was suggested that the LPL protein, perhaps independent of its catalytic activity, may be influencing vascular tone, perhaps by altering factors such as the release of nitric oxide. The finding of decreased blood pressure in carriers of the S447X further strengthens this idea, particularly as these were in part independent of its effects on TG. As LPL is anchored to the vascular endothelium through HSPG, it is possible that this may somehow alter cell signaling, or endothelial cell biology. Clearly more studies are needed to examine these mechanisms.

Although unlikely, it remains possible that the S447X variant is in linkage disequilibrium with another variant responsible for the changes in blood pressure. *In vitro* analysis, or analysis in a transgenic system, will be required to directly examine whether the alterations in blood pressure are caused by expression of the S447X variant, or whether it is perhaps a marker of an alteration in a nearby gene to which blood pressure is really linked.

Hypertension is associated with an increased risk of CAD[65,67]. Increased blood pressure is significantly correlated with atherosclerosis[10]. Each 20 mm Hg increase in systolic blood pressure has been associated with a greater than two-fold increased severity of atherosclerosis. These findings were nearly double those of a 10 mg/dL increase in plasma cholesterol and greater than double the effect of 5 pack-years smoking[395]. A 5 mm Hg decrease in blood pressure for a period of 5 years has been suggested to result in a 34% decrease in the incidence of stroke[396]. The 5-10 mm Hg increased in S447X carriers described here might therefore be expected to significantly impact the risk of CAD, particularly when extrapolated throughout an individual's lifetime. These data thus suggest an additional mechanism whereby the S447X variant may reduce vascular disease risk, independent of plasma lipid levels.

5.6 Discussion

In summary, we have examined the catalytic activity and protein secretion of three common *LPL* cSNPs (the D9N, N291S and S447X) *in vitro*, which have been shown to be associated with altered LPL levels or plasma lipid and lipoprotein concentrations *in vivo*. The N291S variant manifested with a significant reduction in catalytic activity and specific activity. The D9N species gave a marginal but significant reduction in activity that was paralleled by a decreased secretion of the enzyme, resulting in no change in specific activity. The overall catalytic activity of the S447X truncated variant was shown to be normal, but there was an increase in the secretion of monomeric LPL. These initial studies provided the first standardized *in vitro* validation of the functional effects of these SNPs, and have provided clues as to how the phenotypic effects associated with them might arise.

Following initial reports of the N291S cSNP, questions were raised as to its functional effects. It had previously been shown that the effects of LPL defects may become exaggerated during an environmental challenge. Therefore we examined the function of the N291S in response to an oral fat load. This study suggested that normolipidemic carriers of the N291S cSNP have an abnormal postprandial response to an oral fat load, and provided further *in vivo* evidence as to the functional nature of this cSNP in the *LPL* gene. Normolipidemic N291S carriers had a significantly greater chylomicron postprandial response to a fat load test compared with normolipidemic controls. This would suggest that other environmental factors such as diet influence the expression of this cSNP and may unmask a significant partial lipolytic defect, which may not be evident in the fasting state.

Although this was a small preliminary study, it was the first to demonstrate such a functional defect in carriers of this variant. Subsequent studies have confirmed the findings of an increased post-prandial response in carriers of the N291S[278,318]. Additionally, in studies examining the effects of the other two variants, no difference in postprandial response has been demonstrated for carriers of the D9N[278], while carriers of the S447X have been shown to have a decreased postprandial response[273].

The studies on the –93 promoter SNP were the first to show that the –93g allele, which occurs with high frequency in the South African Black population, is in linkage disequilibrium with the D9N cSNP in Caucasian populations and is associated with mildly lower TG, independent of the D9N variant. Furthermore, these studies have shown that the D9N cSNP is

capable of raising TG, even in the presence of this beneficial variant. These findings are consistent with the decreased LPL secretion shown for this variant.

Subsequent studies have confirmed our findings that the N291S and D9N are not seen at a high frequency in Chinese or Japanese populations[397,398]. Furthermore, several groups have now shown that the D9N and –93 are in linkage disequilibrium in Caucasian populations[272,380] and are seen at a higher frequency but not in linkage disequilibrium in Blacks[320,399]. The –93g allele has been shown to be associated with lower TG[380,399] and reduced post-prandial lipemia[399], independent of the D9N, and the D9N to be associated with increased TG on this background[380].

The studies on the S447X variant have provided additional evidence of its effects on plasma TG. The data also suggests that carriers of this variant have a reduced atherosclerotic risk. The observation of reduced blood pressure in carriers of the S447X is a novel finding. Previous studies have suggested that blood pressure is linked to the *LPL* locus in some families[386,387], but there has been little direct evidence to suggest that LPL itself may regulate blood pressure. What proportion of this effect occurs indirectly, through LPL-induced alterations in plasma TG levels[400], and how much occurs through direct effects of the LPL protein at the vascular endothelium, will require further study. The data suggest that both mechanisms are likely to play a role.

Recently, several cumulative or meta-analyses have been reported for these variants[146,342,376,401]. Each of these has examined different parameters. All have come to essentially similar conclusions. The most recent and comprehensive meta-analysis has provided information on both lipid levels and CAD in carriers of these three cSNPs. The D9N was associated with significantly increased TG and decreased HDL-C, as was the N291S[146]. The S447X variant, in contrast, was associated with decreased TG and increased HDL-C. Both the D9N and N291S had increased odds ratios for CAD (1.4, 1.2 respectively), although neither was statistically significant. The population attributable fraction for ischemic heart disease associated with the D9N variant has been estimated at 3%[322]. These meta-analyses have included several general population studies, and it is quite possible that in populations with other risk factors, such as increased BMI or diabetes, that the effects of these variants will become increased. The S447X, on the other hand, was associated with a significantly reduced risk of CAD (OR=0.8, p=0.02). It has been estimated that approximately 9% of CAD in the general population is prevented by this variant[334]. Taken together, these data suggest that the three common *LPL*

coding variants have functional effects on plasma lipid levels and contribute significantly to the population risk of CAD.

Chapter 6: Identification of the *ABCA1* gene as the underlying cause of Tangier Disease and some forms of Familial Hypoalphalipoproteinemia

The data presented in this chapter has been published in two manuscripts:

Brooks-Wilson A.*, Marcil M.*, **Clee S. M.**, Zhang L.-H., Roomp K., van Dam M., Yu L., Brewer C., Collins J. A., Molhuizen H.O.F., Loubser O., Ouelette B.F.F., Fichter K., Ashbourne-Excoffon K.J.D., Sensen C.W., Scherer S., Mott S., Denis M., Martindale D., Frohlich J., Morgan K., Koop B., Pimstone S., Kastelein J.J.P., Genest J. Jr., and Hayden M.R. Mutations in the ATP binding cassette (ABC1) transporter gene in Tangier disease and Familial HDL Deficiency (FHA). *Nat. Genet.* 1999 **22**:336-345.

Marcil M.*, Brooks-Wilson A.*, **Clee S. M.***, Roomp K., Zhang L.-H., Brewer C., Collins J. A., Loubser O., Ouelette B.F.F., Fichter K., Yu L., Mott S., Denis M., Martindale D., Koop B., Pimstone S., Kastelein J.J.P., Genest J. Jr., and Hayden M.R. Mutations in the ABC1 gene in familial HDL deficiency with defective cholesterol efflux. *Lancet* 1999 **354**(9187):1341-6.

*equal contribution

This work has also been published in abstract form

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Preface

The data from this chapter serves as an introduction to the next two chapters on the *ABCA1* gene. The cloning of a disease gene requires a large group effort. My contributions to this project were as follows: I made several intellectual contributions to the cloning throughout its stages; I tested all of the initial exonic primer pairs to ensure that they amplified bands of the expected size, aiding in the deduction of the human *ABCA1* genomic structure; and once potential mutations were identified, I designed and implemented all assays to confirm the variants noted by sequencing were mutations not polymorphisms. This involved the validation of the sequence change by RFLP, confirmation of its co-segregation with affected status in the family, and its absence from control individuals. The data from all figures presented in this chapter was generated by me, except for: Figure 6.1 which is included for illustrative purposes and the Northern blot of TD1, included as additional evidence of the functionality of the splice mutation.

6.1 Introduction

Epidemiological studies have consistently demonstrated that plasma HDL-C concentration is inversely related to the incidence of CAD[71,78,94,402,403]. Approximately 4% of such patients have low HDL-C as an isolated finding, while another approximately 25% have low HDL-C associated with other lipoprotein abnormalities[23]. Decreased HDL-C levels are the most common lipoprotein abnormality seen in patients with premature CAD[404]. Genetic factors play a key role in regulating HDL levels[55,144,405,406]. Changes in the genes for the apo AI-CIII gene cluster[78], LPL[144], CETP[405], HL[406], and LCAT[55] all significantly contribute to the determination of HDL-C levels in humans[173].

Tangier disease (TD, OMIM 205400) is a rare form of genetic HDL deficiency, not due to genes known to be involved in HDL metabolism. TD was the first genetic HDL deficiency to be described, reported in two siblings from Tangier Island, in Chesapeake Bay, Virginia[174]. It is an autosomal recessive disorder, diagnosed in approximately 60 patients worldwide, and associated with almost complete absence of HDL-C and apoAI. Since then, it has been clearly established that TD is not due to a defect in a plasma enzyme or structural protein of HDL, but rather due to a defect involving intracellular trafficking of cholesterol[179,407-411]. Defective removal of cellular cholesterol and phospholipids and a marked deficiency in HDL mediated efflux of intracellular cholesterol has been demonstrated in TD fibroblasts[179,408,409,411]. TD patients thus accumulate cholesterol esters, resulting in the clinical hallmarks of the disorder, including enlarged yellow/orange tonsils, hepatosplenomegaly, peripheral neuropathy, and deposits in the rectal mucosa[407]. Though TD is rare, defining its molecular basis could identify pathways relevant for cholesterol efflux in the general population.

A more common form of genetic HDL deficiency (familial hypoalphalipoproteinemia, FHA or familial HDL deficiency, FHD) has been described in patients with dominantly inherited low plasma HDL cholesterol, usually below the 5th percentile (OMIM 604091), but an absence of clinical manifestations of TD[178,179]. Recently, it has been demonstrated that some patients with FHA have reductions in cellular cholesterol efflux[179] resulting in cholesterol-depleted HDL particles that are rapidly catabolized[179,412-415]. These patients have no obvious environmental factors associated with this lipid phenotype, are not diabetic or hypertriglyceridemic, and do not have other known causes of severe HDL deficiency. In contrast to persons with TD, who have

HDL-C <0.2 mmol/L, patients with FHA usually have HDL-C levels between 0.4 – 0.9 mmol/L[179].

To understand the molecular mechanisms underlying these forms of HDL deficiency, we performed genetic analyses of two TD families and of four large French Canadian families with FHA. The genetic defect in TD was unknown, but had recently been localized to chromosome 9q31 by us and others[182]. We also showed linkage of FHA to the same region. The biology and biochemistry of TD suggested signaling molecules were good candidates. Activation of protein kinases has been shown to increase efflux in TD cells[187], and activation of protein kinase C has been shown to have an essential role in promoting cholesterol efflux[416]. Brefeldin inhibits intracellular vesicular trafficking and cholesterol efflux, essentially reproducing the effect TD[411]. These findings suggested that the defect in TD should lie upstream of these signaling cascades.

Candidate genes in this region included the LPA receptor which stimulates phospholipase C, that is necessary for normal cholesterol efflux, (which is impaired in TD[417]) and a protein (RGS3) which regulates G protein signaling[418], also potentially involved in cholesterol efflux mechanisms (Figure 6.1). However, previous work by our group had excluded both of these genes. Additional genetic localization identified the ATP binding cassette transporter *ABC1* as a potential candidate in this region, as other ABC transporters had been shown to be involved in the transport of lipids[419-422]. The *ABC1* gene has since been renamed *ABCA1,* and so will be referred to as such throughout the text.

6.2 Methods

6.2.1 Patient selection

The probands in the TD families identified in lipid clinics in Vancouver and Amsterdam were diagnosed as suffering from TD based on clinical and biochemical data. The previously undescribed Dutch proband with TD (III:01 in TD-1), presented with an acute myocardial infarction at the age of 38. He had a marked deficiency of HDL-C (<0.1 mmol/L) and exhibited clinical features of TD, as previously described. DNA was also collected from multiple family members of another previously described proband with TD (TD-2)[423]. The presence of an HDL cholesterol of <5th percentile in 1st and 2nd degree relatives of both patients allowed us to identify potential heterozygotes in both kindreds and facilitated linkage analysis. Both TD probands had evidence of a marked deficiency of cholesterol efflux.

Study subjects with FHA were selected from the Cardiology Clinic of the Clinical Research Institute of Montréal and the lipid clinic in Amsterdam. Four FHA families of French Canadian descent and one of Dutch descent were ascertained. The main criterion was an HDL-C level <5th percentile and TG <95th percentile in the proband, and a first-degree relative with the same lipid abnormality. The patients did not have diabetes, or any other known cause of low HDL-C. In each family at least 1 member with low HDL-C showed a marked decrease in apoAI-mediated cholesterol efflux of 40-50% as compared to fibroblasts from normal controls. Low HDL-C is inherited as an autosomal dominant trait in all five families. A history of premature CAD was present in 2 of the families. The four French Canadian families were included in the linkage analysis, whereas the Dutch family (FHA5) was identified during the sequencing of *ABCA1* and was only included in mutation detection.

6.2.2 Biochemical studies

Blood was withdrawn in EDTA-containing tubes for plasma lipid and apolipoprotein analyses, and storage at –80°C. Leukocytes were isolated from the buffy coat for DNA extraction. Lipoprotein measurement was performed on fresh plasma by the respective hospital lipid laboratories in Amsterdam and Montreal[408]. For the measurement of lipoprotein lipids, cholesterol and TG levels were determined in total plasma, plasma at density d<1.006 g/mL obtained after preparative ultracentrifugation, before and after precipitation with dextran manganese. Apolipoprotein measurement was performed by nephelometry for apoAI and apoB.

6.2.3 DNA sequencing and analysis

Four bacterial artificial chromosomes (BACs) in total spanning 800 kb that tested positive by PCR for sequences near both the 5' and 3' ends of the *ABCA1* mRNA were selected for high throughput genomic sequencing at the Canadian Genetic Diseases Network core facility in Victoria. Briefly, a sublibrary was first constructed from each of the BAC DNAs. The BAC DNA was isolated and randomly sheared by nebulization. The sheared DNA was then size fractionated by agarose gel electrophoresis and fragments above 2 kb were collected, treated with Mung Bean nuclease followed by T4 DNA polymerase and klenow enzyme to ensure blunt-ends,

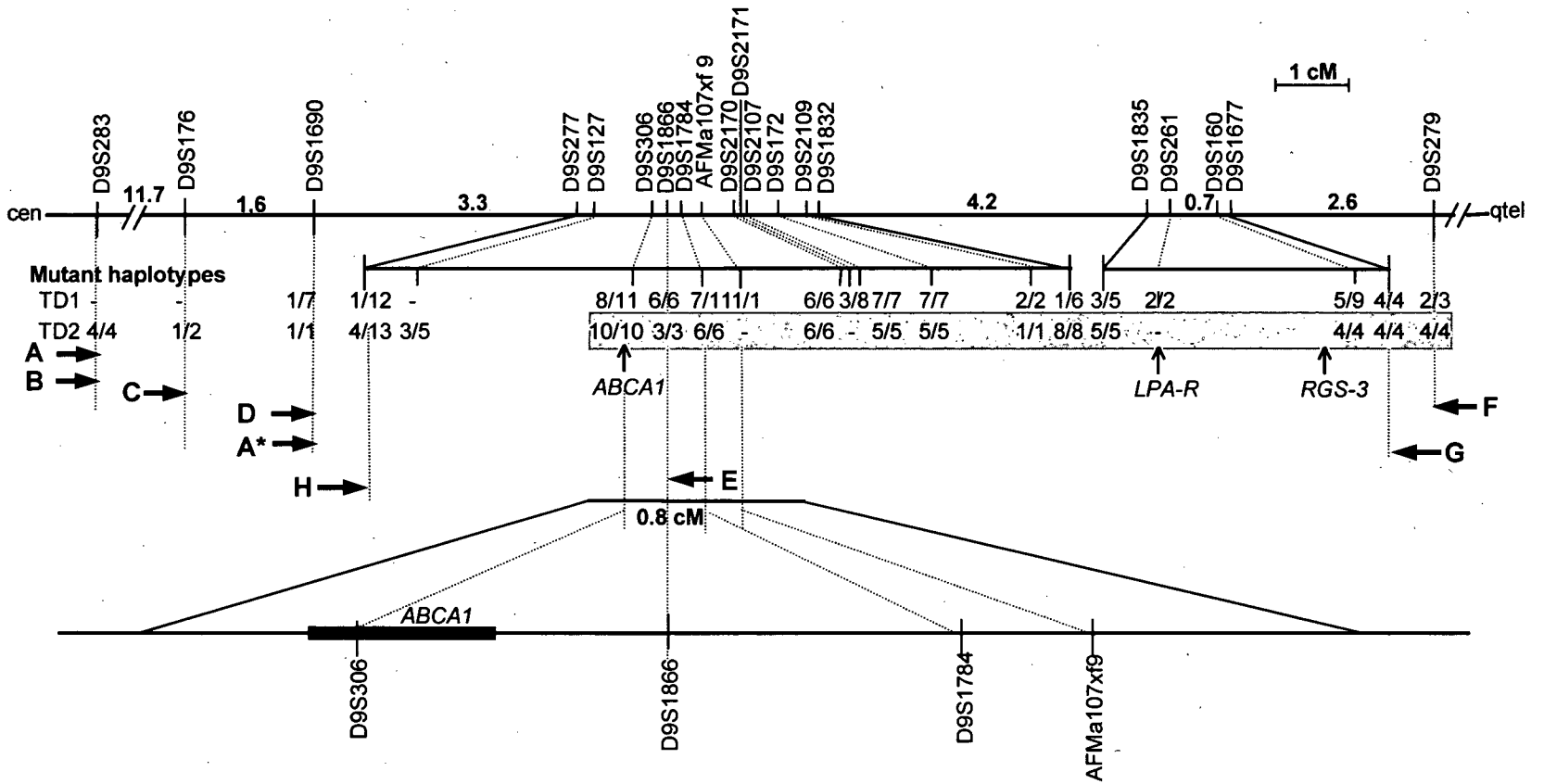

Figure 6.1. Genetic map of 9q31.
A total of 22 polymorphic CA microsatellite markers were mapped to the contig and used in haplotype analysis in TD-1 and TD-2 (distances between some pairs are given in cM). The mutant haplotypes for probands in TD-1 and –2 indicate a significant region of homozygosity in TD-2 (grey bar), while the proband in TD-1 has 2 different mutant haplotypes. The candidate region can be narrowed to the region of homozygosity for CA markers in proband TD2. A critical crossover at D9S1690 in TD-1 (A*) also provides a centromeric boundary for the region containing the putative gene. Meiotic recombinations in the FHA families (A-H) refine the minimal critical region to less than 1.5 cM between D9S277 and D9S1866. The heterozygosity at D9S127, which breaks a continuous region of homozygosity in TD-2, further refines the region to less than 1 cM. Combined genetic data located the gene to a ~200 kb region between D9S1866 and D9S306 within which *ABCA1* (black bar) mapped. The physical locations of three candidate genes in this region including the lysophosphatidic receptor (LPA-R)[417], RGS-3[418] and the *ABCA1* gene are shown.

and cloned into *Sma*I-cut M13mp19. Random clones were sequenced with an ABI373 or 377 sequencer and fluorescently labeled primers (ABI). DNAStar software was used for gel trace analysis and contig assembly.

The human (accession number AJ012376.1) and mouse *ABCA1* mRNA sequences (accession number X75926) were retrieved from GenBank, and multiple sequence alignments were performed using ClustalW Version 1.7 (via the BCM search launcher with the default parameter). Estimated splice site locations in the human sequence were deduced by comparison of human cDNA sequence with mouse genomic organization (accession number X75926). Exonic primers were designed for use in direct BAC DNA sequencing by the BigDye terminator method in cases where exon boundaries were not found by the shotgun method. Human *ABCA1* cDNA and genomic sequence contigs were compared by BLAST and aligned using Sequencher (Genecodes). Splice junctions were thus confirmed in the human genomic sequence. Forward and reverse PCR primers flanking each exon were then designed for use in mutation detection. Generally, primers were located within 50 to 70 bp of the splice junction.

Amplification of exons in genomic DNA was performed in 60 μL in 1.5 mM $MgCl_2$, 220 μM dNTPs and 0.5 μM of each primer. Samples were heated to 95 °C for 3 minutes and amplified with 35 cycles of 95°C, 10 seconds; 57°C, 30 seconds; 72°C, 30 seconds, with a final extension step of 10 minutes at 72°C. Ten microlitres of product was analyzed on a 2% agarose gel. Twenty microlitres of the remaining product was cleaned for sequencing using the polyethylene glycol 8000 protocol[424]. Cycle sequencing was performed on the ABI 373 using BigDye Terminators (ABI) with appropriate exon flanking primers. Sequences were assembled using Sequencher.

The *Caenorhabditis elegans ABCA1* orthologue was identified with BLAST (version 2.08) using the wild-type protein sequence as a query, with the default parameter except for doing an organism filter for *C. elegans*.

6.2.4 Reverse transcription (RT)-PCR amplification and sequence analysis

Total RNA was isolated from the cultured fibroblasts of TD and FHA patients, and reverse transcribed with an oligo $d(T)_{18}$ primer, and cDNA was amplified using primers derived from the published human *ABCA1* cDNA sequence[181]. Six sets of primer pairs were designed to amplify each cDNA sample, generating six DNA fragments which were sequentially overlapped

and spanned 1 to 6880 bp of the *ABCA1* cDNA, numbered according to the order of the published human cDNA sequence (GenBank accession number AJ012376)[181]. The fragments amplified encompassed nucleotides 1-1065, 946-2139, 2037-3270, 3189-4483, 4381-5677, and 5608-6880.

6.2.5 Northern blot analysis

Twenty micrograms of total fibroblast RNA samples were resolved by electrophoresis on a denaturing agarose (1.2% (w/v)) gel in the presence of 7% formaldehyde, and transferred to Hybond N nylon membranes (Amersham). The filters were probed with ^{32}P-labeled human *ABCA1* cDNA probes as indicated.

6.2.6 Genotyping of mutations

Subsequent to the initial publications of the cloning of this gene, it has been shown that the published human cDNA sequence[181] is missing the first exon and part of the second exon, including 60 amino acids of coding sequence. Thus, the numbering of all mutations has been revised from what was published to reflect that based on the full length transcript[184], with amino acids numbered 1-2261 and exons numbered 1-50.

Individuals were genotyped by restriction fragment length polymorphism (RFLP) assays, according to the methods specified in Table 6.1. PCR reactions were carried out in 100 µL volumes. Digestions were carried out for two hours, except for the A2185G (Q597R), which was digested for 3 hours, at the temperature specified by the manufacturer. For the splice site mutation, a mismatch strategy was employed, whereby a single nucleotide mismatch is incorporated into the PCR product (shown in bold in the primer) to generate a restriction site in combination with the wildtype allele, but not the variant allele.

6.3 Linkage analysis and establishment of a physical map

Initial linkage analysis in TD gave a maximal peak lod score of 6.49 at D9S1832 and 6.22 at D9S277 with linkage to all markers in an approximately 10 cM interval. We established a YAC contig spanning the 10 cM in this region of 9q31 (Figure 6.1). Recombination with the most proximal marker, D9S1690 was seen in II:09 in TD-1 (A* in Fig 6.1) providing a centromeric boundary for the disease gene. The proband in TD-2 was the offspring of a first-

Table 6.1. RFLP methods for *ABCA1* mutation detection

Mutation	Family	Exon	Primers: forward reverse	Enzyme	Units enzyme	% agarose gel for resolution	Fragments: wildtype mutant
T4824C	TD1	31	5'CTGCCAGGCAGGGGAGGAAGAGTG 5'GAAAGTGACTCACTTGTGGAGGA	*Hga*I	4	1.5	194 134,60
IVS25:G+1C	TD1	intron 25	5'ATGTCTCTGCTATCTCCAACCTCAT 5'GCTATATATTCCGACAGTCAGCCACTTAAC**G**TA	*Rsa*I	5	2	205,32 237
A2185G	TD2	14	5'AAAGGGGCTTGGTAAGGGTA 5'CATGCACATGCACACACATA	*Aci*I	8	2	215,145 185,145,30
Del L 693	FHA1	15	5'CTTTCTGCGGGTGATGAGCCGGTCAAT 5'CCTTAGCCCGTGTTGAGCTA	*Ear*I	4	2	151,59,48,39 210,48,39
C6825T	FHA2	49	5'GGGTTCCCAGGGTTCAGTAT 5'GATCAGGAATTCAAGCACCAA	*Rsa*I	5	1.5	332,104 436
Del E,D 1893,94	FHA3	42	5'CCTGTAAATGCAAAGCTATCTCCTCT 5'CGTCAACTCCTTGATTTCTAAGATGT	-	-	3	117 111
C3120T	FHA4	18	5'CTGCATGGAGGAGGAACCCACCCACTTGA 5'GGGATCAGCATGGTTTCCTA	*Dde*I	5	2	242,21 175,67,21
T3667C	FHA5	23	5'CCAGTGCTTACCCCTGCTAA 5'AACAGAGCAGGGAGATGGTG	*Nla*III	5	2	239,134,46,28 239,180,28

cousin marriage. We postulated that it was most likely that this proband would be homozygous for a mutation while the proband in the Dutch family was likely to be a compound heterozygote. The TD-2 proband was homozygous for all markers distal to D9S127 but was heterozygous at D9S127 and at markers centromeric to it (Figure 6.1). This narrowed the centromeric boundary. A maximum lod score of 9.67 at a recombination fraction of 0 was detected at D9S277 in family members from the four FHA pedigrees of French Canadian origin. Multipoint linkage analysis provided maximal support for the FHA gene being located near markers D9S277 and D9S306. Eight separate meiotic recombination events were seen (Figure 6.1, A-H), clearly indicating that the minimal genomic region containing the disease locus was a region of approximately 1.5 cM between markers D9S277 and D9S1866.

TD and FHA had been considered distinct disorders, with different clinical and biochemical characteristics. Though the genes for these disorders mapped to the same region, it was not certain whether FHA and TD were due to mutations in the same gene or due to mutations in different genes in the same region. The overlapping genetic data, pointing to the same genomic region, strongly suggested that FHA may in fact be allelic to TD. Combining the genetic data from FHA and TD together provided a minimal region between D9S306 and D9S1866.

The *ABCA1* transporter gene had previously been mapped to 9q31 but its precise physical location had not been determined[181]. Fine mapping refined the localization of *ABCA1* to this region; indeed, D9S306 is located within an intron of this gene. ABCA1 is a member of the ATP binding cassette transporters, a super family of conserved proteins involved in membrane transport of diverse substrates including amino acids, peptides, vitamins and notably steroid hormones[181,425,426]. Thus we assessed *ABCA1* as a candidate gene within our region.

6.4 Mutation detection in TD

The sequencing of BACs containing the *ABCA1* gene revealed its genomic organization. Mutations were searched for by several methods. Northern blot analysis of patient fibroblast RNA and Southern blot analysis of patient genomic DNA were assessed for altered transcripts or major DNA rearrangements, respectively. In addition, RT-PCR products spanning the whole gene were sequenced. Finally, all *ABCA1* exons were amplified individually and subjected to DNA sequencing.

RT-PCR and sequence analysis of TD-1 revealed a T to C substitution in the TD-1 proband, which would predict a substitution of arginine for cysteine at residue 1477. This mutation was confirmed to be heterozygous by RFLP analysis and then sequencing exon 31 of the *ABCA1* gene from genomic DNA of TD-1. There was complete segregation of the mutation with the phenotype of decreased HDL-C levels on one side of this family (Figure 6.2). This point mutation was not seen on over 200 normal chromosomes from unaffected persons of Dutch descent, or in 250 chromosomes of other Western European descent.

Northern blot analysis of fibroblast RNA from TD-1, using a cDNA probe encompassing exons 2 – 50 of *ABCA1*; revealed a normal sized (~8 kb), and a truncated transcript not visible in control RNA or in RNA from other patients with HDL-C deficiency (Figure 6.2 (B)). Furthermore, Northern blot analysis using probes encompassing specific regions of the cDNA revealed that the mutant transcript was detected with cDNA probes encompassing exons 2 to 50 (a), 2 to 42 (b), 2 to 23 (c), much more faintly with a probe spanning exon 24 to 30 (d) and not with probes encompassing exons 31 – 42 (e), or exons 43 to 50 (f). Sequence analysis of the complete coding region of TD-1 in RT-PCR products did not reveal a sequence alteration that could account for this finding. Furthermore, DNA analysis by Southern blot did not reveal any major rearrangements. Genomic sequencing revealed a G→C substitution at the first nucleotide of intron 25 (IVS25 +1 G→C). This would disrupt the consensus for the splice donor signal. This variant was assessed by others in our group, and shown to cosegregate with the low HDL-C phenotype in the proband's maternal branch, and to be absent from over 400 control chromosomes.

RT-PCR analysis of fibroblast RNA from the proband in TD-2 revealed a homozygous nucleotide change of A to G at nucleotide 2185 in exon 14, resulting in a substitution of arginine for a conserved glutamine at residue 597 (Q597R). Segregation analysis of the mutation revealed complete concordance between the mutation and the low HDL-C phenotype (Figure 6.3). Homozygosity for the A2185G mutation in the TD-2 proband was consistent with our expectation of a disease causing mutation in this consanguineous family. This mutation was not observed in over 400 control chromosomes of individuals of Western European ancestry.

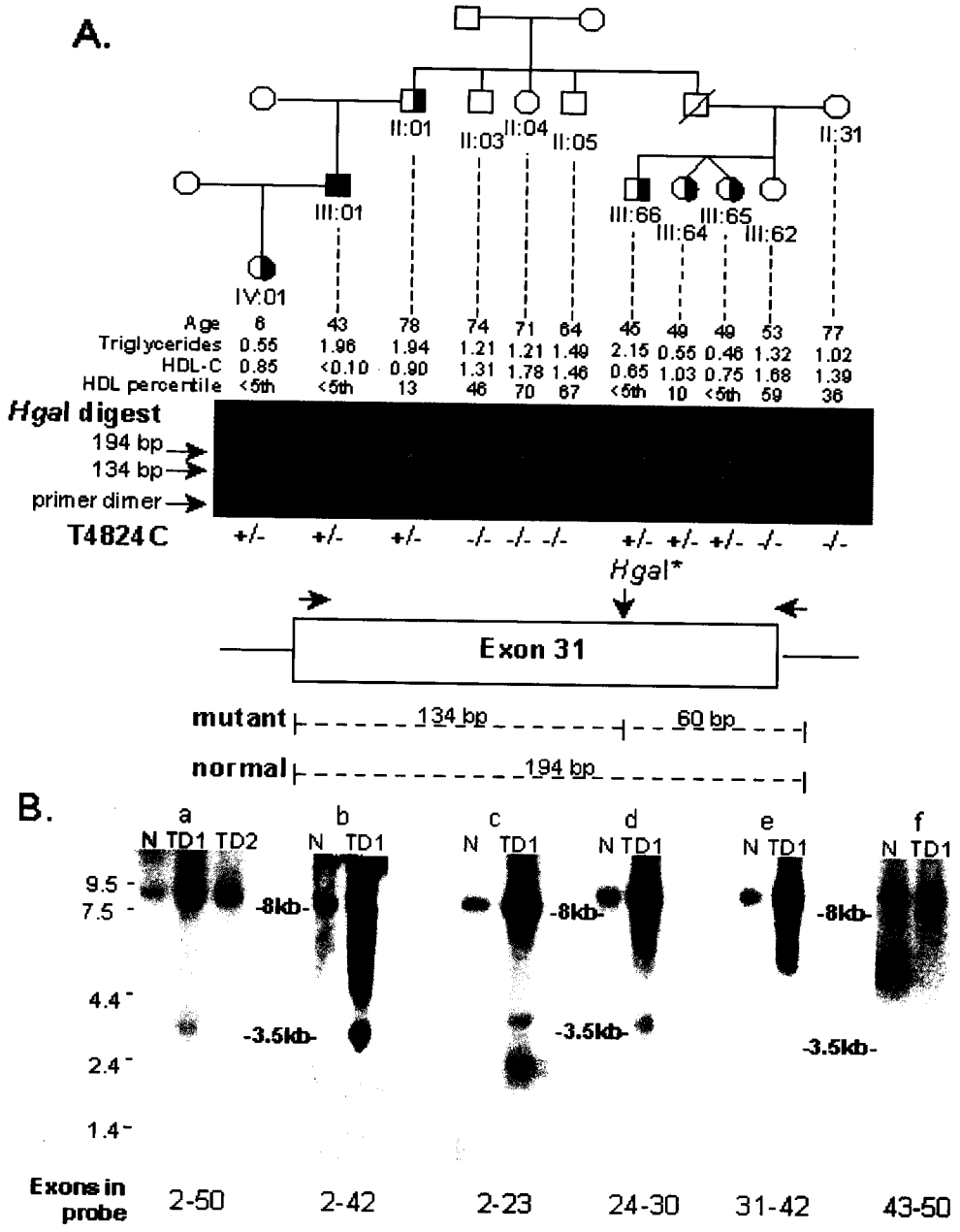

Figure 6.2. Segregation of the C1477R mutation in TD-1.
(A) The paternal branch of TD-1. Circles denote females; squares denote males. A diagonal line through the symbol denotes a deceased individual. Shading of the symbols (half or solid black) corresponds to the presence of reduced HDL-C (solid black for the TD proband, half black for the relatives with reduced HDL-C). The presence of the T4824C mutation (+) was assayed by restriction enzyme digestion with *Hga*I, which cuts only the mutant (*) allele. Thus, in the absence of the mutation, only the 194 bp PCR product is observed, while in its presence the PCR product is cleaved into fragments of 134 bp and 60 bp. The proband (individual III:01) was observed to be heterozygous (+/-) for this mutation (as indicated by both the 194 bp and 134 bp bands), as were his daughter, father, and three paternal cousins. A fourth cousin and three of the father's siblings were not carriers of this mutation. **(B)** Northern blot analysis with probes spanning the complete *ABCA1* gene reveal the expected ~8 kb transcript and in addition an ~3.5 kb truncated transcript only seen in the proband TD-1 and not in TD-2 or a control (N). This was detected by probes spanning exons 2-50 (a), 2-42 (b), 2-23 (c), and 24-309 (d), but not with probes spanning exons 31-42 (e) or 43-50 (f).

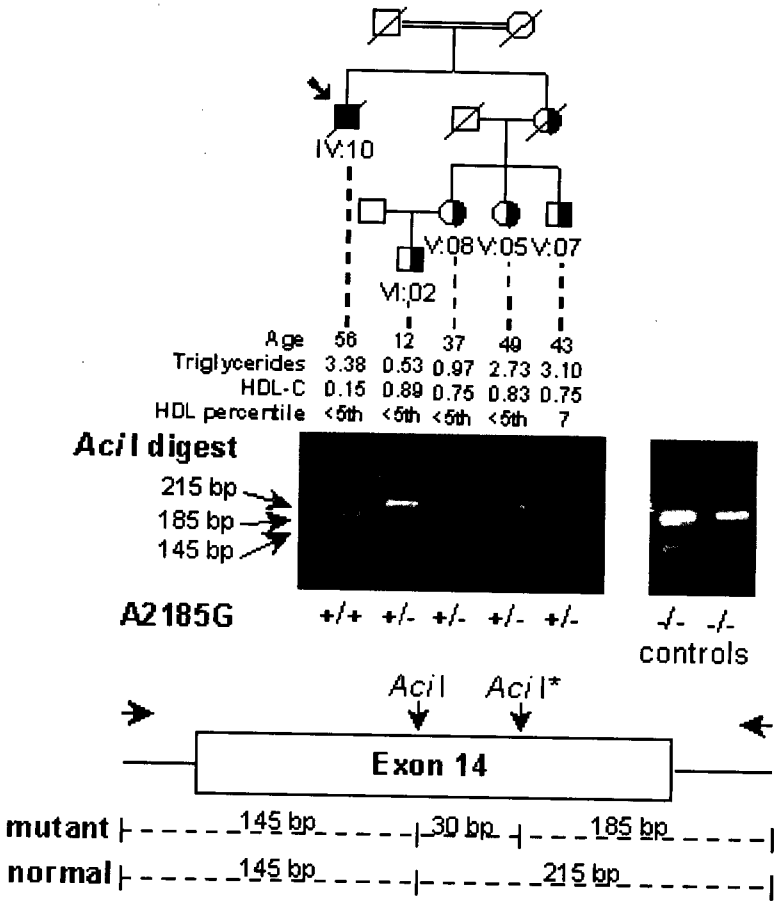

Figure 6.3. Segregation of the Q597R mutation in TD-2.
The double horizontal line denotes the consanguineous mating. The remainder of the symbols are as described in Figure 6.2. The presence of the A2185G mutation (indicated by +) was assayed by restriction enzyme digestion with *AciI*. The 260 bp PCR product has one invariant *AciI* recognition site. A second site (*AciI**) is created by the A2185G mutation. The wildtype allele is thus cleaved to fragments of 215 bp and 145 bp, while the mutant allele (G-allele) is cleaved to fragments of 185 bp, 145 bp and 30 bp. The proband (individual IV:10), the product of a consanguineous mating, was homozygous for the A2185G mutation (+/+), as evidenced by the presence of only the 185 bp and 145 bp bands, while four other family members are heterozygous carriers of this mutation (both the 215 bp and 185 bp fragments were present). Two unaffected individuals (-/-), with only the 215 bp and 145 bp bands are shown for comparison.

6.5 Mutation detection in FHA families

All FHA probands were also subjected to cDNA and genomic sequencing of *ABCA1*. We detected a heterozygous deletion of three nucleotides in the RT-PCR sequence of individual III:01 of FHA-1, resulting in a loss of nucleotides 2472-2474 and deletion of a leucine (ΔL693). This leucine is conserved in mouse and *C. elegans*. RFLP analysis confirmed that this mutation segregated with the phenotype of HDL-C deficiency (Figure 6.4).

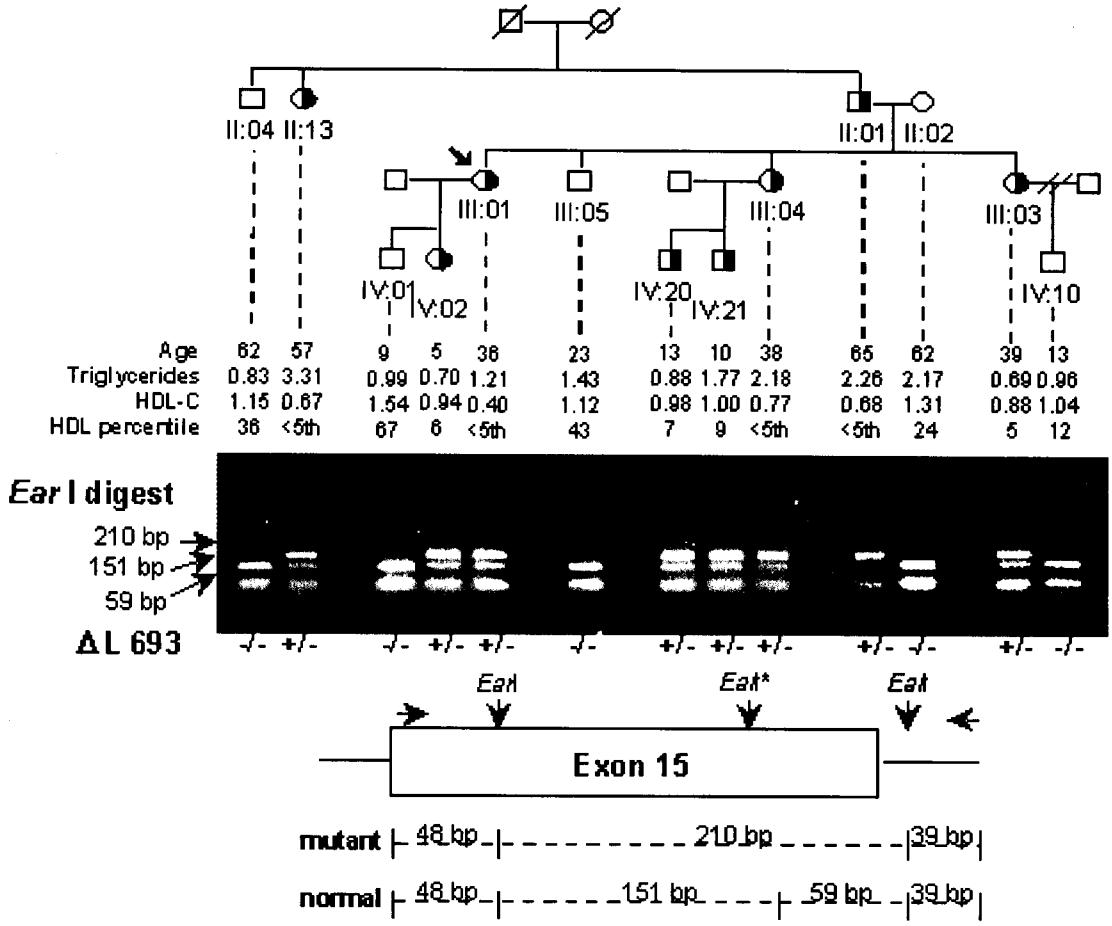

Figure 6.4. Segregation of the ΔL693 mutation in FHA-1.
The symbols are as defined in the previous 2 figures. A double slash through a horizontal line denotes a divorce. Two invariant *Ear*I restriction sites are present within the 297 bp PCR product while a third site is present in the wild-type allele only (*Ear*I*). The presence of the mutant allele is thus distinguished by the presence of a 210 bp fragment (+), while the normal allele produces a 151 bp fragment (-). The proband of this family (III:01) is heterozygous for this mutation, as indicated by the presence of both the 210 and 151 bp bands.

The RT-PCR product and genomic DNA of the FHA-2 proband detected a heterozygous C and T peak at position 6825 within exon 49 of this individual. This alteration converts Arg2144 to a TGA stop codon (R2144X), causing a truncation which excludes the last 118 amino acids of the ABCA1 protein. This alteration abolishes an *Rsa*I restriction site, which allowed confirmation that the mutation co-segregates with the low HDL-C trait in this family (FHA-2) (Figure 6.5).

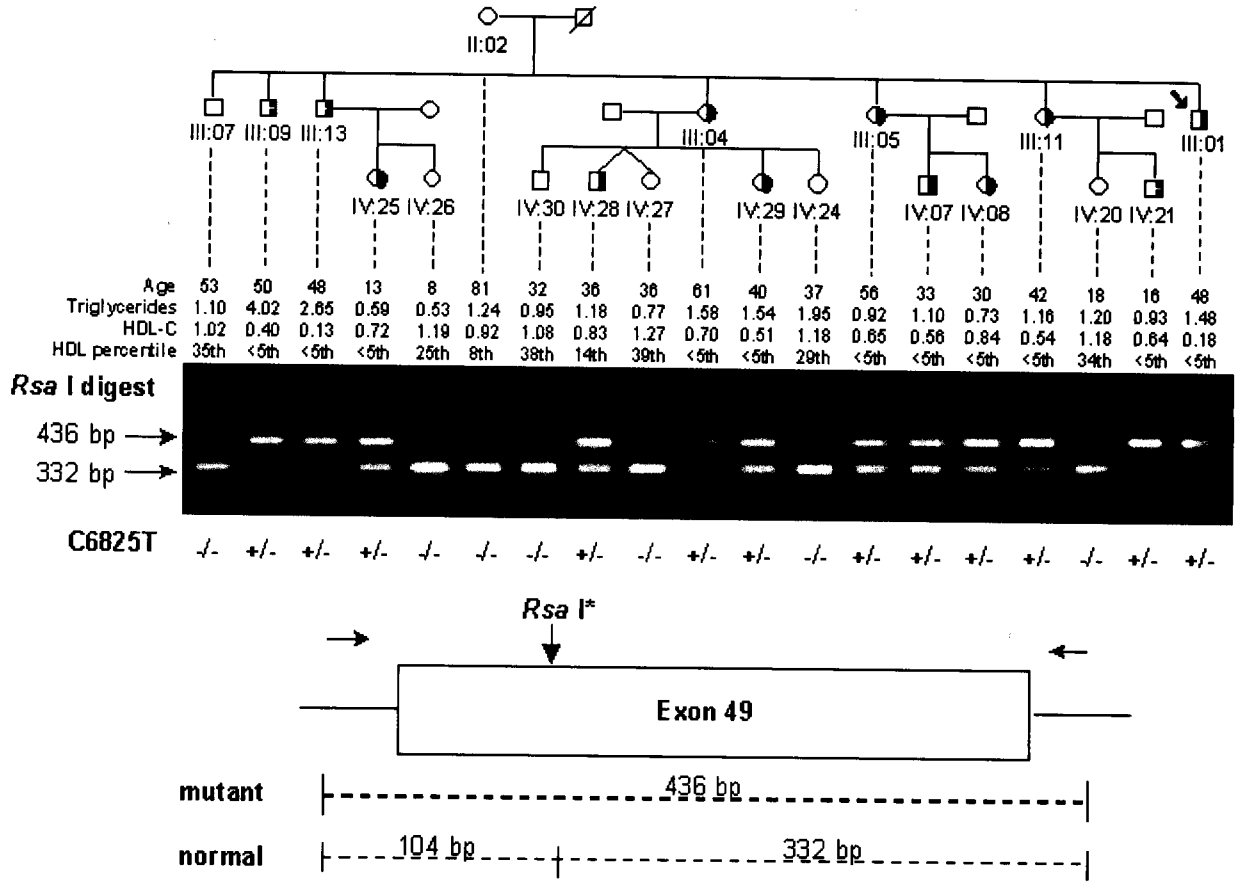

Figure 6.5. Segregation of the R2144X mutation in FHA-2.
The symbols are as defined in the previous figures. Presence of the mutation (+) was assayed by restriction enzyme digestion with *Rsa*I, which cuts only the wildtype allele. In the absence of the mutation, the 436 bp PCR product is cleaved into 332 and 104 bp fragments, while in its presence the full length PCR product is retained. The proband (individual III:01) is heterozygous for this mutation, as are five of his siblings and six of his nieces and nephews.

A mutation was also detected in the RT-PCR product and genomic DNA of the proband of FHA-3. This alteration, a 6 bp deletion of nucleotides 6073-6078 within exon 42, results in an inframe deletion of amino acids 1893 (glutamate, E) and 1894 (aspartate, D) and was evident in PCR products directly resolved on agarose or polyacrylamide gels. Amino acids 1893 and 1894 are in a region of the ABCA1 protein that is conserved between human, mouse and *C. elegans*, indicating that it is of functional importance. This heterozygous mutation co-segregated with low HDL-C in pedigree FHA-3 (Figure 6.6).

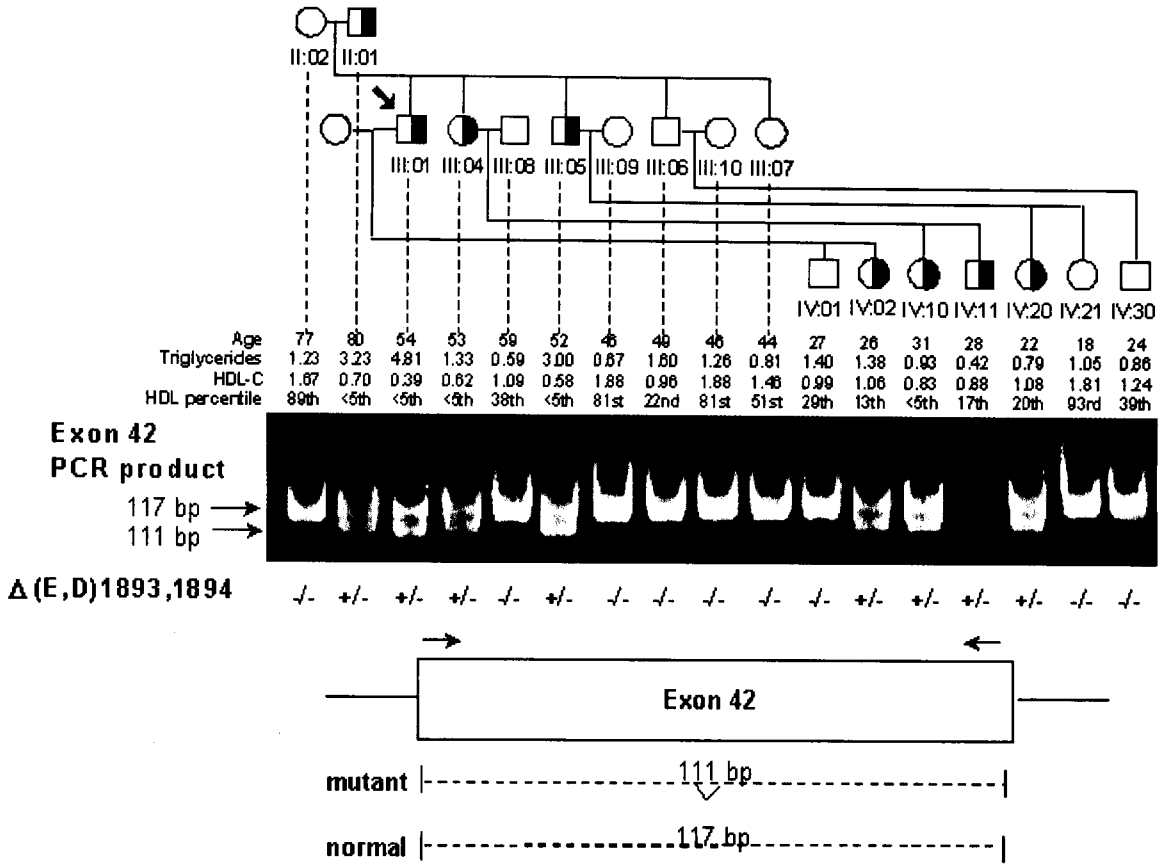

Figure 6.6. Segregation of the Δ(E,D) 1893,1894 mutation in FHA-3.
The symbols are as described in the previous figures. The presence of the mutation (+) was assayed by fragment size determination on a 10% polyacrylamide gel. The deleted allele is observed as a 111 bp PCR fragment, the wildtype allele as a 117 bp PCR fragment. The proband (III:01) was heterozygous for this mutation (as indicated by the presence of two bands), as were his father, sister, brother and four of his nieces and nephews. The genotype of individual IV:11 was determined on a separate gel.

A C to T transition was found in exon 19 of the proband from family FHA-4, detected as a double, heterozygous peak at nucleotide 3120. This changes an arginine to a stop codon (R909X) and predicts a truncation of over half of the ABCA1 protein. The substitution creates a *Dde*I site that is assayable by RFLP analysis in PCR product of exon 19. This mutation co-segregated with the phenotype of low HDL-C in FHA-4 (Figure 6.7).

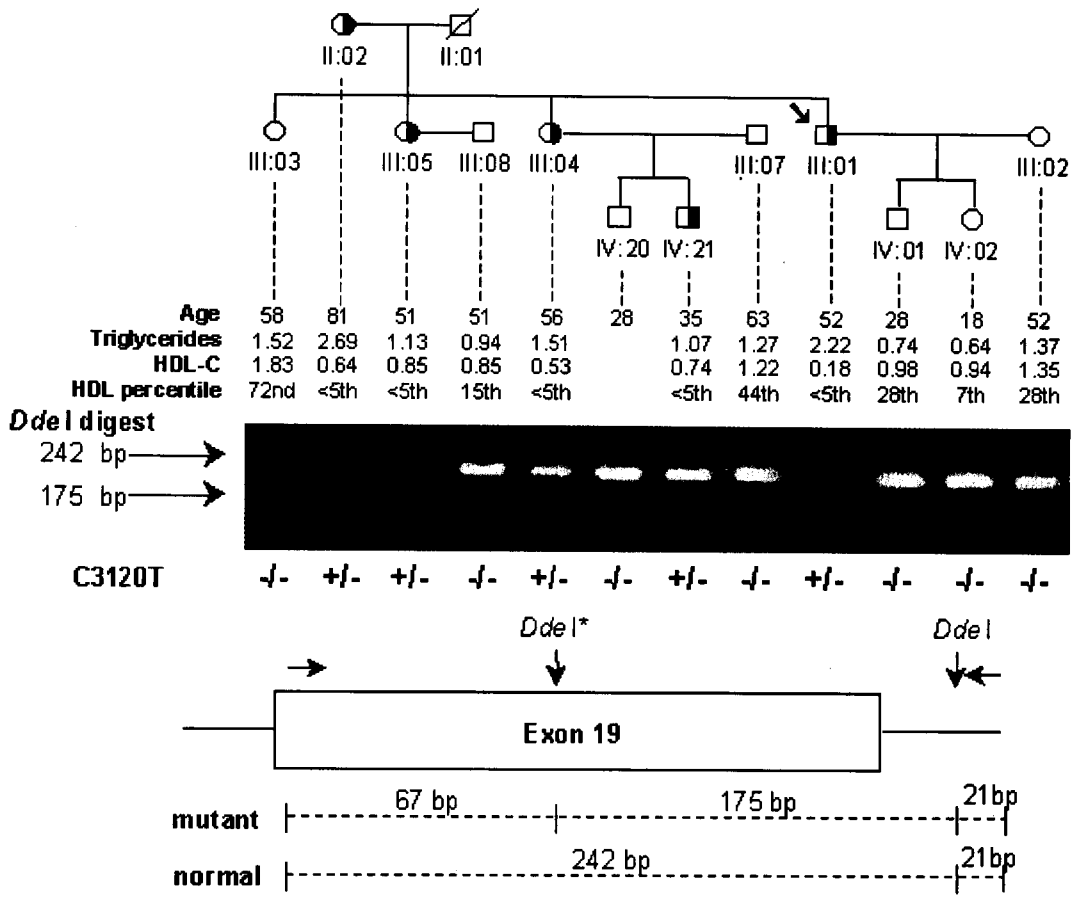

Figure 6.7. Segregation of the R909X mutation in family FHA- 4.
The symbols are as defined in the previous figures. The presence of the mutation creates an additional *Dde* I site within the 263 bp PCR product. Thus the wildtype allele is distinguished by the presence of a 242 bp fragment (-), while the mutant allele displays a 175 bp fragment (+). Note that the genotype of individual III:05 was confirmed in a separate assay.

A T to C transition was found in genomic DNA of the proband of FHA-5. It was detected as a double, heterozygous peak at nucleotide 3667 in exon 23. This changes a methionine to a threonine at amino acid 1091 of *ABCA1* (M1091T). Amino acid 1091 is conserved between human and mouse ABCA1, and in a *C. elegans* homologue, indicating that it is likely of functional importance. The substitution removes an *Nla*III site that is assayable by RFLP analysis in PCR product of exon 23 (Figure 6.8). The mutation co-segregates with the phenotype of low HDL-C in FHA-5 (Figure 6.8).

None of the mutations seen in any of the FHA families were seen in over 120 chromosomes from unaffected French-Canadians, nor in over 400 chromosomes derived from

Western European controls. Polymorphisms present on control chromosomes in persons with normal HDL levels were also detected in the *ABCA1* gene.

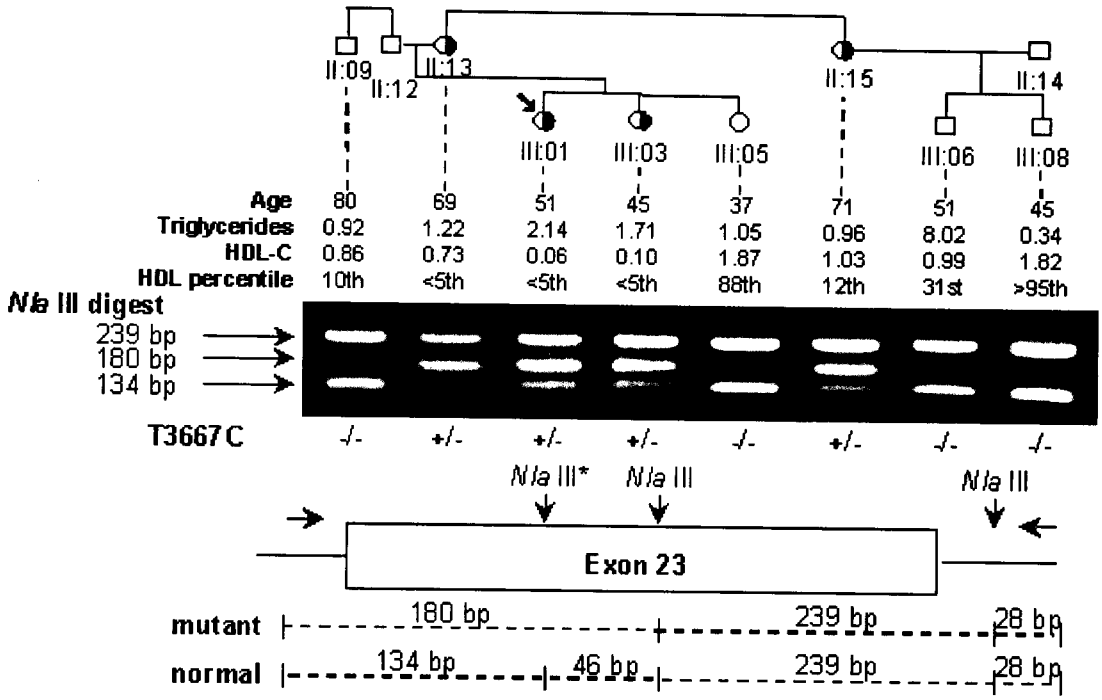

Figure 6.8. Segregation of the M1091T mutation in FHA-5.
The symbols are as defined on the previous figures. The presence of the mutation (+) was assayed by restriction enzyme digestion of exon 23 PCR product with *Nla*III, which cuts at an additional site (*Nla*III*) on the wildtype allele. The wildtype allele is distinguished by the presence of a 134 bp fragment (-), while the mutant allele displays a 180 bp fragment (+).

6.6 Discussion

We independently sought the genes involved in two genetic disorders of HDL deficiency, TD and FHA. Interestingly, both disorders mapped to the same region and mutations in the *ABCA1* transporter gene were seen in both FHA and TD, indicating they are allelic.

ABCA1 is a member of the ATP-binding transporter superfamily which is involved in energy-dependent transport of many substrates across membranes[426] and has distinguishing motifs from other ATP binding proteins including two ATP binding segments and two transmembrane domains[426] (Figure 6.10). Interestingly, no orthologues of the *ABCA1* subfamily have been found in bacteria or yeast[427], suggesting that genes from this family diverged in multicellular organisms and evolved to develop highly specialized functions. Here we have

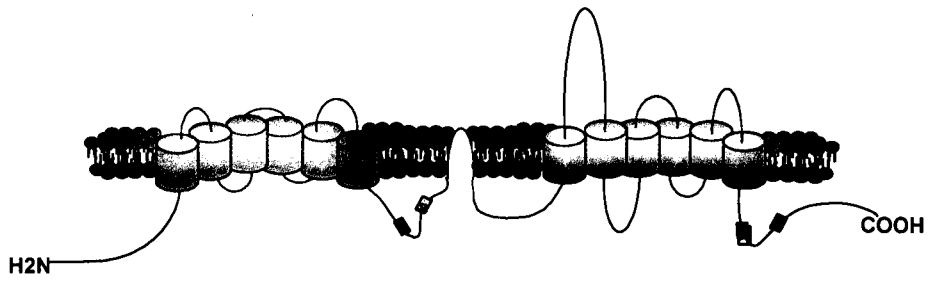

Figure 6.9. Structure of ABCA1.
ABCA1 consists of two transmembrane domains (TM), each composed of six membrane-spanning segments (green), and two ATP binding cassette motifs (ABC), characterized by the Walker A and Walker B signature sequences (orange). A large N-terminal domain exists prior to the first membrane spanning segment (~600 aa). Most loops connecting the membrane spanning segments are small, with the exception of the first loop in the second TM domain. A highly hydrophobic region following the first ATP binding cassette has been described[181], which may contact or insert into the plasma membrane. The ABCA subfamily of transporters is characterized by the domain arrangement: TM-ABC-TM-ABC.

shown that the ABCA1 transporter is crucial for intracellular cholesterol transport, hence we named it the cholesterol efflux regulatory protein (CERP).

TD and FHA now join the growing list of genetic diseases due to defects in the ABC group of proteins including cystic fibrosis[428], adrenoleukodystrophy[429], Zellweger syndrome[430], progressive familial intrahepatic cholestatis[431], pseudoxanthoma elasticum[432,433], and different eye disorders including Stargardt disease[434], autosomal recessive retinitis pigmentosa[435], and cone-rod dystrophy[436].

Patients with TD had been distinguished from patients with FHA by virtue of their apparently different modes of inheritance. TD is an autosomal recessive disorder (OMIM 205400) while FHA is inherited as an autosomal dominant trait (OMIM 604091). Furthermore, patients with TD have obvious evidence for intracellular cholesterol accumulation that is not seen in FHA patients. It is now evident that TD heterozygotes do have reduced HDL-C levels, and that the same mechanisms underlie the HDL deficiency of TD and FHA. The more severe phenotype in TD represents loss of function from both alleles of the *ABCA1* transporter gene. Cholesterol ester storage in TD must occur because of a complete functional deficiency in ABCA1. The *ABCA1* gene plays a crucial role in intracellular cholesterol trafficking in monocytes and fibroblasts, cells shown to have defective cholesterol efflux in TD, but must also play a role in other tissues such as the nervous system and the cornea where defects in transport result in peripheral neuropathy and corneal opacities.

These findings have significance for the understanding of mechanisms leading to premature atherosclerosis. TD homozygotes develop premature CAD (as seen in the proband of TD-1). There is evidence that heterozygotes for TD may also be at increased risk for premature

vascular disease[437,438], and preliminary evidence for premature atherosclerosis in FHA (e.g. the proband in FHA-2 had a coronary artery bypass graft at 46 years and the proband in FHA-3 had evidence for CAD around 50 years of age). This highlights the importance of intracellular cholesterol transport and efflux as an important mechanism in atherogenesis. Interestingly, the proband of TD-2 whose efflux defect was less severe than TD-1 had no evidence for CAD by 62 when he died of unrelated causes, providing preliminary evidence for a relationship between the degree of cholesterol efflux (perhaps mediated in part by the nature of the mutation) and the likelihood of atherosclerosis. It has been suggested that peripheral cell events determine the net flux of cholesterol to the liver[439]. It is likely *ABCA1* is a key determinant of this process.

HDL-C deficiency is heterogeneous in nature. Delineation of the genetic basis of TD and FHA underlies the importance of this particular pathway in intracellular cholesterol transport, and could lead to new approaches to treatment of HDL deficiency.

At the same time as we reported that *ABCA1* mutations were the underlying cause of TD and FHA, two other groups also reported mutations in *ABCA1* were responsible for TD[51,54]. Since then, at least 35 mutations have been identified within this gene worldwide. To date, no common mutations present in multiple families have been identified, with the majority of the mutations being identified in only one family. In addition, *ABCA1* mutations are not a common cause of low HDL-C or CAD in the general population. Each of the mutations described here has been screened in greater than 300, and in most cases greater than 400, Dutch individuals with low HDL-C and/or premature CAD, but none of the mutations have been identified in any of these individuals. The identification of numerous individuals with *ABCA1* mutations will allow us to characterize the phenotypic effects of ABCA1 deficiency.

Chapter 7: HDL cholesterol levels and coronary artery disease in heterozygotes for ABCA1 mutations are predicted by cholesterol efflux levels and influenced by age

The work presented in this chapter has been published in

Hayden M. R., **Clee S. M.**, Brooks-Wilson A., Genest Jr. J., Attie A. and Kastelein J.J.P. Cholesterol Efflux Regulatory Protein (CERP), Tangier Disease and Familial HDL Deficiency. *Current Opinion in Lipidology* 2000 **11**:117-122.

Clee S. M., Kastelein J.J.P., van Dam M., Marcil M., Roomp K., Zwarts K.Y., Collins J.A., Roelants R., Tamasawa N., Stulc T., Suda T., Ceska R., Boucher B., Rondeau C., DeSouich C., Brooks-Wilson A., Molhuizen H.O.F., Frohlich J., Genest J. Jr., and Hayden M.R. HDL levels and coronary artery disease in heterozygotes for *ABCA1* mutations are predicted by levels of cholesterol efflux and are influenced by age. *Journal of Clinical Investigation* 2000 **106**:1263-1270.

This work was also presented and published in abstract form

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Clee S.M., et al. *Oral presentation*, 73rd Scientific Sessions of the American Heart Association, New Orleans LA Nov. 12-15, 2000. *Circulation* 2000 102 (18):II-31.

Preface

The work presented in this chapter describes information from many families. The families described in Chapter 6 were identified and collected by M. van Dam, R. Roelants, H. Molhuizen, M. Marcil, C. de Souich, J. Kastelein, and J. Genest Jr. Additional families have been identified, collected and information provided by N. Tamasawa, T. Suda, J. Frohlich, T. Stulc, and R. Ceska. All family information was collected, recorded and verified in Vancouver by J. Collins. Mutation detection in these families was from sequencing performed by K. Roomp under the direction of A. Brooks-Wilson. B. Boucher and C. Rondeau performed the efflux assays described in this chapter.

7.1 Introduction

In Chapter 6, data was provided suggesting that heterozygosity for mutations in the *ABCA1* gene is associated with reduced HDL-C. However, the phenotype of heterozygosity for mutations in the *ABCA1* gene has not been clearly defined. As many factors, both genetic and environmental, influence plasma HDL–C levels and contribute to low HDL-C values[173,440], unambiguous identification of heterozygotes from TD families was impossible until the identification of the *ABCA1* gene. Individuals from Tangier disease kindreds presumed to be heterozygous have shown a range of phenotypes, and much overlap with unaffected individuals has been seen[441-443], possibly reflecting the fact that some individuals had been misclassified. Indeed, the inability to uniquely identify heterozygous individuals created difficulty in mapping the gene for TD[444]. Studies in obligate heterozygotes have also been limited to small numbers[437] often within a single family[441], and thus restricted in the ability to analyze the phenotypic expression with different mutations, and over a range of ages. Furthermore, conflicting results about the CAD risk in TD families have been reported[441]. The data from Chapter 6 suggested that the degree of cholesterol efflux defect may influence the likelihood of CAD.

Subsequent to the cloning of the *ABCA1* gene with the families described in Chapter 6, we have identified three additional TD and one additional FHA family and their corresponding mutations. Thus, we have now identified a large cohort of individuals in whom heterozygosity has been defined by mutation identification in the *ABCA1* gene. For the first time it is therefore now possible to characterize the phenotype in mutation-defined heterozygotes and to compare this to a large number of unaffected (i.e. without *ABCA1* mutations) family members, controlling for other genetic and environmental factors. We have also now been able to directly investigate of the relationship between variations in efflux of cholesterol from peripheral cells to plasma HDL-C levels and risk of CAD[63,64], directly testing the hypothesis that decreased reverse cholesterol transport results in increased atherosclerosis.

7.2 Methods

7.2.1 Identification of subjects

Subjects heterozygous for mutations in the *ABCA1* gene were individuals identified from the seven TD and FHA families described in Chapter 6. In addition, heterozygous individuals from three subsequently identified TD families (TD3-5) of Dutch, Czech and Japanese origin and

one new Dutch FHA kindred (FHA6) were included. The second mutation has not been identified in the TD4 kindred, however a marker immediately adjacent to *ABCA1* cosegregated with the low HDL-C phenotype. Individuals bearing the affected haplotype were considered heterozygotes. The presence or absence of mutations identified by genomic sequencing of probands from each family was subsequently confirmed in family members by RFLP assays, to define heterozygous and unaffected individuals, respectively.

Tangier disease families have been ascertained on the basis of the clinical features of TD, and all heterozygotes available from each family were included. There has been no selection on the basis of HDL-C levels or CAD status of these individuals. Two of the six FHA probands (FHA2-301 and FHA3-301) were referred to the clinic on the basis of CAD. The remaining probands were identified solely on the basis of low HDL-C. Again, all heterozygotes from the FHA families were included, with no selection for HDL-C levels or CAD.

Our control cohort comprised unaffected members of the 11 families. These individuals share a genetic background with the heterozygotes, and environmental factors are expected to be similar amongst family members. Thus, many additional factors that may influence HDL-C are controlled for, and the phenotypic differences between heterozygotes and unaffected individuals can be largely attributed to variation in *ABCA1* gene activity.

All subjects gave informed consent to their participation in this study, and the genetic analysis protocol was approved by the Ethics committees of the University of British Columbia, the Academic Medical Centre in Amsterdam and the Clinical Research Institute of Montreal (IRCM).

7.2.2 Lipid and cholesterol efflux measurements

Lipid levels in ABCA1 heterozygotes were measured as previously described in Chapter 6, at standardized lipid clinics in Vancouver, Montreal and Amsterdam. LDL cholesterol was calculated by the method of Friedewald[374], modified to account for lipid measurements in mmol/L.

Cellular cholesterol efflux from fibroblast cultures was measured as previously described[179,445,446]. Skin fibroblast cultures were established from 3.0 mm punch biopsies of the forearm of patients and healthy control subjects as described[179].

Efflux studies were carried out for 24 hours in the presence of purified apoAI (10 mg protein / mL medium). Radiolabelled cholesterol released into the medium is expressed as a percentage of total ^{3}H-cholesterol per well (medium + cell). Each experiment was performed in triplicate wells. Cells from at least two healthy control subjects were included as controls in each assay. Each result reflects the mean of multiple different experiments, each done in triplicate. Relative efflux in TD and FHA individuals are expressed as a percentage of the average of the healthy controls included within that experiment. Note that the number of heterozygotes with efflux measurements is less than the number of mutations, as not all TD families have had efflux measured in heterozygous carriers of each mutation.

7.2.3 Statistics

In analysis of the heterozygotes, differences in mean baseline demographics and lipid levels between groups were compared by Student's *t*-test. Comparisons of frequencies either between the male:female ratio or of distributions across various percentile ranges were made using the chi-square test. Analysis of potential interactions between affected status and either sex or BMI were performed using a general linear model. Statistical analysis was performed using Prism (version 3.00, Graphpad Software) and Systat (version 8.0, SPSS Inc.). All values are reported as mean $\pm$ standard deviation.

7.3 *ABCA1* heterozygotes have decreased HDL cholesterol and an increased risk for CAD

We have now identified *ABCA1* mutations in eleven families. A total of 13 mutations have been observed throughout the gene (Figure 7.1). Notably, 3 of the 13 mutations occur in the vicinity of the ATP binding cassette. Only one mutation thus far has been seen in the transmembrane domains (Figure 7.1). Previous studies of other ABC transporters such as CFTR (cystic fibrosis)[428], ABCR (Stargardt's disease)[434], P glycoproteins[447], and MRP2 (Dubin-Johnson syndrome)[448] reveal that the majority of mutations occur within or around the ATP-binding cassette. This suggests that mutations in this domain may impair ATP hydrolysis necessary for transporter activity while mutations in the transmembrane domain may be more likely to impair substrate specificity and/or recognition[449,450].

Our cohort comprised 77 individuals from the 11 families identified as heterozygous for mutations in the *ABCA1* gene. A comparison of mean lipid levels in heterozygotes with mean

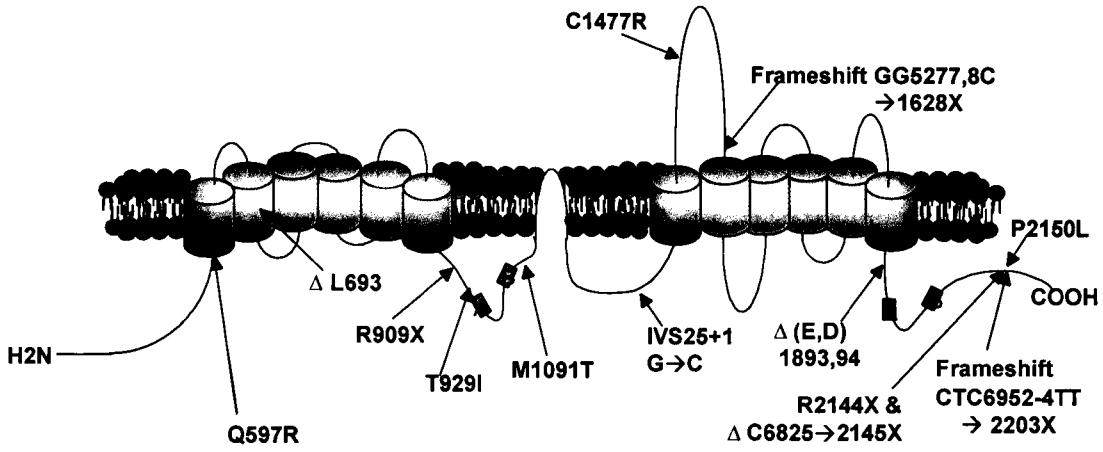

Figure 7.1. Mutations identified in the *ABCA1* gene.
A schematic diagram of the ABCA1 protein (as described in Rigure 6.9), showing the location of the 13 mutations identified within the 11 families. Three of the mutations are observed near the ATP binding cassettes, while only one is observed in a membrane spanning segment. Of note there is also a cluster of 4 mutations at the C-terminus of the protein.

Table 7.1. Characterization of *ABCA1* heterozygotes

	TD Patients	*ABCA1* Heterozygotes	Unaffected family members	P-value heterozygotes vs. unaffected	P-value TD patients vs. unaffected
n	5	77[a]	156[a]		
Age (years)	43.4±9.0	42.5±19.6	39.9±21.0	0.35	0.71
range	31-56	5-81	4-86		
M/F	3/2	33/44	82/74	0.16	0.74
TC (mmol/L)	2.34±1.03	4.52±1.12	4.71±1.07	0.23	**<0.0001**
TG (mmol/L)	1.95±0.97	1.66±1.59	1.20±1.03	**0.03**	0.11
HDL (mmol/L)	0.08±0.05	0.74±0.24	1.31±0.35	**<0.0001**	**<0.0001**
LDL (mmol/L)	1.37±1.02	3.03±0.99	2.84±0.87	0.17	**0.0003**
ApoAI (g/L)	0.03±0.04 (3)	0.92±0.32 (61)	1.43±0.26 (55)	**<0.0001**	**<0.0001**
ApoAII (g/L)	0.10±0.08 (2)	0.35±0.08 (46)	0.39±0.08 (43)	**0.01**	**<0.0001**
ApoB (g/L)	0.89±0.53 (2)	0.93±0.25 (52)	0.94±0.33 (42)	0.88	0.84
CHD ≥ 20 yrs	20% (1/5)	12.9% (8/62)	4.1% (5/122)	**0.03**	0.10
Odds Ratio (95% CI)				3.47 (1.08-11.09)	5.85 (0.55-62.4)
Age of onset	38	48.9±8.6	60.4±12.8	0.08	-

[a] For TC, TG, LDL n=76 for heterozygotes, 153 for unaffected family members

Table 7.2. HDL-C levels by *ABCA1* mutation

family	mutation	HDL in heterozygotes mean ± SD (n)	HDL in unaffected family members mean ± SD (n)	HDL in heterozygotes % of unaffected	Age and sex matched population median[a] mean ± SD	CAD in heterozygotes
FHA1	Del L 693	0.79±0.20 (8)	1.22±0.35 (11)	64.8	1.39±0.08	-
FHA2	R2144X	0.56±0.23 (12)	1.07±0.22 (20)	52.3	1.34±0.19	+
FHA3	Del E,D 1893,94	0.77±0.24 (8)	1.44±0.38 (9)	53.5	1.30±0.17	+
FHA4	R909X	0.59±0.26 (5)	1.04±0.37 (9)	56.5	1.39±0.24	-
FHA5	M1091T	0.48±0.48 (4)	1.37±0.43 (6)	35.0	1.56±0.05	+
FHA6	P2150L	0.61±0.07 (7)	1.05 (1)	58.1	1.30±0.22	+
TD1	ivs25+1G-->C	0.78±0.06 (4)	1.35±0.29 (70)	57.8	1.22±0.22	-
TD4	del C 6825-->2145X	0.91±0.10 (2)	1.00±0.05 (3)	91.0	1.31±0.16	-
TD5	CTC6952-4TT-->2203X	0.80±0.20 (3)	1.65 (1)	48.5	1.39±0.19	-
TD1	C1477R	0.82±0.18 (9)	1.35±0.29 (70)	60.7	1.37±0.14	+
TD2	Q597R	0.82±0.07 (5)	none available	-	1.39±0.17	-
TD3	T929I	1.01±0.18 (8)	1.48±0.42 (26)	68.2	1.33±0.19	-
TD4	unidentified	0.74±0.05 (2)	1.00±0.05 (3)	73.5	1.49±0.09	+

[a] Calculated based on mean the age and sex specific 50th percentile levels in the LRC population

levels in all available family members without *ABCA1* mutations, hereafter referred to as unaffected family members (n=156), is presented in Table 7.1. As predicted, heterozygotes have an approximate 40-45% decrease in HDL-C and apoAI and a mild (approximately 10%) decrease in apoAII compared to unaffected family members. Mean TG levels were increased by approximately 40% in heterozygotes compared to unaffected family members, and were further increased in TD patients. Unlike TD patients, there was no significant decrease in either total cholesterol or LDL-C in heterozygotes, and apoB levels were not different in heterozygotes from controls. Mean HDL-C levels in carriers of each of the mutations were similarly reduced by approximately 40-50% compared to unaffected family members (Table 7.2).

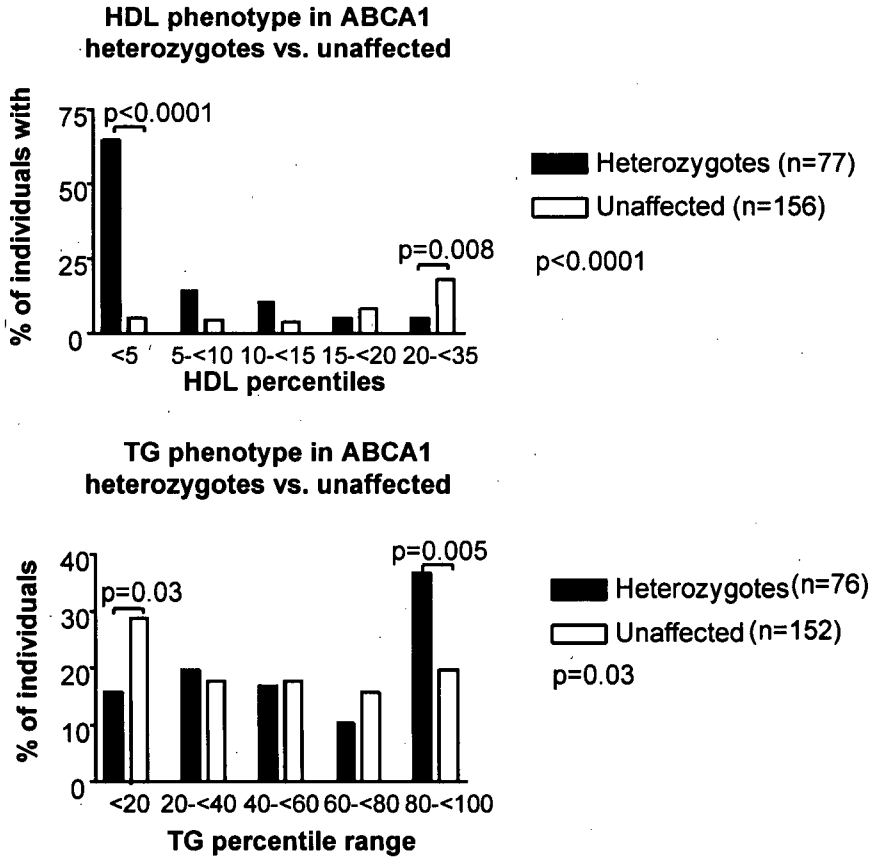

Figure 7.2. HDL-C and TG percentiles in *ABCA1* heterozygotes.
The percent of heterozygotes or unaffected family members with HDL-C and TG within a given range of percentiles for age and sex, based on the Lipid Research Clinics (LRC) criteria[451] are shown. While a majority of heterozygotes (black bars) have HDL-C <5th percentile for age and sex, a broad distribution of HDL-C levels was seen in the heterozygotes, extending up to the 31st percentile in one individual. This is in marked contrast to the percentage of unaffected family members (white bars) with low HDL-C. There is much overlap in the distribution of TG between heterozygotes and unaffected family members, although a larger portion of heterozygotes have TG ≥80th percentile for age and sex, while a smaller percentage have TG<20th percentile.

We further examined the heterozygote phenotype by calculating the percentage of individuals falling within a given range of age and sex specific percentiles[451,452]. Much variability in the heterozygote phenotype was evident. As shown in Figure 7.2, although a significantly higher percentage of heterozygotes had HDL-C less than the 5th percentile for age and sex compared to unaffected controls (65% vs. 5%, $p<0.0001$), 5% of heterozygotes had HDL-C greater than the 20th percentile, with HDL-C ranging up to the 31st percentile for age and sex. Thus in some individuals clearly the phenotype is less severe. A broad distribution of TG levels was also evident (Figure 7.2). A significantly lower percentage of heterozygous individuals had TG below the 20th percentile for age and sex ($p=0.03$), and a significantly larger percentage had TG >80th percentile ($p=0.005$) compared to unaffected family members, but substantial overlap between the two distributions was seen.

Table 7.3. Coronary artery disease in *ABCA1* heterozygotes

Individual	Mutation	exon	disease (age of onset)	other risk factors
TD proband				
TD1	C1477R, ivs25+1G-->C	30, intron 25	CHD (38)	-
***ABCA1* heterozygotes**				
TD4-201	unidentified	-	MI (<58)	-
FHA5-215	M1091T	22	MI (61)	-
FHA5-303	M1091T	22	CHD (<45)	-
TD1-363	C1477R	30	MI (51)	-
FHA3-301	Del(E,D) 1893,94	41	PVD (<54)	smoker, BMI 31.7
FHA3-305	Del(E,D) 1893,94	41	CHD (44)	ex-smoker
FHA6-201	P2150L	48	CVA (36), fatal MI (58)	-
FHA2-301	R2144X	48	CAD (42), PTCA (47), femoral angioplasty (48), CABG (<50)	hypertensive
Unaffected family members				
FHA5-212	none	-	AP (62)	-
TD3-109	none	-	TIA (80)	diabetic
FHA2-315	none	-	MI (51)	BMI 37
TD1-205	none	-	MI (62)	-
TD1-216	none	-	AP (47)	-

Another important question is whether individuals heterozygous for *ABCA1* mutations are at an increased risk of developing CAD. Studies on obligate TD heterozygotes have reported

conflicting findings[437,441]. In our large cohort, symptomatic vascular disease was over three times as frequent in the adult heterozygotes as in unaffected family members (Table 7.1). Interestingly, the presentation of vascular disease was generally more severe in the heterozygotes than their unaffected family members (Table 7.3). Heterozygotes had myocardial infarctions (five, one fatal) and severe vascular disease requiring multiple interventions, whereas in unaffected individuals, CAD was manifest as angina in two cases and as a transient ischemic attack at the age of 80 in another. Furthermore, the mean age of onset was on average a decade earlier in heterozygotes compared to unaffected controls (Table 7.1).

7.4 Cholesterol efflux, HDL cholesterol levels and CAD

We next sought to directly assess the relationship between cholesterol efflux levels, HDL-C and CAD. We have previously shown (Chapter 6) that individuals heterozygous for *ABCA1* mutations have decreased cholesterol efflux, however the extent to which variations in cholesterol efflux are directly related to HDL-C levels is unknown. Relative cholesterol efflux in individuals heterozygous for an *ABCA1* mutation was plotted against the mean HDL-C levels observed in the carriers of that mutation, expressed as a percentage of the unaffected members within that family (Figure 7.3). Efflux measures were not available in heterozygotes of some mutations from TD families where efflux has only been measured in the TD probands.

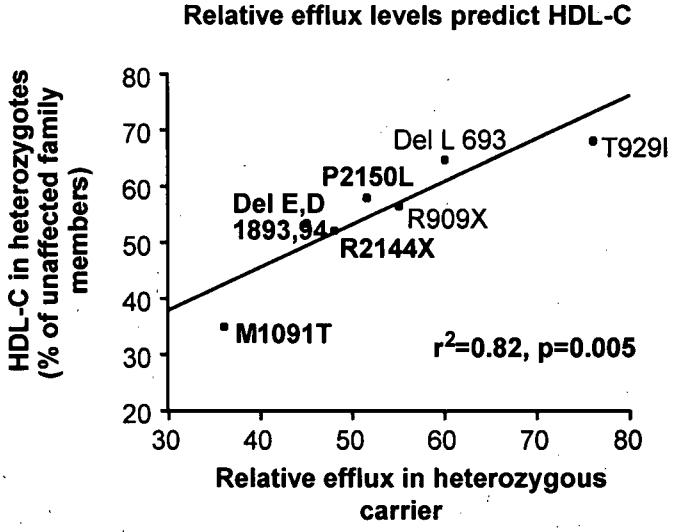

Figure 7.3. Mean HDL-C in *ABCA1* heterozygotes is correlated with cholesterol efflux.
Average HDL-C in the heterozygotes for each mutation (expressed as a percentage of the mean HDL-C in the unaffected members of that family) are plotted against the efflux levels measured in a heterozygous carrier of each mutation. Efflux levels are highly correlated with levels of HDL-C and are associated with 82% of the variation in HDL-C. Individuals from families with CAD are shown in bold.

Cholesterol efflux levels associated with each mutation strongly predict the corresponding HDL-C levels in our families, accounting for 82% of the variation in HDL-C (r^2=0.82, p=0.005). Furthermore, in one large family (FHA2), where efflux has been measured in three independent heterozygotes, an r^2 value of 0.81 was obtained when individual plasma HDL-C levels were plotted against individual efflux measurements. Using the regression equation of mean HDL-C levels in the heterozygotes on the efflux level of the heterozygous carrier (p=0.02), we can estimate the relationship between expected changes in ABCA1 efflux activity and HDL-C levels. From this we would predict that each 8% change in efflux levels would be associated with a 0.1 mmol/L change in HDL-C.

Relative cholesterol efflux levels are also related to CAD within the family. Families with clearest evidence for premature CAD had individuals with the lowest cholesterol efflux (Table 7.2, bold on Figure 7.3). These data suggest that the level of residual ABCA1 function is a critical determinant of both HDL-C levels and susceptibility to CAD.

7.5 *ABCA1* mutation type and location do not influence the severity of phenotype in heterozygous individuals

We have previously noted that the phenotypic presentation of our FHA heterozygotes was more severe than that of our TD heterozygotes[453]. However, we initially noted more deletions and premature truncations of the protein in our FHA families than our TD families in Chapter 6. Thus, with our identification of several different *ABCA1* mutations, and as residual ABCA1 activity is an important predictor of severity of the phenotype, we sought to examine whether the nature of the mutation influenced the phenotypic expression of mutations in the *ABCA1* gene. Severe mutations were defined as deletions, those that caused premature truncation of the protein or disrupted natural splicing of the protein, and would be expected to result in a non-functional allele. Missense mutations, on the other hand, result in the substitution of only a single amino acid and may result in a protein product that still retains partial activity.

Lipid levels were compared in heterozygous carriers of severe and missense mutations. While there was a trend to decreased HDL-C levels in carriers of severe compared to missense mutations, this did not reach significance (0.78$\pm$0.26 vs. 0.70$\pm$0.23, p=0.18). A range of HDL-C levels in individual missense and severe mutations were observed (Table 7.2). No significant

differences in TG were evident between carriers of missense and severe mutations (1.77±2.15 vs. 1.55±1.01, p=0.58). Interestingly, the M1091T missense mutation is the most severe mutation both by effects on efflux and HDL-C levels, with a more severe phenotype than even early truncations of the protein (e.g. R909X).

We next examined whether mutations in certain domains of the protein were more likely to be associated with a given clinical phenotype. The site of mutation (e.g. N-terminal or C-terminal) within the ABCA1 protein did not influence the phenotype (Figure 7.4). The presence of CAD is seen in carriers of mutations throughout the protein. Patients with mutations on both alleles (TD) manifest with splenomegaly alone or in association with CAD (TD1, Figure 7.5), regardless of the location of their mutations. Thus the phenotype appears to be mutation specific, and most likely dependent on remaining ABCA1 function of the wildtype allele and residual function of the mutant allele, similar to what has been shown for mutations in *ABCR*, a close homologue of *ABCA1*[454].

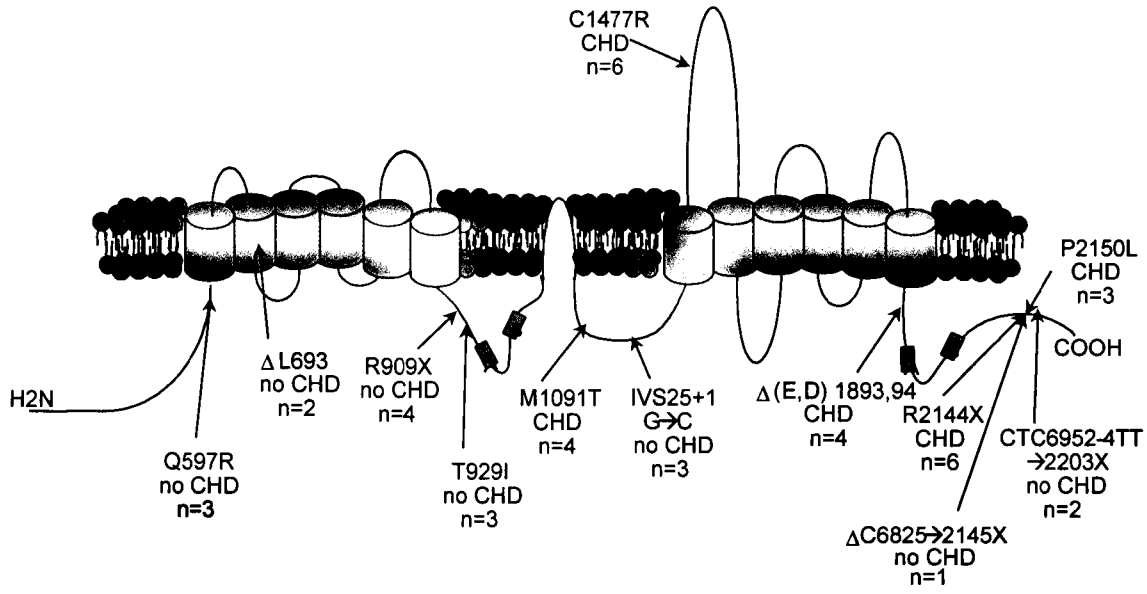

Figure 7.4. Mutations in *ABCA1* and the presence of CAD.
A schematic diagram of the ABCA1 protein (as described in Figure 6.9), illustrating the location of mutations in the heterozygotes and the presence of CAD in carriers of that mutation. The number of heterozygotes aged 40 years or greater is included below each mutation, to illustrate how many individuals are of an age where CAD may have developed. The number of unaffected family members >40 yrs is 69.

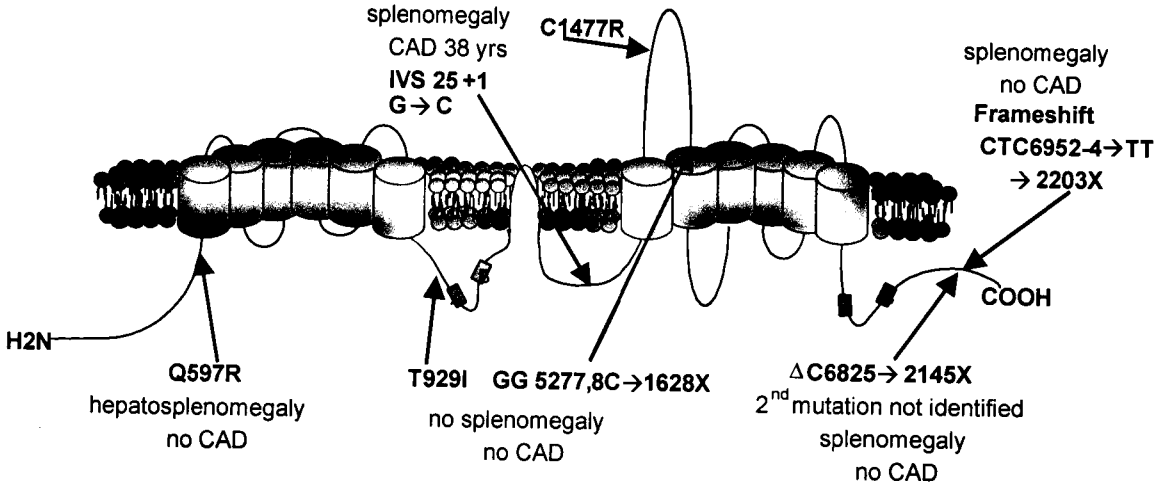

Figure 7.5. Mutations in TD and the clinical phenotype.
The clinical phenotype of each of the five TD probands is shown along with the location of their mutations. No pattern between the location of the mutations and the presence or absence of CAD, splenomegaly or hepatomegaly is observed to date.

7.6 The phenotype of mutations in the ABCA1 gene is modified by age

One factor influencing phenotypic expression that became apparent in our families was age. This was first brought to our attention in two of the families initially investigated (Figure 7.6). In family FHA3, while heterozygous individuals in older generations all had HDL-C levels $<5^{th}$ percentile for age and sex, those in the youngest generation had a much more variable phenotype, with HDL-C ranging up to the 20^{th} percentile. In family FHA1 the same pattern was observed.

We compared the distribution of individuals across HDL-C percentile ranges in those <30 vs. 30-<70 years (Figure 7.7). A significantly larger percentage of individuals 30-70 years of age had HDL-C$<5^{th}$ percentile than those <30 years. Mean HDL-C decreases in heterozygotes greater than 30 years of age compared to those less than 30 years of age, whereas there is no significant change in unaffected controls (Table 7.4). Similar results are seen in males and females separately and are seen at both pre- and post-menopausal ages in women (Figure 7.8). Triglycerides increase with age in both heterozygotes and unaffected family members (Table 7.4).

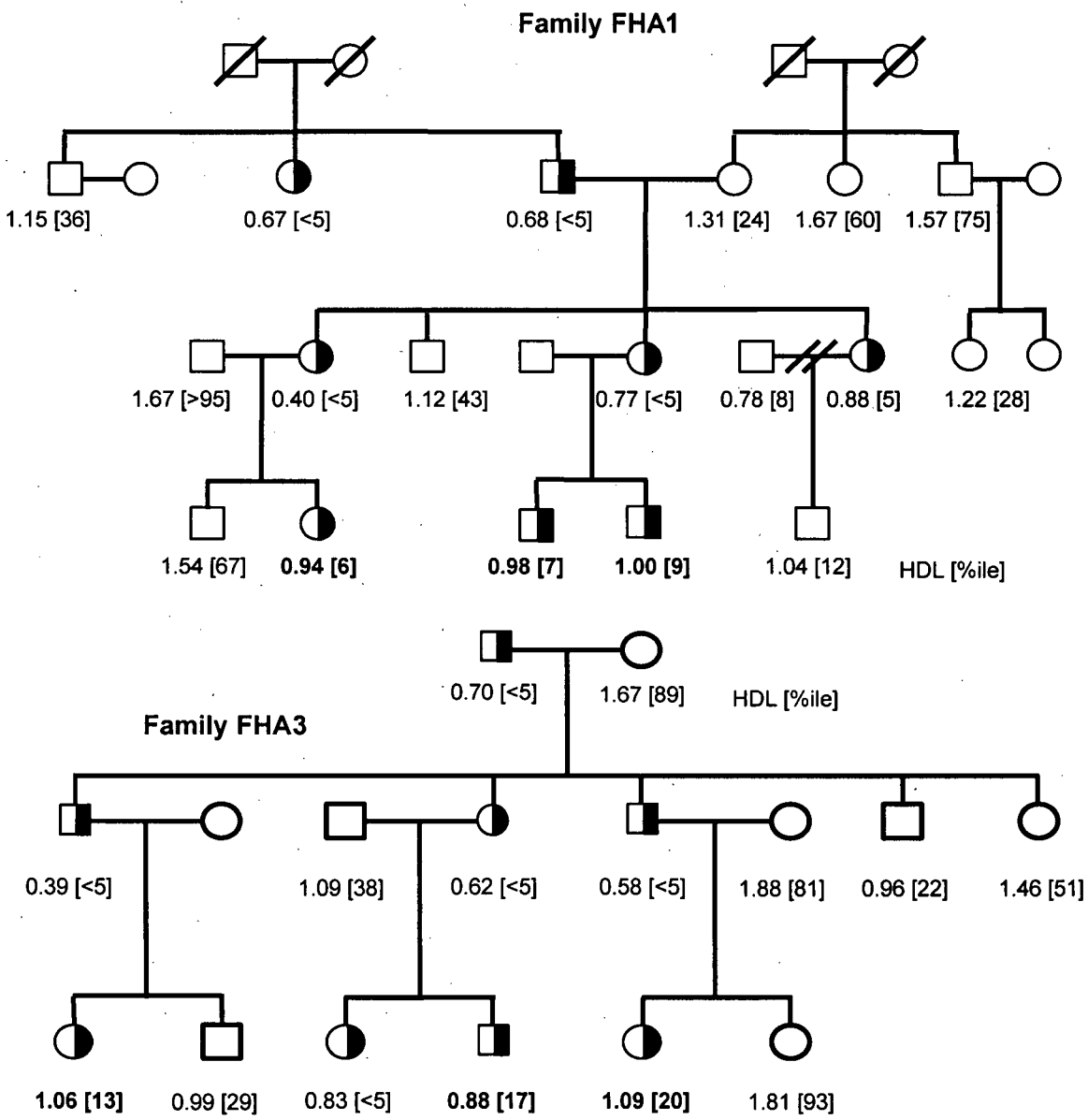

Figure 7.6. HDL-C in families FHA1 and FHA3.
The pedigree symbols are as described in the figures in Chapter 6. The data below each symbol indicates the individual's HDL-C level [percentile value]. The phenotype of HDL-C < 5th percentile for age and sex is more penetrant in the older individuals in each of these families. Younger individuals heterozygous for *ABCA1* mutations more often have HDL-C that is >5th percentile.

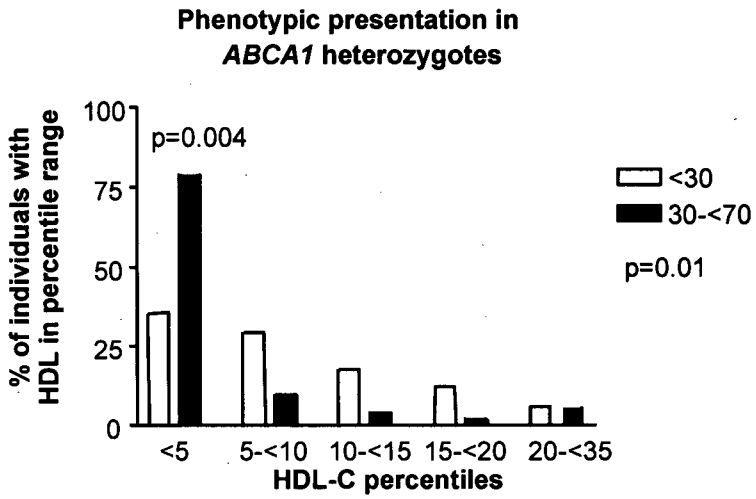

Figure 7.7. HDL-C percentiles by age in *ABCA1* heterozygotes.
The percentage of individuals less than 30 years of age (white bars) and from 30 to less than 70 years of age (black bars) with HDL-C levels in a given percentile range are plotted. Younger individuals have a far broader distribution of HDL-C levels, clearly indicating that the impact of ABCA1 on HDL-C levels is influenced by age, and more obvious in older individuals. A significantly smaller percentage of younger individuals have HDL-C <5th percentile.

Table 7.4. Mean HDL and TG by age in *ABCA1* heterozygotes

	Heterozygotes mean±SD (n)	**Unaffected** mean±SD (n)	**P-value Heterozygotes vs. Unaffected**
HDL (mmol/L)			
<30	0.91±0.16 (17)	1.26±0.29 (51)	**<0.0001**
≥30	0.66±0.24 (52)	1.32±0.36 (90)	**<0.0001**
Change	-0.25	+0.06	0.21
p-value <30 vs. ≥30	**0.0002**	0.23	
TG (mmol/L)			
<30	1.07±0.96 (16)	0.88±0.45 (51)	0.26
≥30	1.84±1.79 (52)	1.36±1.24 (87)	*0.07*
Change	+0.77	+0.48	0.97
p-value <30 vs. ≥30	**0.03**	**0.001**	

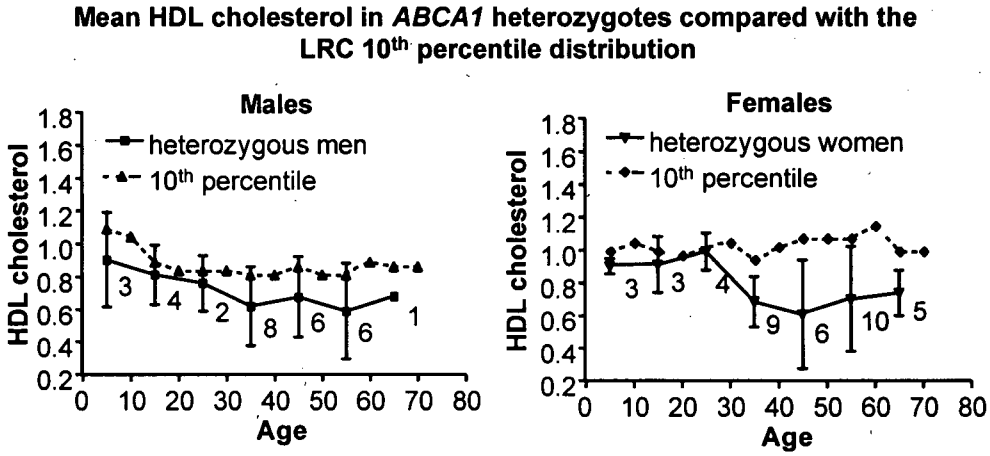

Figure 7.8. Mean HDL-C in *ABCA1* heterozygotes by age.
The mean HDL-C in heterozygous males and females in 10 yr age groups (plotted at the half-way point of each decade; squares and solid line) are shown compared to the 10th percentile distribution in the Lipid Research Clinics (LRC) population[451] (triangles or diamonds and dashed line). Error bars represent the standard deviation of each mean. The number of individuals in each group is shown under each data point. Beyond the age of 30, mean HDL-C levels in heterozygotes fall much lower than the 10th percentile distribution, while less than 30 years of age, mean HDL-C levels in the heterozygotes more closely approximate the 10th percentile distribution, in both males and females.

7.7 Assessment of the influences of gender and BMI on the phenotypic expression of ABCA1 mutations

Females are known to have elevated HDL-C and decreased TG compared to males[452]. Thus, we sought to address whether the phenotype of *ABCA1* heterozygotes was influenced by gender. HDL-C is significantly lower than unaffected controls in both heterozygous males and females (0.70±0.24 vs. 1.21±0.29, $p<0.0001$; 0.76±0.25 vs. 1.41±0.38, $p<0.0001$, respectively). This was reflected in decreased apoAI (0.92±0.27 vs. 1.36±0.22, $p<0.0001$; 0.92±0.36 vs. 1.49±0.28, $p<0.0001$ in males and females, respectively), and a trend towards a mild decrease in apoAII in both males and females compared to unaffected family members (0.35±0.08 vs. 0.40±0.09, $p=0.08$; 0.35±0.09 vs. 0.39±0.07, $p=0.06$, respectively). TG are higher in both male (2.07±2.16 vs. 1.30±1.30, $p=0.02$) and female (1.34±0.86 vs. 1.09±0.63, $p=0.08$) heterozygotes compared to unaffected family members.

Another factor known to influence HDL-C and TG levels is BMI[455]. The entire cohort was divided into tertiles of BMI. The mean HDL-C and TG levels of heterozygotes and unaffected individuals by BMI tertile are shown in Figure 7.9. BMI had a significant effect on both HDL-C and TG in both heterozygotes and controls ($p\leq0.0001$). The effect of BMI on HDL-C and TG was more severe in heterozygotes than in controls, being evident at lower BMIs

(mid-tertile) in heterozygotes. A raised BMI was more obviously associated with changes in HDL-C and TG in heterozygotes compared to controls. However, neither effect reached statistical significance. HDL-C was reduced in heterozygotes compared to controls in all BMI tertiles ($p<0.0001$ in each tertile). While TG were increased in all BMI tertiles in heterozygotes compared to unaffected family members, this difference was only significant in the middle BMI tertile ($p=0.009$).

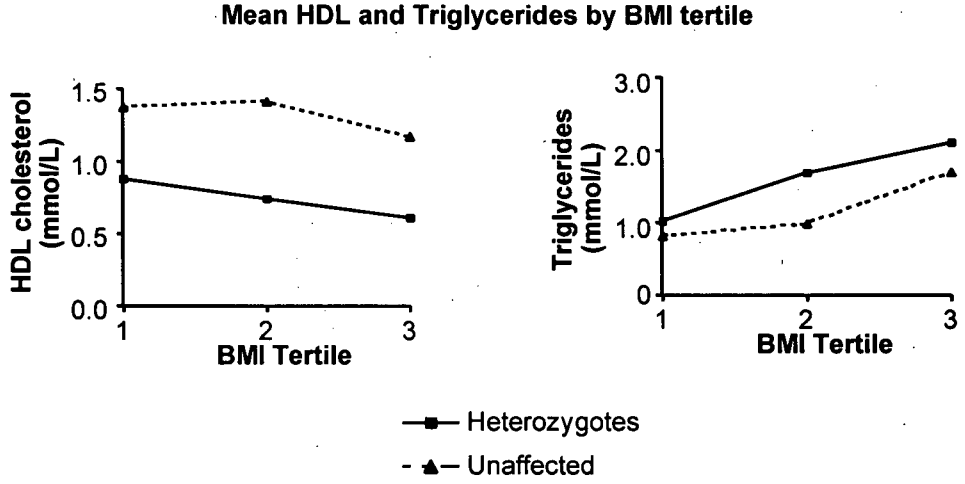

Figure 7.9. Mean HDL-C and TG levels by BMI.
The mean HDL-C and TG levels in heterozygotes (solid line and squares) and unaffected family members (dashed line and triangles) of the individuals falling within each tertile of BMI are shown. The tertiles of BMI correspond to the following values: 1- BMI <21.4; 2- 21.4≤BMI≤25.1; 3- BMI>25.1. The effects of BMI on decreasing HDL-C and increasing TG are more evident in heterozygotes, being seen in the second as well as third tertiles. In unaffected family members, the effects of BMI are primarily manifest in the highest (3rd) BMI tertile.

7.8 Discussion

The reverse transport of cholesterol from peripheral cells to sites of catabolism, first described by Glomset[56], has been hypothesized to be the primary mechanism whereby HDL-C is antiatherogenic. However, there has been little direct evidence that changes in this pathway are associated with changes in HDL-C levels and susceptibility to CAD[95]. Specifically, there has been no direct evidence linking efflux of cholesterol from peripheral cells, the initial step of the reverse cholesterol transport pathway, to CAD.

With the identification of the ABCA1 protein as a key initiator of the efflux pathway, it has now been possible to directly relate cholesterol efflux, HDL-C levels and CAD. For the first time we have been able to describe the phenotype in heterozygotes for different mutations in the

ABCA1 gene in a large cohort where diagnosis has been made by mutation identification. Furthermore, we have been able to compare this to a cohort of unaffected family members, allowing us to control, at least in part, for other genetic and environmental influences.

Here we have shown that *ABCA1* heterozygotes have an approximate 50% decrease in HDL-C and apoAI, and a mild but significant decrease in apoAII. In addition, heterozygotes have increased TG, but in contrast to TD patients, have no significant change in total or LDL cholesterol. The changes in HDL-C, apoAI, and TG were gene-dose dependent, suggesting they are directly related to ABCA1 function. Furthermore, heterozygotes have an over three-fold increased risk of developing CAD, and younger average age of onset compared to unaffected individuals. Furthermore, those heterozygotes with most severe deficiency in efflux had a higher frequency of CAD. It should be noted, however, that the absolute number of CAD cases is small and two of the sixty-two adult heterozygotes were identified on the basis of their CAD. Thus additional studies examining the extent of CAD in a randomly ascertained heterozygote population will be important to confirm these findings.

Mutations are observed throughout the protein. Interestingly, the severity of the phenotype observed in the heterozygotes appeared to be mutation-dependent, but there was no obvious relationship between the site of mutation and the phenotype, either in TD probands or in relationship to CAD in the family. There was a trend towards lower HDL-C in carriers of the severe mutations, causing truncations or null alleles, compared to carriers of missense mutations. One notable exception is the M1091T missense mutation, which had the most severe phenotype, with marked reductions in HDL-C and efflux in affected family members, suggesting that this mutation may act in a dominant-negative fashion, downregulating the function of the wildtype allele. Another interesting finding is the small cluster of mutations at the very C-terminal region of the protein, which suggests that this region must be critical to ABCA1 function.

The severe HDL deficiency in *ABCA1* heterozygotes suggests that residual cholesterol efflux is the major determinant of HDL-C levels. A report has recently appeared, correlating efflux with HDL-C levels in a small number (n=9) of heterozygotes from one family[203]. Our results extend these findings to multiple families, directly linking residual ABCA1 efflux activity to HDL-C levels and now also to the risk of CAD. From the regression equation of mean HDL-C on efflux, we would predict that each 8% increase in relative efflux is associated with a 0.1 mmol/L increase in HDL-C levels. Alternatively, a 50% increase in ABCA1-mediated

cholesterol efflux would be predicted to result in a 30% increase in HDL-C in a normal 40-year-old male. Although these numbers may not directly extrapolate to what is observed in a general population where other genetic and environmental factors have not been controlled for, these data nonetheless suggest that relatively small changes in ABCA1 function may have a significant impact on plasma HDL-C levels. Furthermore, the data presented here suggest that variations in efflux due to variations in ABCA1 function directly reflect not only plasma HDL-C levels but also CAD susceptibility, thus providing direct validation of the reverse cholesterol transport hypothesis and validation of ABCA1 as a therapeutic target to raise HDL-C and protect against atherosclerosis.

We have also shown that the phenotype in *ABCA1* heterozygotes is age modulated. From 20 years of age there is a small but definite increase in HDL-C with advancing age that is obviously absent in the heterozygotes. One explanation for this finding is that there is normally an age-related increase in ABCA1 function, which is not seen in heterozygotes, perhaps because the remaining functioning allele has already been maximally upregulated secondary to an increase in intracellular cholesterol. This would exaggerate the phenotype in older age groups. There is some evidence for an age-modulated increase of the ABC transporters[456]. Further evidence of a potential age-related increase in ABCA1 function comes from the observation that the percentage of apoAI found in the preβ_1 subfraction of HDL, the predominant cholesterol acceptors, decreases with age[457], suggesting increased formation of mature α-migrating HDL particles with age. Clearly additional experiments directly assessing the impact of age on ABCA1 function are needed to address this.

Here we have shown that heterozygotes for *ABCA1* mutations have age modulated decreases in HDL-C with significantly increased risk for CAD. Furthermore, this phenotype was highly correlated with efflux, clearly demonstrating that impairment of reverse cholesterol transport is associated with decreased plasma HDL-C and increased atherogenesis. These findings are important in that an increased atherogenic risk in heterozygotes has not been previously recognized[440]. In conclusion, our data suggests that therapies designed to specifically increase ABCA1 function should be associated with increased plasma HDL-C and protection against atherosclerosis.

Chapter 8: Single nucleotide polymorphism analysis of the ABCA1 gene

The work presented in this chapter is published in part in

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The work has also been published in abstract form

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Clee S.M., et al. *Poster presentation*, 73rd Scientific Sessions of the American Heart Association, New Orleans LA Nov. 12-15, 2000. *Circulation* 2000 102 (18):II-278.

Preface

I have designed and coordinated this study, and analyzed and interpreted the results presented herein. The REGRESS study and all its subsequent studies have been coordinated by Dr. W. Jukema, and the data set maintained in The Netherlands. Dr. A. Zwinderman has acted as the statistician for the study. I have directed the analysis, but he has performed the statistics in this chapter. The subsequent between-group comparisons were performed by me, when the data was available. Genotyping of some SNPs was performed under the guidance of Dr. H. Molhuizen and Dr. M. van Dam in the group of Dr. J. Kastelein. High throughput genotyping of some other variants with the TaqMan assay was performed by Dr. J. Engert in Dr. T. Hudson's group. The efflux assays described in this chapter were performed by M. van Wijland. The SNPs were identified during the sequencing of *ABCA1* performed by K. Roomp, under the direction of Dr. A. Brooks-Wilson. The replication study for the R219K was performed by K. Zwarts, under my direction. Some DNA samples for the replication study were provided by Dr. J. Genest, Jr.

8.1 Introduction

The work described in Chapter 7 has shown that individuals heterozygous for mutations in the *ABCA1* gene have decreased HDL-C, increased TG, and an approximately 3-fold increased risk of CAD[202]. Specific mutations associated with complete or near complete loss of ABCA1 function are not found at a high frequency in patients presenting with low HDL-C (Chapter 6), and are not likely to be a common cause of low HDL-C in the general population. Thus, their impact on plasma lipid levels and CAD at the population level is predicted to be fairly small.

During the sequencing of *ABCA1* described in Chapter 6, several SNPs were identified. Additional SNPs were identified during the sequencing of the additional probands described in Chapter 7. We have shown in Chapter 5 that common *LPL* cSNPs are associated with altered lipid levels and CAD risk. These cSNPs may have large population effects, despite relatively small effects themselves. As several SNPs were identified throughout the *ABCA1* gene, and as the extent to which common variation in the *ABCA1* gene influences these phenotypes in the general population is uncertain, we have sought to address whether variants having milder effects on ABCA1 function influence plasma lipid levels and risk of CAD. As cSNPs that change amino acids (non-synonymous) are the ones most likely to directly influence ABCA1 function, we have focused initially on those ten cSNPs.

8.2 Methods

8.2.1 Identification of SNPs

SNPs in the *ABCA1* gene were identified during the complete genomic sequencing of 16 unrelated TD and FHA probands[52,445]. This includes two additional TD probands from Japan, identified subsequent to the analysis in Chapter 7, and three individuals who did not possess *ABCA1* mutations. Variants that were also observed in unrelated, unaffected individuals or that did not co-segregate with the low HDL-C phenotype were assumed to be SNPs. In addition, BAC clones spanning the entire region have been sequenced, as described in Chapter 6, and sites identified as heterozygous, or different to the consensus obtained from the other individuals sequenced were also identified as polymorphisms. The SNPs are numbered from the nucleotide described as position 1[184], naming the first exon number 1. This is the same nucleotide numbering system as was used in Chapters 6 and 7. As a standardized nomenclature for all

variants, the allele that was more frequent in Caucasians was designated A, while the variant (less frequent) allele was designated B.

8.2.2 Subjects

To assess the frequency with which the cSNPs are found and to examine possible differences in frequency between individuals with and without CAD or low HDL-C, we genotyped cohorts of Dutch subjects with low HDL-C[340,405,458] or premature CAD[459-461] obtained from previously described populations[460,461]. Low HDL-C was defined as HDL-C less than the 10th percentile, while the premature CAD cohort was comprised of individuals who had manifest CAD before the age of 50. Dutch control subjects were taken from a large population based study designed to assess the effects of various risk factors on CAD[340,462]. French Canadian subjects were a random sample of individuals ascertained as part of routine healthcare. All subjects gave informed consent.

To assess the effects of these SNPs on lipid levels and CAD, we studied a cohort of 804 Dutch men who participated in the Regression Growth Evaluation Statin Study (REGRESS), which has previously been described in detail[463]. Study participants were males less than 70 years of age with at least one coronary artery with a stenosis of greater than 50% as assessed by coronary angiography. Inclusion criteria included plasma total cholesterol between 4 and 8 mmol/L (155 to 310 mg/dL) and plasma TG concentration less than 4 mmol/L (350 mg/dL). HDL-C levels were not used as a selection criterion. The phenotypic effects of the cSNPs were examined in relationship to baseline lipid parameters. Several genetic studies have previously been performed on this cohort[141,144,272,464,465]. The REGRESS and its DNA substudies were approved by all seven institutional review boards of the participating centres and by their medical ethics committees.

For replication studies we have genotyped three small cohorts available in the lab. As reliable, standardized information on CAD was not available on all cohorts, we have not included CAD in the replication analysis. These cohorts comprised: individuals of European descent with familial hypercholesterolemia seen at the lipid clinic at St. Paul's Hospital in Vancouver (described in Section 5.5); a group of French Canadians with CAD and low HDL-C (less than 0.86 mmol/L); and a random sample of French Canadians without clinical manifestations of CAD, unselected for plasma lipid levels. All known individuals who were

diabetic, had the apoE2 allele, BMI>30 or TG>5mmol/L were excluded. Comparisons were performed on a case-control basis to avoid stratification by ethnicity or other demographic factors. For each BB identified, an AA individual matched for age, sex and BMI from the same cohort was selected without regard to lipid levels. All individuals gave informed consent.

For studies of the ethnic frequencies of the R219K variant, the Cantonese and South African Black individuals described in Chapter 5 were also genotyped.

8.2.3 Coronary artery disease measurements

Computer-assisted quantitative coronary angiography was carried out as previously described as part of the REGRESS protocol[463]. The mean segment diameter (MSD) measures the average luminal diameter along the vessel, reflecting diffuse atherosclerotic differences. The minimum obstruction diameter (MOD) represents the smallest vessel diameter at an obstructed site, assessing focal atherosclerotic changes (Figure 8.1). Larger MSD and MOD measurements therefore reflect less vessel occlusion. Events during the study (death, MI, unscheduled PTCA or CABG, and stroke/TIA) were also examined.

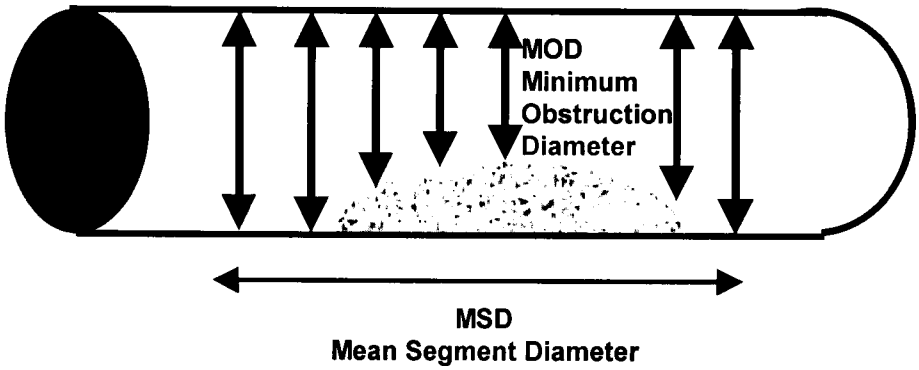

Figure 8.1. Measures of atherosclerosis in REGRESS.
The means segment diameter (MSD) measures the average unobstructed diameter along the length of the vessel. The minimum obstruction diameter (MOD) measures the minimum unobstructed diameter at the site of an obstruction.

8.2.4 cSNP screening

For each variant, we identified a restriction enzyme whose cleavage pattern was altered by the variant for development of an RFLP assay. If no suitable enzyme was found, a mismatch strategy was employed, whereby a single nucleotide mismatch was incorporated into the PCR primer, creating a restriction site in combination with either the wildtype or variant allele, similar

Table 8.1. Methods for RFLP screening of *ABCA1* cSNPs

Variant	"B" allele	Forward oligo (5'-->3')[a] Reverse oligo (5'-->3')[a]	Annealing temp. (C)	Enzyme	Products (bp): A allele B allele	% agarose gel for resolution
G1051A (R219K)	K	GTATTTTTGCAAGGCTACCAGTTACATTTGACAA GATTGGCTTCAGGATGTCCATGTTGGAA	60	*EcoN* I	177 107,70	1.5
T1591C (V399A)	A	GCTGCTGTGATGGGGTATCT ACCTCACTCACACCTGGGAA	57	*Hph* I	117,103,48,33 220,48,33	1.5
G2706A (V771M)	M	CAAGTGAGTGCTTGGGATTG TGCTTTTATTCAGGGACTCCA	57	*BsaA* I	98, 252 350	2
A2715C (T774P)	P	GTGATCCCAGCGTGGTGTTTGTCTT GAAAGGCCAGAGGTACTCACAGCGAAGATCTTGAG**G**G	55	*Hph* I	56,69,95 56,161	2
G2723C (K776N)	N	TCGTTTTATTCAGGGACTCCA CAAGTGAGTGCTTGGGATTG	55	*Bgl* II	269,80 349	2
G2868A (V825I)	I	CCCATGCACTGCAGAGATTC GCAAATTCAAATTTCTCCAGG	57	*Bsa* I	149, 237 386	2
A3044G (I883M)	M	GAGAAGAGCCACCCTGGTTCCAACCAGAAGAG**G**AT AAGGCAGGAGACATCGCTT	55	*EcoR* V	94,35 129	2.5
G3911C (E1172D)	D	GAGCAGTTCTGATGCTGGCCTGGGCAGCGACCA**C**GA TCTGCACCTCTCCTCCTCTG	55	*BssS* I	104, 37 141	2
G5155A (R1587K)	K	CAGCTTGGGAAGATTTATGACAGGACTGGACAC**G**A ATGCCCCTGCCAACTTAC	55	*BssS* I	114, 31 145	2
C5587G (S1731C)	C	GTGCAATTACGTTGTCCCTGCCACACT CCATACAGCAAAAGTAGAAGGGCTAGCACA	60	*Mnl* I	82,35 117	3

[a] Bold indicates mismatch in oligo to create restriction site

to the strategy used for mutation assessment and to detect the *LPL* cSNPs. The specific conditions of all assays are described in Table 8.1. All PCR reactions were carried out in 50 μL volumes, in the presence of 1x PCR buffer, 1.5 μM $MgCl_2$ (Life Technologies), and 200μM each dNTP. Thermocycling parameters for all assays were as follows: 95°C 3 minutes; 35 cycles of denaturation at 95°C 10 seconds, annealing for 30 seconds at the temperature specified in Table 8.1, and elongation for 30 seconds at 72°C; and ended with a final elongation at 72°C for 10 minutes. Digestions (15-20 μL PCR product) were carried out in the manufacturer's buffer (New England Biolabs) for 2 hours at the temperature specified by the manufacturer, and products were resolved on agarose gels (Table 8.1).

8.2.5 Genotyping with the TaqMan® assay

To facilitate the mass screening of some variants, TaqMan® based assays[466,467] were developed for the genotyping of the V399A, V771M, T774P, I883M and E1172D cSNPs in the Dutch populations. In this one-tube assay, two fluorogenic hybridization probes (one for each allele) are labeled with different fluorescent reporter dyes (FAM or TET) at their 5' terminus and a common quencher dye (TAMRA) at their 3' terminus. These probes are cleaved by the 5' nuclease activity of Taq polymerase during PCR amplification. This cleavage separates the reporter from the quencher dye and generates an increase in reporter fluorescence. By using two different reporter dyes, cleavage of allele-specific probes can be detected in a single PCR. The difference in the measured fluorescence intensity between the two TaqMan probes allows for accurate allele calling when compared to known genotype standards included on each plate.

PCR amplifications with flanking sets of primers (300nM) in the presence of two TaqMan probes (25nM each) and 4.5 mM $MgCl_2$ were performed using the following thermocycling protocol: initial denaturation at 96°C for 10 minutes, followed by 39 cycles of 96°C for 30 seconds, 63°C for 1 minute and 72°C for 15 seconds, followed by a final extension at 72°C for 10 minutes. Each plate included controls (no DNA template) as well as the known genotype standards. Fluorescence quantification and genotype determination were performed on a Perkin Elmer LS50B or ABI Prism 7700 Sequence Detector. The fluorescence from each reaction was normalized to the signal from the no-template controls[468].

8.2.6 Cellular cholesterol efflux

Cholesterol efflux has been measured in a series of Dutch individuals with HDL-C less than the 5th percentile for age and sex. As these assays were performed in Amsterdam, the protocol is slightly modified from that presented in Chapter 7. Fibroblasts from a 3.0 mm punch biopsy were cultured in 24 well plates until confluence, washed with PBS containing 0.2% (wt/vol) fatty acid free BSA, 1.2 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS-BSA), and loaded with ^{3}H-cholesterol (0.5μCi/ml) for 24 h at 37°C in efflux medium (DMEM with 25 mM HEPES, 4 mM glutamine and 0.2% fatty acid free BSA). The final concentration of cholesterol was 30 μg/ml. After loading, the cells were washed four times with PBS-BSA and incubated with 5μg/ml ApoAI in efflux medium for 20 h at 37°C. The amount of cholesterol in medium and cells was determined by liquid scintillation counting. Each experiment was performed in triplicate. Measurements are reported as the percentage efflux relative to the average of two healthy controls included within the same experiment. All individuals had efflux in the normal range (>60% of controls).

8.2.7 Statistics

The baseline characteristics of the patients in the three genotypes (AA, AB, BB) were compared using one-way ANOVA and the chi-square test, where appropriate. In cases where the BB genotype was rare, we also compared AA versus the combined group AB+BB. Subsequent comparisons between carriers and non-carriers were made using a t-test. P-values unadjusted for multiple comparisons are presented to allow the reader to reach their own conclusions regarding the significance. The cumulative event incidence was compared using the logrank test, and the event-free durations were plotted in Kaplan-Meier curves. The relationships between age and HDL-C or efflux were investigated using a linear regression model, and the slopes of the regression lines compared using covariance analysis (the interaction between age and genotype).

Randomization to placebo and pravastatin was assessed by chi-square analysis and was equivalent in all genotypic groups for all variants except the R1587K, where a lower proportion of carriers was randomized to pravastatin treatment. In addition, the change in MOD and MSD and events (the three variables measured during the trial and thus following randomization) were analyzed for the placebo and pravastatin subgroups separately. Similar genotypic effects for each of the variants were observed in the treatment subgroups. Thus, the combined results are

presented. All lipid levels are reported in mmol/L, and all values are reported as the mean ± standard deviation.

The population attributable risk (PAR) is calculated from the sum of each genotype frequency multiplied by its genotype relative risk in relation to the allele with the least risk. For the R219K, the genotype relative risks are thus calculated relative to the BB genotype (i.e. the BB genotype has relative risk = 1). The PAR is then calculated as: [this sum – 1]/ this sum.

For replication studies, BB and AA individuals were compared by one-tailed t-test to test for the specific differences in mean lipid levels seen in the REGRESS cohort. Although each cohort was small, statistical power was increased by combining the results in a meta-analysis, using the program Meta 5.3, freely available on internet (www.fu-berlin.de/gesund/gesu_engl/meta_e.htm).

8.3 Identification of cSNPs within the *ABCA1* gene

In the course of mutation detection within the *ABCA1* gene in TD and FHA families, we have obtained complete coding sequence information on the *ABCA1* gene of 16 unrelated individuals. Over 50 SNPs have been identified (Figure 8.2). A total of 16 polymorphisms within the coding region (cSNPs) have been identified. The frequency of 16 cSNPs in the 6.8 kb coding region yields an estimate of 1 cSNP approximately every 425 bp. This frequency is quite consistent with that seen in other genes[306,469,470]. It has been estimated that sequencing 16 individuals yields a 97% chance of identifying a variant present at a frequency of greater than or equal to 10%[471]. Since we have sequenced an equivalent number of individuals, it is likely that all the common variants have now been identified, with the possible exception of population-specific variants.

8.3.1 Frequencies of the cSNPs

As non-synonymous cSNPs (those that change amino acids) are the most likely to be associated with functional effects, we have chosen to focus on the 10 cSNPs that directly result in amino acid changes within the protein (Figure 8.2). We have examined the frequency of these cSNPs in several populations (Table 8.2) and also report the frequency of the synonymous

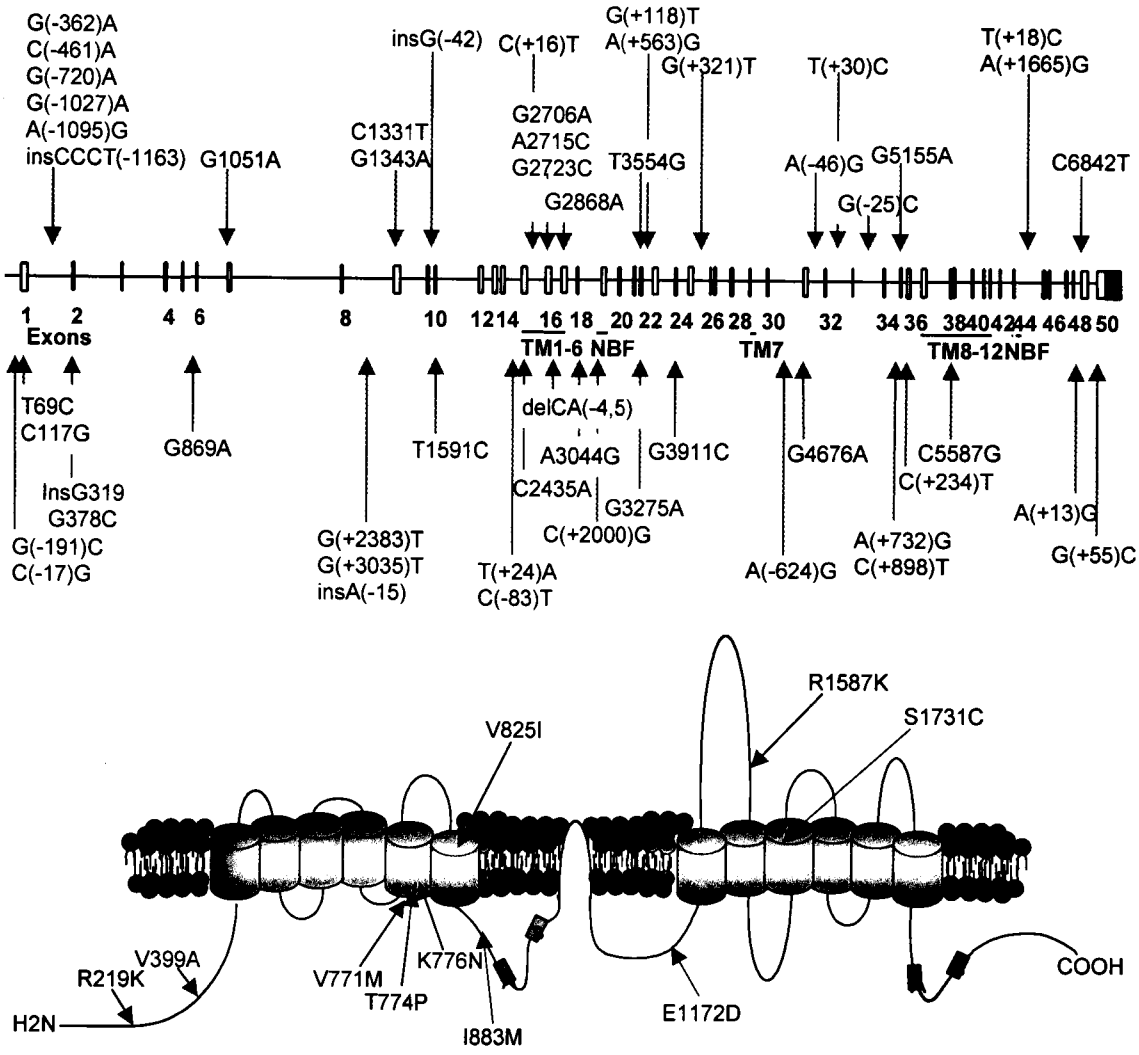

Figure 8.2. *ABCA1* SNPs.
The upper part of the figure illustrates a schematic of the *ABCA1* gene, showing all SNPs identified. Those located within introns are numbered as – when counted from the 3' end of the intron back into the intron or + from the 5' end of the intron, moving forward through the intron. Numerous SNPs have been identified. The locations of the non-synonymous cSNPs are shown on the protein diagram in the lower part. These generally occur at locations distinct from the mutations (as shown in Figure 7.1).

cSNPs derived from sequencing. All variants were observed at frequencies not different from those expected under Hardy-Weinberg equilibrium, except as discussed in subsequent sections. Strikingly, the I883M variant was found at nearly double the frequency in the Dutch premature CAD population compared to controls (p=0.007), although this difference was not found found between the REGRESS population and controls. As the REGRESS cohort includes individuals with CAD up to age 70, whereas the premature CAD cohort includes only

individuals with CAD before the age of 50, this suggests that this variant may be associated specifically with premature CAD. Furthermore, in the low HDL population, the E1172D variant was found at approximately 1/5 the frequency of the controls ($p=0.04$), suggesting it may be associated with increased HDL-C levels. The frequency of all variants was generally similar between the Dutch populations studied.

There were large differences in frequency between the Dutch and French Canadian control samples for some variants. For example, the T774P variant had a frequency in the French Canadian populations approximately 8 times higher than the average Dutch frequency, and the S1731C variant was only seen in French Canadian subjects. This emphasizes the importance of using controls derived from the same population when comparing frequencies, as differences in frequency can be large even within Caucasian populations.

8.3.2 cSNPs are located at sites distinct from and are less conserved than missense mutations

As can be seen in Figure 8.2, the non-synonymous cSNPs are distributed throughout the protein. However, this distribution is not random. These variants are found at locations distant from the ATP binding cassette regions, the N-terminal segment just prior to the first membrane spanning region, and the far C-terminus where mutations cluster (Figure 7.1). Of note, there is a small cluster of cSNPs at the beginning of the fifth membrane-spanning domain, suggesting that this may be a region of the protein not critical for proper ABCA1 function, where increased variation may be tolerated.

To assess the potential importance of the sites altered by these cSNPs, we have examined the conservation of these residues at both the nucleotide and amino acid levels in various species and in the two other ABCA family members most closely related to ABCA1, namely ABCA7[472] and ABCA4 (ABCR[472], Table 8.3). Nearly all of the missense mutations (described in references[51-53,202,444,445]) are conserved at the nucleotide level and over half at the amino acid level through to the *C. elegans* orthologue, suggesting they disrupt sites critical for normal ABC transporter function. In contrast, only five of the cSNPs are conserved at the nucleotide and amino acid levels in the chicken. Only 3 of these nucleotides and none of the amino acids are

Table 8.2. Frequencies of *ABCA1* cSNPs in CAD, low HDL and control populations

Nucleotide Change	Amino Acid Change	Exon	"B" allele	*REGRESS* Carrier Freq.	*REGRESS* Allele Freq.	*REGRESS* N[a]	*Dutch low HDL* Carrier Freq.	*Dutch low HDL* Allele Freq.	*Dutch low HDL* N	*Dutch premature CAD* Carrier Freq.	*Dutch premature CAD* Allele Freq.	*Dutch premature CAD* N	*Dutch control* Carrier Freq.	*Dutch control* Allele Freq.	*Dutch control* N	*French Canadian* Carrier Freq.	*French Canadian* Allele Freq.	*French Canadian* N
Non-synonymous																		
G1051A	R219K	7	A	46.3	0.254	1588	39.9	0.227	546	44.1	0.247	826	47.5	0.249	686	48.9	0.287	94
T1591C	V399A	11	C	1.6	0.008	1098	1.2	0.006	164	1.3	0.007	150	1.0	0.005	416	0	0	88
G2706A	V771M	16	A	5.8	0.029	1270	6.3	0.031	318	6.3	0.031	796	3.9	0.020	406	12.8	0.064	94
A2715C	T774P	16	C	0.6	0.003	1250	1.3	0.006	154	1.3	0.006	156	0.6	0.003	326	14.6	0.073	82
G2723C	K776N	16	C	0.5	0.003	1106	0	0	178	0.6	0.003	360	1.9	0.009	318	0	0	88
G2868A	V825I	17	A	15.7	0.081	1364	-	-	-	-	-	-	-	-	-	-	-	-
A3044G	I883M	18	G	23.8	0.136	840	19.7	0.102	264	45.9	0.262	122	26.8	0.153	314	25.0	0.125	88
G3911C	E1172D	24	C	5.3	0.026	1288	1.3	0.007	150	6.6	0.033	822	8.2	0.041	440	10.3	0.051	78
G5155A	R1587K	35	A	44.3	0.259	1566	46.1	0.271	542	50.0	0.302	808	51.1	0.285	446	46.7	0.278	90
C5587G	S1731C	38	G	0	0	558	0	0	182	0	0	746	0	0	158	2.2	0.011	92
Synonymous				***From Sequencing***														
G869A	none	6	A	62.5	0.38	32												
C1331T	none	9	T	31.3	0.19	32												
G1343A	none	9	A	25	0.133	32												
T3554G	none	22	G	12.5	0.059	32												
G4676A	none	30	G	6.3	0.06	32												
C6842T	none	49	T	6.3	0.033	32												

[a] N refers to the number of alleles screened; frequencies refer to the frequency of the B allele

Table 8.3. Comparison of conservation amongst *ABCA1*-related proteins of cSNPs compared to missense mutations

	Nucleotide							Amino acid						
Variant	**human**	**mouse**	**chicken**	**ABCA7**	**ABCA4**	***C.elegans***		**Variant**	**human**	**mouse**	**chicken**	**ABCA7**	**ABCA4**	***C.elegans***
cSNPs														
G1051A	G	G	A	A	C	-[b]		R219K	R	R	K	P	Q	-
T1591C	T	T	T	T	C	T		V399A	V	V	V	V	A	T
G2706A	G	G	G	G	A	T		V771M	V	V	V	L	M	T
A2715C	A	T	T	G	G	C		T774P	T	S	S	G	E	G
G2723C	G	G	G	G	G	T		K776N	K	K	K	R	K	R
G2868A	G	G	G	T	A	A		V825I	V	V	A	S	M	L
A3044G	A	G	C	-	A	G		I883M	I	V	P	D	I	A
G3911C	G	A	A	C	G	-		E1172D	E	E	E	G	P	-
G5155A	G	A	-	A	A	G		R1587K	R	K	K	Q	E	F
C5587G	C	C	C	C	C	C		S1731C	S	S	S	A	S	H
Missense mutations*														
C2154T	C	C	C	C	C	G		R587W	R	R	R	R	R	R
G2164C	G	G	G	G	G	C		W590S	W	W	W	W	W	F
A2185G	A	A	A	A	A	A		Q597R	Q	Q	Q	Q	Q	-
C3181T	C	C	C	C	C	C		T929I	T	T	T	T	T	T
A3199G	A	A	A	A	A	A		N935S	N	N	N	N	N	N
C3205T	C	C	C	C	C	C		A937V	A	A	A	A	A	A
T3667C	T	T	T	T	T	T		M1091T	M	M	M	L	M	M
G4260A	G	G	G	G	A	G		D1289L	D	D	D	Q	A	D
T4824C	T	T	-	T	T	G		C1477R	C	C	C	C	C	V
A5793C	A	A	A	A	A	A		N1800H	N	N	N	S	N	W
C6844T	C	C	C	C	C	T		P2150L	P	P	P	S	P	-

[a] ABCA4=ABCR

[b] - corresponds to a gap in the alignment

* described by us in this thesis and in references[51-53,202,444,445]

conserved through to the *C. elegans* orthologue. Additionally, we would predict that those cSNPs showing a lower degree of conservation might be less likely to be important for proper protein function.

8.4 The *ABCA1* R219K cSNP is associated with altered lipoprotein levels and reduced coronary artery disease

8.4.1 The R219K polymorphism is associated with a decreased severity of CAD

The G1051A polymorphism results in the substitution of a lysine for arginine at amino acid 219 of the ABCA1 protein (R219K). The allele frequency of the lysine, or "B", allele was 25.4%, and its carrier frequency was 46.3% in the REGRESS population (Table 8.2).

The B allele of the R219K polymorphism was associated with decreased severity of CAD (Table 8.4). The mean segment diameter (MSD, diffuse atherosclerosis) increased from 2.70±0.37 to 2.77±0.37 to 2.78±0.40 (p=0.02 by ANOVA) from AA to AB to BB. The minimum obstruction diameter (MOD, focal atherosclerosis) increased from 1.73±0.35 to 1.81±0.35 to 1.85±0.35 (p=0.004 by ANOVA).

Table 8.4. Coronary artery disease in R219K carriers compared to controls

R219K	AA	AB	BB	Carriers AB+BB	P-value AB vs. AA	BB vs. AA	AB+BB vs. AA
n	424	330	36	366			
MSD	2.70±0.37	2.77±0.37	2.78±0.40	2.77±0.37	**0.01**	0.22	**0.005**
MOD	1.73±0.35	1.81±0.35	1.85±0.35	1.81±0.35	**0.002**	0.05	**0.001**
MI before trial %(n)	48.3 (205)	47.1 (155)	33.3 (12)	45.8 (167)	0.71	0.12	0.48
events during trial %(n)	17 (71)	13 (41)	11 (4)	12 (45)	*0.10*	0.49	*0.09*
total events (%)	59 (248)	52 (170)	39 (14)	50 (184)	*0.06*[a]	**0.03**[b]	**0.02**[c]

[a] Odds ratio for AB vs. AA=0.75, 95% confidence interval 0.56-1.01
[b] Odds ratio for BB vs. AA=0.45, 95% confidence interval 0.22-0.91
[c] Odds ratio for AB+BB vs. AA=0.72, 95% confidence interval 0.54-0.95

The angiographic data were paralleled by differences in clinical events. A smaller percentage of individuals homozygous for the B allele had had an MI prior to the trial (33% vs.

48%), although this did not reach significance (p=0.12, Table 8.4). Carriers had 29% less events (death, MI, unscheduled PTCA or CABG, stroke, TIA) during the study compared to non-carriers (Figure 8.3, p=0.07). Furthermore, total events (individuals with either a prior MI or an event during the trial) were significantly reduced in BB compared to AA individuals (39% vs. 59%, p=0.03; odds ratio for BB=0.45, 95% confidence interval 0.22-0.91). Conversely, this translates to a two-fold increased risk (odds ratio=2.2, 95% confidence interval 1.1-4.4) for AA individuals relative to BB.

There were no significant differences in demographic factors that may account for this, including blood pressure (systolic and diastolic), plasma glucose levels, or smoking behaviour between the genotypes. Body mass index was slightly higher in heterozygotes compared to either homozygous genotype, and thus seems unlikely to be responsible for the gene-dose dependent trends observed.

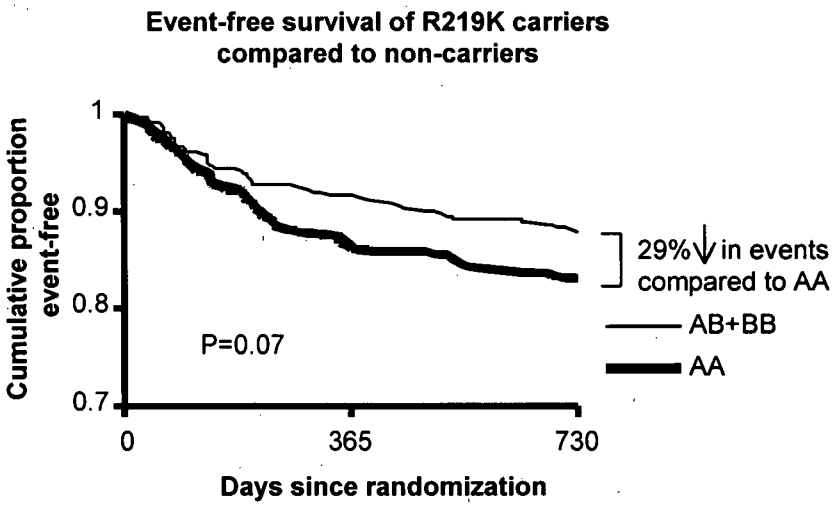

Figure 8.3. Event-free survival in the REGRESS trial.
Event-free survival curves for carriers (AB+BB, thin line) and non-carriers (AA, thick line) of the R219K. The curves represent the cumulative proportion of the cohort that have remained event-free during the 2-year trial. Carriers of the variant have a 29% increased event-free survival compared with non-carriers.

From the increased relative risk associated with the AB and AA genotypes compared to the BB, the population attributable risk was calculated. For the R219K variant, the population attributable risk is 5.3%, suggesting that the frequency of CAD events would be 5.3% lower if all individuals carried the BB genotype.

If the B-allele of the R219K variant is protective against CAD, we might expect its frequency to be reduced in this cohort that was selected for CAD. Indeed, the genotype

frequencies observed for this variant are not consistent with Hardy-Weinberg equilibrium (p=0.004). Fewer BB individuals were observed than would be expected (observed=36, expected=51, p=0.04), suggesting a preferential exclusion of BB individuals from this cohort.

8.4.2 Association of the R219K polymorphism with plasma lipid levels

TG were significantly lower in the carriers of the B allele (1.42±0.49 (BB) vs. 1.78±0.78 (AB) vs. 1.84±0.77 (AA), p=0.007, Table 8.5). As we have previously shown that decreased ABCA1 function is associated with increased TG (Chapter 7)[202], this suggests that the R219K variant may be associated with a gain of ABCA1 function, and that modulation of TG may be a mechanism whereby ABCA1 activity influences risk of CAD. In contrast, there were no differences in mean HDL-C levels between the genotypes (Table 8.5).

Table 8.5. Baseline demographics and lipid levels in the REGRESS cohort by R219K genotype

R219K	AA	AB	BB	AB+BB	P-value		
					AB vs. AA	BB vs. AA	AB+BB vs. AA
n	424	330	36	366			
Age (years)	57±8	55±8	57±7	55±8	**0.0007**	1.00	**0.03**
BMI (kg/m^2)	25.8±2.6	26.3±2.7	25.5±2.3	26.2±2.7	**0.01**	0.50	*0.09*
TC (mmol/L)	6.02±0.86	6.07±0.89	5.89±0.85	6.06±0.89	0.44	0.38	0.60
HDL-C (mmol/L)	0.92±0.22	0.93±0.23	0.92±0.20	0.93±0.23	0.54	1.00	0.81
LDL-C (mmol/L)	4.27±0.75	4.35±0.83	4.33±0.82	4.35±0.83	*0.17*	0.65	*0.19*
TG (mmol/L)	1.84±0.77	1.78±0.78	1.42±0.49	1.74±0.76	0.29	**0.001**	*0.08*

8.4.3 Age modifies the phenotypic expression of the R219K variant in the REGRESS population

The phenotype of individuals heterozygous for *ABCA1* mutations becomes more pronounced in older individuals (Chapter 7)[202]. To explore the apparent lack of effect of the R219K on HDL-C, we examined HDL-C levels in age-defined subgroups. In individuals less than the median age of the cohort (56.7 years), carriers had a trend towards increased HDL-C

compared to non-carriers (p=0.12, Table 8.6) that was no longer evident in those above the median age.

We then performed a linear regression analysis of HDL-C and age. In AA individuals, HDL-C increased significantly with age (p=0.0004). In contrast, this relationship was not apparent in carriers (p=0.66, Figure 8.4), such that the HDL-C difference between the genotypes was lost in the older individuals (p-value comparing slopes=0.04).

Table 8.6. Lipid levels and CAD above and below the median age in R219K carriers and controls

	< median		> median		P-value	P-value	
	n	mean±SD	n	mean±SD	< vs. > median	AB+BB vs. AA < median	AB+BB vs. AA > median
AB+BB							
TC (mmol/L)	193	6.22±0.91	172	5.87±0.82	**0.0001**	0.22	0.43
HDL-C (mmol/L)	192	0.91±0.22	171	0.94±0.23	0.21	*0.12*	0.37
LDL-C (mmol/L)	192	4.49±0.84	171	4.19±0.78	**0.0005**	**0.03**	0.57
TG (mmol/L)	193	1.82±0.79	172	1.65±0.72	**0.03**	**0.02**	0.85
MSD	193	2.79±0.37	171	2.75±0.37	0.30	*0.18*	**0.01**
MOD	193	1.83±0.36	171	1.78±0.34	*0.18*	*0.09*	**0.006**
AA							
TC (mmol/L)	207	6.11±0.86	217	5.94±0.84	**0.04**		
HDL-C (mmol/L)	206	0.88±0.20	214	0.96±0.24	**0.0002**		
LDL-C (mmol/L)	205	4.32±0.77	214	4.23±0.72	0.22		
TG (mmol/L)	206	2.02±0.82	217	1.67±0.67	**<0.0001**		
MSD	205	2.75±0.36	217	2.65±0.38	**0.006**		
MOD	205	1.77±0.34	217	1.69±0.35	**<0.0001**		

The changes in HDL-C with age are matched by trends in cholesterol efflux with age (Figure 8.4). We have genotyped this variant in the cohort of Dutch individuals in whom we have measured efflux. In R219K AA individuals (n=16), cholesterol efflux increases with age, whereas in AB and BB individuals (n=22) efflux decreases with age (p-value comparing slopes=0.07). Thus, the differential age-related changes in HDL-C seen in the different genotypes are consistent with similar functional changes in ABCA1 activity. In younger

individuals, cholesterol efflux and HDL-C are increased in BB compared to AA individuals, which suggests that the R219K variant may be especially protective against premature CAD.

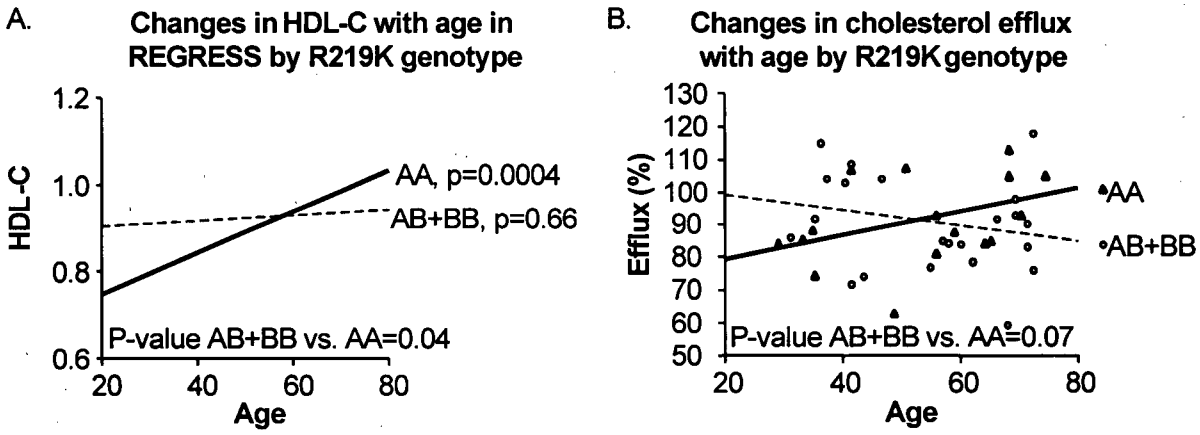

Figure 8.4. Changes in HDL-C and efflux with age, by R219K genotype.
(A) The left panel depicts the relationship between HDL-C and age in R219K carriers (AB+BB, dashed line) compared to non-carriers (AA, solid line). HDL-C increases significantly with age (p=0.0004) in the non-carriers but not in carriers (p=0.66). **(B)** The right panel depicts the relationship between cholesterol efflux and age in carriers (open circles and dashed line) and non-carriers (black triangles and solid line). Consistent with the findings for HDL-C, cholesterol efflux increases with age in non-carriers but not in carriers.

8.4.4 Age subgroup analysis indicates CAD progresses more slowly in R219K carriers compared to non-carriers

Interesting differences between the genotypes in the MSD and MOD were also observed in the age defined groups (Table 8.6). In the non-carriers, MSD and MOD decrease significantly with age (p=0.006, p<0.0001, respectively), reflecting increased atherosclerosis in the older

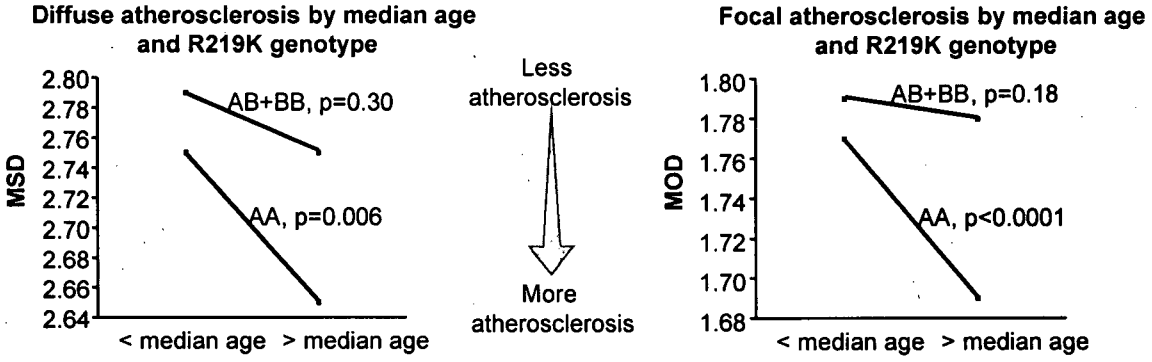

Figure 8.5. Changes in atherosclerosis with age.
Change in diffuse (MSD) and focal (MOD) atherosclerosis by median age in carriers (AB+BB) and non-carriers (AA) of the R219K. The average MSD and MOD in carriers and non-carriers above and below the median age is plotted. In non-carriers both MSD and MOD decrease significantly with age, reflecting progression of atherosclerosis. In carriers, the decrease in MSD and MOD with age is much less, suggesting a slower progression of atherosclerosis.

individuals (Table 8.6, Figure 8.5). In contrast, in carriers of the R219K variant (AB+BB) these measurements do not significantly change with age (p=0.30, p=0.18, respectively). As the total number of carriers (366) is similar to the number of non-carriers (422), this is unlikely to be related to differences in statistical power between the two groups. Thus, vascular disease progresses more slowly with age in carriers of the R219K compared to non-carriers.

8.4.5 Replication cohorts show the R219K variant is associated with decreased TG and increased HDL-C

One limitation of association studies is the subsequent inability to replicate the findings. To replicate the relationship observed between the R219K variant and plasma lipid levels, we have genotyped this variant in three small additional Caucasian cohorts available within the lab. To control for other factors that may influence lipid levels, we performed the analyses in a case-control fashion. For every BB individual identified, an AA individual matched for age, sex and BMI was selected from the same cohort.

Table 8.7. Mean HDL-C and TG in replication case-control cohorts

R219K	BB	AA	Number BB-AA pairs	P-value
HDL-C				
European FH	1.27±0.29	1.15±0.28	24	0.08
French Canadian CAD	0.78±0.05	0.70±0.06	6	0.02
French Canadian no CAD	1.59±0.28	1.38±0.38	4	0.20
All cohorts combined				**0.02**
Triglycerides				
European FH	1.55±0.83	1.77±0.80	24	0.18
French Canadian CAD	2.38±0.97	3.94±2.16	6	0.07
French Canadian no CAD	1.26±0.09	1.75±0.93	4	0.17
All cohorts combined				*0.08*

The mean plasma HDL-C and TG levels in the BB-AA pairs from each cohort are shown in Table 8.7. In each of the cohorts HDL-C is increased 10-15% in BB compared to AA individuals, regardless of the presence or absence of CAD in the cohort. Furthermore, TG are reduced in BB individuals in each of the cohorts compared to their matched AA pairs. Due to the small number of pairs in each cohort, comparisons within each cohort did not reach statistical significance. However, as trends were evident in each of the cohorts, we combined the results in a meta analysis to increase statistical power. Here we show that HDL-C is significantly increased in homozygous carriers compared to non-carriers ($p=0.02$), as was seen in the younger REGRESS individuals. Furthermore, there is a strong trend to decreased TG in BB individuals compared to AA individuals, which likely did not reach significance due to the increased variability of TG compared to HDL-C.

8.4.6 R219K is found at increased frequencies in Cantonese and Black individuals

To examine the relevance of this cSNP to populations of other ethnic origins, we examined its frequency in the Cantonese and South African Black populations described in Chapter 5. Remarkably, this variant is much more common in individuals of Cantonese and South African Black descent where it is the predominant, "wildtype", allele. Populations of Asian and African origins have been shown to have increased HDL-C, decreased TG and a decreased risk of CAD compared to Caucasian populations[382,473-478]. As this difference parallels the phenotype of the

Table 8.8. Frequency of the R219K cSNP in ABCA1 in different ethnic cohorts

	South African Black[a]	Cantonese	Dutch[a]	P-value South African vs. Dutch	P-value Cantonese vs. Dutch
AA % (n)	5.6 (5)	37.2 (137)	52.5 (180)		
AB % (n)	68.9 (62)	50.5 (186)	45.2 (155)	**<0.0001**	**<0.0001**
BB % (n)	25.6 (23)	12.2 (45)	2.3 (8)		
n	90	368	343		
carrier freq.	94.4	62.8	47.5	**<0.0001**	**<0.0001**
allele freq.	0.60	0.38	0.249	**<0.0001**	**<0.0001**

[a] Not consistent with Hardy Weinberg equilibrium ($p<0.001$)

R219K allele, this suggests that the increased frequency of this variant may in part account for the increased HDL, decreased TG and decreased CAD observed in these populations compared to Caucasians. Furthermore, the number of heterozygotes observed in these populations is in excess compared to that predicted under Hardy-Weinberg equilibrium (significant in the South African Black and randomly ascertained Dutch groups, $p<0.001$, $p<0.001$; $p=0.13$ in Cantonese), suggesting there may be a selective advantage for the B allele.

8.5 Other ABCA1 cSNPs have moderate effects on plasma lipids and CAD

8.5.1 Common ABCA1 cSNPs influence plasma lipid levels and risk of CAD

Carriers of the V771M (G2706A, n=37) had decreased focal atherosclerosis (MOD) compared to non-carriers (1.89 ± 0.38 vs. 1.76 ± 0.35, $p=0.05$, Table 8.9). A trend to less diffuse atherosclerosis (increased MSD) was also observed in carriers of this variant compared to non-carriers (2.83 ± 0.49 vs. 2.73 ± 0.37, $p=0.13$). Carriers of the V771M variant, had no difference in HDL-C compared to non-carriers, and a marginally significant interaction between age and genotype on HDL-C levels was noted ($p=0.05$). However, all but 2 carriers of the V771M are also carriers of the R219K, which was associated with decreased CAD.

Carriers of the G2858A (V825I, n=103 AB + 4 BB) had no obvious differences in lipid levels or baseline MSD or MOD (Table 8.9). Carriers did, however, have a significantly increased number of events during the trial (44% vs. 33%, $p=0.0008$; OR=2.31, 95% confidence interval 1.41-3.83).

Although there were no differences in mean lipid levels between the I883M genotypes (Table 8.9), homozygous BB individuals (n=14) have increased progression in MOD (mean change of 0.53 ± 0.79 vs. 0.11 ± 0.25, $p<0.001$). BB individuals had a coronary events rate double that of the AA individuals (n=320; 21.4% vs. 10.6%), although this was not statistically significant ($p=0.19$), likely due to the small number of BB individuals. This variant was seen at a significantly increased frequency in the premature CAD population (Table 8.2, odds ratio for CAD in carriers of this variant= 2.16, 95% confidence interval 1.16-4.03, $p=0.02$). These findings contrast with those of a very recent report that suggests that homozygous carriers (n=16) of this cSNP have increased HDL-C compared to a cohort of non-carriers and heterozygotes[479].

It should be noted that the genotype frequencies of this variant in both the REGRESS and Dutch control populations were not consistent ($p<0.01$) with those predicted under Hardy-Weinberg

Table 8.9. Lipid levels and CAD in carriers of common *ABCA1* cSNPs

				P-values vs. AA		
	AA	**AB**	**BB**	**AB**	**BB**	**AB+BB**
V771M						
n	598	37	0			
TC (mmol/L)	6.05±0.88	6.18±0.88	-	0.38	-	-
HDL-C (mmol/L)	0.92±0.22	0.91±0.20	-	0.58	-	-
LDL-C (mmol/L)	4.32±0.79	4.38±0.85	-	0.64	-	-
TG (mmol/L)	1.78±0.76	1.98±0.79	-	0.11	-	-
MOD	1.76±0.35	1.89±0.38	-	**0.05**	-	-
MSD	2.73±0.37	2.83±0.49	-	0.13	-	-
V825I						
n	575	103	4			
TC (mmol/L)	6.04±0.90	6.03±0.84	6.52±0.73	0.92	0.29	0.17
HDL-C (mmol/L)	0.93±0.22	0.91±0.23	0.91±0.34	0.40	0.86	0.42
LDL-C (mmol/L)	4.30±0.81	4.29±0.72	4.73±0.60	0.91	0.29	0.90
TG (mmol/L)	1.80±0.76	1.85±0.85	1.97±0.49	0.55	0.66	0.49
MOD	1.77±0.35	1.72±0.35	2.13±0.66	0.18	**0.04**	0.39
MSD	2.75±0.38	2.69±0.38	2.98±0.27	0.14	0.23	0.21
I883M						
n	320	86	14			
TC (mmol/L)	5.93±0.88	5.98±0.85	6.04±0.97	0.64	0.65	0.62
HDL-C (mmol/L)	0.91±0.21	0.91±0.22	0.91±0.17	1.00	1.00	0.97
LDL-C (mmol/L)	4.21±0.81	4.26±0.76	4.43±0.80	0.61	0.32	0.38
TG (mmol/L)	1.82±0.75	1.78±0.76	1.55±0.86	0.66	*0.19*	0.42
MOD	1.75±0.36	1.74±0.38	1.71±0.35	0.82	0.68	0.71
MSD	2.73±0.36	2.69±0.37	2.73±0.46	0.36	1.00	0.41
R1587K						
n	433	288	58			
TC (mmol/L)	6.04±0.86	6.03±0.92	6.07±0.78	0.88	0.80	0.94
HDL-C (mmol/L)	0.94±0.23	0.91±0.23	0.86±0.16	*0.09*	**0.01**	**0.03**
LDL-C (mmol/L)	4.29±0.77	4.32±0.83	4.35±0.73	0.62	0.58	0.51
TG (mmol/L)	1.81±0.78	1.76±0.75	1.91±0.80	0.39	0.36	0.77
MOD	1.76±0.37	1.77±0.34	1.79±0.37	0.71	0.56	0.75
MSD	2.74±0.36	2.73±0.40	2.73±0.36	0.73	0.84	0.64

equilibrium, with too few heterozygotes being observed in both cases, suggestive of this variant conferring a selective disadvantage.

Table 8.10. Rare *ABCA1* cSNPs in REGRESS

	AB	AA	P-value AB vs. AA
V399A			
n	9	540	
TC (mmol/L)	5.94±0.73	6.05±0.87	0.71
HDL-C (mmol/L)	1.03±0.28	0.92±0.23	*0.15*
LDL-C (mmol/L)	4.14±0.80	4.31±0.79	0.52
TG (mmol/L)	1.71±0.63	1.82±0.78	0.68
MOD	1.92±0.32	1.73±0.35	0.13
MSD	2.73±0.40	2.71±0.37	0.89
T774P			
n	4	621	
TC (mmol/L)	5.42±0.84	6.04±0.89	0.15
HDL-C (mmol/L)	0.85±0.07	0.93±0.22	0.50
LDL-C (mmol/L)	3.71±0.86	4.29±0.77	0.13
TG (mmol/L)	1.90±1.04	1.82±0.77	0.84
MOD	1.63±0.31	1.76±0.36	0.47
MSD	2.85±0.34	2.73±0.37	0.52
K776N			
n	3	546	
TC (mmol/L)	6.83±0.31	6.03±0.85	0.10
HDL-C (mmol/L)	0.94±0.28	0.93±0.22	0.93
LDL-C (mmol/L)	4.87±0.47	4.31±0.77	0.21
TG (mmol/L)	2.25±0.94	1.76±0.76	0.26
MOD	1.92±0.33	1.78±0.34	0.48
MSD	2.95±0.48	2.76±0.37	0.36
E1172D			
n	34	610	
TC (mmol/L)	5.90±0.76	6.02±0.88	0.41
HDL-C (mmol/L)	0.93±0.23	0.94±0.23	0.89
LDL-C (mmol/L)	4.14±0.67	4.29±0.80	0.28
TG (mmol/L)	1.80±0.90	1.77±0.76	0.80
MOD	1.80±0.39	1.77±0.36	0.67
MSD	2.78±0.35	2.74±0.37	0.42

Carriers of the R1587K (AB, BB) have decreased HDL-C compared to non-carriers in an allele-dose dependent trend (0.86±0.16, 0.91±0.23, 0.94±0.23 for BB, AB, AA, p=0.03; Table 8.9). On multiple regression analysis including age, BMI, smoking, and TG as covariates, the R1587K genotype remains a significant predictor of HDL-C (p=0.03). However, no significant differences in CAD or events during the trial were evident in carriers compared to non-carriers.

8.5.2 Rare ABCA1 cSNPs may also influence plasma lipid levels and risk of CAD

No homozygous carriers (BB) were detected for any of these cSNPs. Carriers of the V399A (AB, n=9), however, had a trend to higher HDL-C (1.03±0.28 vs. 0.92±0.23, p=0.15) compared to individuals who were AA at this site (n=540, Table 8.10). Interestingly, no coronary events were observed in the AB group (vs. 14% in AA's), although due to the small numbers this was not significant. Carriers had half the frequency of a positive family history of CAD (22.2% vs. 49.4%, p=0.18). Furthermore, consistent with this data, carriers had a trend to increased baseline MOD (1.92±0.32 vs. 1.73±0.35, p=0.13) and to less progression in MSD (-0.05±0.10 vs. 0.08±0.19, p=0.16) during the trial. However, as the number of carriers was small, firm conclusions regarding the relationship of this variant to increased HDL-C and decreased CAD cannot be drawn. Carriers of the other three rare variants, A2715C (T774P, n=4), G2723C (K776N, n=3) and G3911C (E1172D, n=34) showed no significant differences in lipid levels or CAD compared to respective non-carriers (Table 8.10).

8.5.3 The C5587G (S1731C) cSNP

No carriers of the S1731C were detected in the REGRESS population, and this cSNP has so far only been seen in French Canadian individuals. This variant was initially found in one of our FHA families (FHA2). The presence of this variant in individuals heterozygous for the R2144X *ABCA1* mutation, was associated with further significantly decreased HDL-C compared to R2144X carriers without this polymorphism (0.16±0.04, n=2 vs. 0.64±0.14, n=10; p=0.0009). In unaffected family members, while carriers of the S1731C (n=6) had slightly lower HDL-C compared to non-carriers (n=14, 1.03±0.22 vs. 1.09±0.23), this was not statistically significant. Interestingly, the control individual in whom this variant was also seen (Table 8.2) also had low plasma HDL-C (0.72 mmol/L). The serine residue altered by this polymorphism is a potential site of phosphorylation, and it is possible that the introduction of a new cysteine may disrupt

cysteine bonding of the native ABCA1 protein. These data suggest that this variant may have functional consequences which may be associated with significant alterations in lipid levels in the French Canadian population.

8.6 Linkage disequilibrium between cSNPs

We have examined whether linkage disequilibrium between the cSNPs exists by pair-wise association. Table 8.11 gives the p-values for each Chi-square analysis of pair-wise genotype distributions. It is evident that several of the variants are seen more often in combination with others than would be expected by chance. In particular, the V771M and K776N variants were almost completely seen in carriers of the R219K variant (all K776N carriers (n=3) and all except 2 V771M carriers (total n=37) were also carriers of the R219K).

Table 8.11. Pairwise associations between non-synonymous cSNPs in the REGRESS population

	R219K	V399A	V771M	T774P	K776N	V825I	I883M	E1172D	R1587K
R219K	-								
V399A	0.345	-							
V771M	**<0.001**	0.296	-						
T774P	0.411	1.000	1.000	-					
K776N	**0.023**	1.000	1.000	1.000	-				
V825I	0.164	0.585	0.050	0.710	0.351	-			
I883M	**0.002**	0.539	0.495	0.731	1.000	**<0.001**	-		
E1172D	0.397	1.000	1.000	0.155	1.000	0.112	**0.023**	-	
R1587K	**<0.001**	0.646	0.249	0.709	0.559	0.058	0.101	**<0.001**	-

8.6.1 The phenotypic effects of the R219K are independent of other cSNPs

The V771M and K776N cSNPs are most commonly found in individuals carrying the R219K. If all V771M and K776N carriers are excluded, the results for the R219K variant are unaltered. The mean MOD remains increased in carriers (n=329) compared to non-carriers (n=422, 1.80±0.35 vs. 1.73±0.35, p=0.006), as does MSD (2.76±0.36 vs. 2.70±0.37, p=0.02). Mean TG levels are significantly lower in carriers compared to non-carriers (1.71±0.75 vs. 1.84±0.77, p=0.02), and mean HDL-C levels remain unchanged.

Two common cSNPs (I883M and R1587K) are also often seen in carriers of the R219K. From the subgroup of individuals for whom complete genotypes were available, we have

identified R219K carriers who do not also carry either the I883M and R1587K cSNPs (n=62), and compared them to the group of individuals who do not carry any of the three variants (n=116). Again, the findings remain unchanged (Table 8.12). MSD is significantly increased in R219K carriers compared to non-carriers (2.81±0.37 vs. 2.69±0.36, p=0.04). MOD is increased (1.78±0.39 vs. 1.73±0.38), although this finding did not retain statistical significance in this smaller cohort. In addition, TG remain significantly decreased in carriers (1.67±0.76 vs. 1.97±0.74, p=0.02). Thus, the effects of the R219K variant described herein are not due to other cSNPs which are found in association with it.

8.6.2 Other cSNPs in linkage disequilibrium

The V825I cSNP was found to be in linkage disequilibrium with the I883M (Table 8.11). The relative risk of the V825I carriers adjusted for I883M genotype is 2.31 (95% confidence interval 0.78-6.85), however likely due to the much smaller numbers, this no longer remains significant (p=0.13). As the effects of the I883M variant were only evident in BB individuals, the number of individuals is too small to correct for V825I genotype.

The E1172D cSNP was found exclusively in carriers of the R1587K variant. We have examined the effects of the R1587K, excluding carriers of the E1172D (n=34). A trend to decreasing HDL with dosage of the R1587K B allele was still evident (0.87±0.18 in BB, 0.92±0.23 in AB, 0.94±0.23 in AA, p=0.19). It is likely this no longer remains significant due to the fact that the number of BB individuals (n=29) is decreased by 50%. No significant differences in TG or CAD were unmasked by the exclusion of this variant. In addition, no significant differences in lipid levels or CAD were observed for E1172D/R1587K double heterozygotes compared to R1587K heterozygotes without the E1172D. Thus, the effects of the R1587K are not due to the non-functional E1172D variant, with which it is in linkage disequilibrium. Furthermore, when the I883M and R219K carriers are removed from this group, carriers of the R1587K still have reduced HDL-C, although likely due to the large reduction in numbers this was no longer significant (Table 8.12).

As the I883M appeared to have effects only in BB individuals, exclusion of the R1587K and R219K variants is not useful, as the number of BBs is too small for analysis independent of the ABs.

Table 8.12. Phenotypic analysis of individuals who are carriers of only one of the three most common cSNPs compared to non-carriers of all three variants

	wildtype	R219K only		R1587K only		I883M only	
	AA [a]	AB+BB	P vs. AA	AB+BB	P vs. AA	AB+BB	P vs. AA
n	116	62		77		23	
TG (mmol/L)	1.97±0.74	1.67±0.76	**0.02**	1.79±0.71	0.09	1.64±0.72	**0.05**
HDL-C (mmol/L)	0.90±0.19	0.92±0.23	0.54	0.87±0.18	0.27	0.93±0.24	0.51
MSD	2.69±0.36	2.81±0.37	**0.04**	2.67±0.34	0.70	2.63±0.32	0.46
MOD	1.73±0.38	1.78±0.39	0.40	1.70±0.31	0.56	1.60±0.28	0.12
events	14% (16)	7% (4)	0.21	8% (6)	0.25	9% (2)	0.73

[a] Individuals who do not have either the R219K, R1587K or I883M variants

8.7 Discussion

This chapter describes a complete cSNP analysis of the *ABCA1* gene, providing evidence that common genetic variation within *ABCA1* is associated with altered plasma lipid levels and risk of CAD (summarized in Table 8.13). The R219K variant, with a carrier frequency of 46% in Caucasian populations, is associated with a decreased severity of CAD, assessed in multiple ways. Angiographically, this was manifest as both decreased focal and diffuse atherosclerosis. Atherosclerosis progresses more slowly in carriers of the R219K. Coronary events were also decreased in carriers, who exhibited a prolonged event-free survival. Carriers of this variant

Table 8.13. Associations of ABCA1 cSNPs with altered lipid levels and risk of CAD

Variant	P-value	Variant	P-value
Increased HDL-C		***Decreased severity of CAD***	
R219K	0.02	R219K	<0.01
V399A	0.15	V399A	0.13
		V771M	0.05
Decreased HDL-C			
R1587K	0.03	***Increased severity of CAD***	
S1731C	<0.001	V825I	<0.001
		I883M	<0.001
Decreased TG			
R219K	0.007		

have a significantly reduced risk of CAD events, and the increased risk associated with the wildtype (A) allele may account for up to 5% of the population risk of coronary events.

Both the finding of decreased TG and of increased HDL-C in younger carriers of the B allele is consistent with the decreased CAD observed in carriers of the variant[79,84,89]. TG levels showed similar trends in our replication groups, and increased HDL-C levels in R219K carriers were observed in our independent populations. We have examined cholesterol efflux levels in a small number of normolipidemic controls but were unable to detect differences between carriers (n=2) and non-carriers (n=4). However, in addition to the small numbers we were also limited in the ability to detect a difference due to the approximate 15% inter-assay coefficient of variation in the efflux assay, which may make it impossible to detect small differences in efflux. The phenotypic effects we describe for this variant are opposite to those observed in individuals heterozygous for *ABCA1* mutations (Chapter 7), suggesting this variant is associated with a gain of normal ABCA1 function. This increased ABCA1 activity would be predicted to increase RCT. Thus one might expect that therapies designed to substantially increase ABCA1 function might have the desirable effects of increasing HDL-C, decreasing TG and decreasing the severity of atherosclerosis and risk of CAD events.

Although we did not observe differences in HDL-C levels in the whole REGRESS cohort, they were observed in our subsequent replication populations and in the younger individuals within REGRESS. The REGRESS cohort was selected for individuals with CAD. Although there was no selection for specific HDL-C levels, HDL-C was low (average in the whole cohort approximately 0.9 mmol/L). Many individuals may have existing low HDL-C, perhaps due to other genetic abnormalities and likely other risk factors predisposing them to CAD. Therefore differences in HDL-C between R219K genotypes may have been masked when not compared to matched individuals. Alternatively, it is possible that stimulation of the RCT pathway may increase the net flux of cholesterol towards the liver without altering steady-state plasma HDL-C levels. This increase in RCT activity may directly reduce the development of atherosclerosis without altering plasma lipid levels. The cohort was also selected for TG< 4 mmol/L, and thus variants associated with more extreme changes in TG may have been selected against. Furthermore, as ABCA1 has also been suggested to have other functions (e.g. in cell

death, or monocyte function[186-189]), variants deemed "non-functional" may still have effects on different phenotypes not examined in this study.

The mechanism underlying the decreased TG in carriers of the R219K variant is unknown. CETP activity results in the equilibration of the core components of lipoprotein particles[38]. Cholesteryl esters (CE) are transferred from HDL to TG-rich lipoproteins, while TG are transferred from TG-rich lipoproteins to HDL. Increased ABCA1 activity, resulting in increased HDL-C, might trigger an increased CE-TG exchange. Hepatic lipase efficiently hydrolyzes the TG component of HDL[480]. Thus increased transfer of TG to HDL may ultimately increase TG catabolism.

It is also possible that changes in HDL metabolism may result in a redistribution of the exchangeable lipoproteins (e.g. apoCII, apoCIII or apoE) between HDL and VLDL. If the apoCIII content of VLDL is decreased, or the apoCII content increased, this may lead to increased LPL activity towards VLDL. This would result in increased TG hydrolysis and decreased plasma TG concentrations.

Alternatively, alterations in ABCA1 activity have been suggested to alter intracellular lipid transport[199,201]. It is possible that other genes involved in intracellular lipid transport and/or TG metabolism may be coordinately regulated with *ABCA1*. Indeed, several genes involved in lipid metabolism have been shown to be differentially regulated in ABCA1 deficient mice[201]. Changes in intracellular cholesterol and phospholipid metabolism triggered by increased ABCA1 activity[53] might lead, for example, to the diversion of fatty acids from TG synthesis to phospholipid synthesis, resulting in decreased TG secretion by the liver and reduced plasma TG levels. Similar mechanisms can be invoked to explain the increased TG in *ABCA1* heterozygotes, only with the equilibrium concentrations shifted the other way. Further detailed analysis of lipoprotein composition and lipoprotein metabolism resulting from alterations in ABCA1 activity will help delineate between these possible mechanisms.

The phenotype in individuals heterozygous for *ABCA1* mutations is modified by age. In heterozygotes, the phenotype is more pronounced in older individuals (Chapter 7). This suggests that there may be an increased demand for ABCA1 activity with age that cannot be produced in heterozygotes. Furthermore, age related increases in expression and activity of P-glycoprotein, another ATP binding cassette transporter, have been described[456,481,482]. Here we show that the R219K polymorphism was also associated with an altered relationship between age and HDL-C.

In AA individuals there was a general increase in cholesterol efflux and HDL-C with age, suggestive of increasing ABCA1 function. However in carriers of the B allele, this age-dependent increase in both HDL-C and efflux was not evident, suggesting this variant is already maximally upregulated and unable to respond to the age-related stimuli. The mechanism of this response will require *in vitro* re-creation of this cSNP to understand its regulation and functioning.

The phenotypic effects of the other cSNPs are evident but less striking (Tables 8.9, 8.10) and need further analysis. The R1587K is a common variant that is associated with decreased HDL-C, although no effects on CAD were observed. The data also suggest that the S1731C, which may be a founder variant within the French Canadian population, is also associated with reduced HDL-C. Carriers of the V399A variant had a mild trend towards increased HDL-C, which was associated with similar trends towards decreased vascular disease. As this variant had a carrier frequency of only 1.6%, the lack of significance of these results is likely due to the small number of carriers. Finally we provide preliminary evidence that the V825I and I883M variants are associated with an increased risk of CAD.

This high frequency of cSNPs emphasizes the importance of verifying that sequence changes observed within the gene are not in fact cSNPs. Of note, the V399A and I883M variants were shown to co-segregate on a mutation-bearing chromosome in one of the initial Tangier families described[51]. The authors suggested that one of these two variants was likely the functional mutation. Yet, here we show that the V399A variant was associated with a trend towards increased HDL. Furthermore, we show that the I883M is a common variant associated with an increased risk of CAD in the homozygous state, although no differences in HDL-C were evident. Neither variant is associated with the marked decrease in HDL-C seen in individuals heterozygous for *ABCA1* mutations. Thus, without proper analysis of missense changes in a large ethnically matched cohort, cSNPs can be inappropriately confused with mutations. Furthermore, as the frequency of cSNPs may vary considerably between populations, comparing data across ethnically distinct cohorts may lead to improper conclusions about frequency differences between populations due to population stratification.

Although cSNPs were detected on average every 425 bp, the distribution of these cSNPs was not random. Interestingly, cSNPs tended to be found further away from known functional sites, such as the ATP binding cassette regions, or suspected functional regions, such as those

where mutations seem to cluster. This suggests that different regions of the protein tolerate more variability. The one exception to this pattern was the I883M variant, located just N-terminal of the first ATP binding cassette region, where several mutations have been shown to occur (between amino acids 909 and 937)[51-54,444,445,479] (and Chapter 7). As this variant was associated with little functional effect, this might perhaps mark the border of the region in which structural alterations significantly impair ATP binding cassette function. Similarly, the region containing the V771M, T774P and K776N variants is unlikely to be critical to ABCA1 function, since a high degree of polymorphism is tolerated without functional effects.

It is expected that some variants will not have any functional effect, and linkage disequilibrium between functional and non-functional sites may further complicate the analysis of individual SNPs[306,483]. Thus, a detailed analysis of multiple sites is required before function can be attributed to a single variant. The findings of this study underscore the importance of examining multiple SNPs at once, so that linkage disequilibrium between variant sites can be examined, and effects not mis-attributed to the wrong variant. While it remains possible that the cSNPs reported here are in linkage disequilibrium with other variants (e.g. intronic or within the promoter or within neighbouring genes), our detailed analysis suggests that, at least within the coding region, the phenotypic effects have been ascribed to the correct variant.

We have shown that the frequent R219K variant is associated with a decreased severity of atherosclerosis, a decreased risk of coronary events, decreased TG, and increased HDL-C, consistent with an ABCA1 gain of function. The protection associated with the B allele of this variant may prevent approximately 5% of CAD events. These effects were independent of any other cSNPs found in association with the R219K, and were seen both in many different measures of CAD and in multiple cohorts. The increased frequency of this variant in Black and Asian cohorts suggests that it may have an even greater impact on lipid levels and CAD within these populations. These findings emphasize the importance of common genetic variation in *ABCA1* in the general population in determining plasma lipid levels and severity of CAD. Furthermore, this data suggests that mechanisms that increase ABCA1 function, as seen with the R219K variant, will have significant therapeutic benefit.

Chapter 9: Discussion and Conclusions

9.1 Summary

The data presented in this thesis consists of studies designed to examine the role of genetic variation in two genes in lipoprotein metabolism, namely lipoprotein lipase and the ATP binding cassette transporter *ABCA1*, in the determination of plasma lipid levels, specifically HDL-C and TG, and in the development of atherosclerosis.

9.1.1 Lipoprotein lipase

Data on the role of LPL in atherosclerosis have been presented from both animal models and human studies. The initial studies in a mouse model of increased LPL provided validation for the use of this animal model (and CETP deficient models in general) in studies of LPL in lipid metabolism and atherosclerosis. The data showed that the positive correlations between LPL on HDL-C observed in humans may be absent in mice, partly because of their lack of plasma CETP activity. When this protein was added to the mouse, the correlations between LPL and HDL-C were restored.

However, the studies also demonstrated that CETP is not the only mechanism influencing this correlation. When mice were placed on a high fat high carbohydrate diet, the correlation between LPL and HDL-C was also restored. This suggests that perhaps because normal mouse chow is low in fat, LPL-mediated hydrolysis of the TG does not produce enough surface remnants to detect a significant increase in HDL-C. When the TG substrate is increased, such as through high fat feeding, the increased surface remnant generation is sufficient to have a detectable impact on HDL-C levels.

These findings provided insight as to potential reasons why LPL did not appear correlated with HDL-C in mice. Understanding these mechanisms validated the mouse as a model system for studies of atherosclerosis, where mice are either fed high fat diets or have targeted-mutations that result in endogenous hypertriglyceridemia and hypercholesterolemia. They also suggested that the role of CETP should be considered when interpreting mouse studies in relation to what we may expect in human populations.

This allowed us to assess the role of LPL in atherosclerosis using mouse models. Data obtained in the apoE deficient model and in a cholesterol-fed C57BL/6 model confirmed the hypotheses that LPL may have dual roles in atherosclerosis dependent upon its site of expression. Specifically, by comparing the atherosclerosis development in mice with reduced

LPL expression in both the plasma and vessel wall with mice with increased LPL expression specifically in the plasma, these studies showed that *increased* plasma LPL activity is associated with a reduction in atherosclerosis, while *decreasing* LPL in the vessel wall led to a similar reduction in atherosclerosis. Interestingly, these differences were no longer evident in complex lesions, suggesting the role of LPL, either in the plasma or the vessel wall, is in the initial stages of lesion formation, namely lipid accumulation within the vessel wall. Once a lesion has progressed to the stage where changes of complexity predominate, increased lipid accumulation may not be visible as increased outward growth of the plaque into the lumen.

The data obtained in the mouse models were further extended into a larger animal model, specifically a naturally occurring LPL deficient feline model. The initial studies in these animals provided a more detailed characterization of the effects of LPL deficiency on plasma lipoproteins. This suggested that the consequences of LPL deficiency in the feline model are similar to those observed in humans. In the atherosclerosis study, although large, complex lesions were induced in these animals, trends toward reduced atherosclerosis in the animals with reduced LPL in both the plasma and vessel wall were still evident. This provided further confirmation of the findings observed in the mice, and additional evidence of the predominant role of vessel wall LPL. These findings suggest that therapies should be designed to specifically increase plasma LPL, without altering its levels within the vessel wall.

Analysis of three *LPL* cSNPs has shown that genetic variation in *LPL* can influence plasma lipid levels and the severity of atherosclerosis in human populations. A careful *in vitro* analysis of these cSNPs showed that each influences the protein in a different fashion. The N291S variant is associated with a reduction in the catalytic activity of the enzyme, despite normal production and secretion. The D9N variant was found to be associated with decreased secretion of a catalytically normal enzyme, resulting in a net reduction of LPL activity. The S447X variant appears to have normal catalytic activity but to be associated with an increased secretion of the monomeric form of the enzyme.

Studies of the N291S variant had shown conflicting results on the impact of this cSNP on plasma lipid levels. The study described in this thesis provides some insight into this. The data were the first to show that carriers of this cSNP have an increased postprandial response, and delayed TG clearance of a fat rich meal, despite the fact that in the fasting state lipids may be within the normal range. This has illustrated that environmental factors interact with *LPL*

genetic variation, and that differences in LPL function may be unmasked when the system is challenged. Thus, the lack of effect in some studies may have been due to their assessment in the basal state.

Studies of a common substitution in the *LPL* promoter identified linkage disequilibrium between this variant and the D9N substitution. Because of ethnic differences in the frequency of these alleles, we were able to investigate the impact of each individually. The data were the first to show that the –93 g allele is associated with decreased plasma TG, suggestive of increased activity. When the D9N variant is in linkage disequilibrium with this allele, however, plasma TG are increased, suggesting that the effects of this cSNP are dominant over those of the promoter substitution. This is consistent with the *in vitro* results, predicting a protein with defective secretion: increasing its production (through the –93g allele) may not alter the amount of enzyme that is secreted from cells. This confirmed that the findings previously reported for the D9N variant are due to the effects of that variant and not of the –93 g allele.

Finally, studies of the S447X cSNP demonstrated that this variant is associated with reduced plasma TG levels, and suggest that it is associated with a decreased risk of CAD. The data also suggest a novel potential mechanism whereby this variant reduces the risk of CAD independent of its effects on plasma lipids, namely, through decreasing blood pressure. The underlying mechanism of this relationship is not yet understood, however its elucidation may provide insight into yet another role of this multifunctional protein. These findings suggest that the S447X variant has multiple beneficial effects, and might have additional therapeutic value compared to the wildtype protein.

9.1.2 ATP binding cassette transporter A1

Chapter 6 of this thesis describes the identification of the ATP binding cassette transporter *ABCA1* gene as a key determinant of plasma HDL-C levels. Mutations in this gene are the underlying cause of Tangier disease, and familial HDL deficiency with reduced cholesterol efflux. Eight different mutations in this gene were identified in TD and FHA families, and shown to cosegregate with the reduced HDL-C in the families. These changes were absent from over 400 control chromosomes, and are thus unlikely to be polymorphisms linked to the disease causing allele.

Analysis of individuals heterozygous for mutations in the *ABCA1* gene has demonstrated that decreased activity of this protein is associated with decreased plasma HDL-C and increased plasma TG. Individuals with mutations in *ABCA1* have a significantly increased risk of coronary events, and an earlier onset of these events than their unaffected relatives. The residual cholesterol efflux in these individuals is a strong predictor of their HDL-C levels, and thus their risk of CAD. Furthermore, the data have shown that age is an important modifier of the phenotype, in that the severity of the phenotype is increased in older individuals.

However, mutations resulting in significant impairment of ABCA1 activity are not a common cause of low HDL-C in the general population. Thus, the question arose as to how frequent variation within the gene influences plasma lipid levels and risk of CAD. Throughout the course of sequencing TD and FHA probands, several SNPs were identified. We have examined the effects of the cSNPs that result in amino acid changes in a cohort of approximately 800 Dutch men with CAD.

This identified the R219K cSNP as a variant having significant beneficial effects. Carriers of this cSNP, like those of the S447X in *LPL*, have significantly decreased plasma TG, increased HDL-C and significantly decreased atherosclerosis. The phenotypic effects of this variant were also modified by age, suggesting *ABCA1* function may naturally increase with age. These findings are consistent with those observed in heterozygotes, where their defect becomes more pronounced with age. Furthermore, the effects of the R219K cSNP appear independent of any other cSNPs with which it is in linkage disequilibrium.

The remainder of the cSNPs showed less consistent effects on plasma lipid levels and CAD. The R1587K and S1731C cSNPs were associated with reduced HDL-C. The V399A cSNP, like the R219K, was associated with trends to increased HDL-C and decreased severity of CAD. In contrast, the V825I and I883M variants were associated with an increased severity of CAD. These findings suggest that *ABCA1* genetic variation may commonly contribute to altered plasma lipid levels and risk of CAD.

9.2 Suggestions for further work

As with any study, seemingly more questions have been raised than have been answered. The work presented in this thesis has raised several additional questions, and suggests many potential avenues for further research.

1. The studies described in this thesis indicate that increasing plasma LPL activity should have a significant therapeutic effect on the risk of CAD. Future studies on potential LPL therapies should therefore investigate ways that LPL may be targeted away from vessel wall. Drugs which increase LPL expression, such as NO-1886[246,247] and PPAR activators[484], will likely increase LPL expression from all tissues in which it is produced. Drugs to be used as anti-atherosclerotic agents should have tissue specific effects. As such, a better knowledge of which tissues contribute to vessel wall LPL is needed. Studies of the trafficking of LPL into vessel wall are also relevant. Is it all derived from macrophages, or if there is increased LPL in plasma, can some of this enter vessel wall? Does dramatically increasing LPL in the plasma result in increased entry into the vessel wall? Studies of the transport of LPL from its sites of synthesis to the vascular endothelium, and whether these processes can also operate in the reverse direction will be important. Is LPL transport across the vascular endothelium unidirectional, or does it occur in both directions? As an alternate therapeutic strategy, could LPL secretion be inhibited in a tissue specific manner (e.g. in macrophages)?

Additional studies in mice should be designed to investigate the involvement of macrophage LPL in atherosclerosis. Is it purely structural? Or, is catalytic activity necessary? Such studies could have consequences for new therapeutics, in that knowing whether any LPL in the vessel wall is bad, or whether it is only that which has catalytic activity that is important, will help determine whether increased inactive LPL (such as monomeric LPL) will have negative consequences. Gene therapy or viral-mediated gene transfer may be the best approach both to study these questions, and as a way to increase LPL activity from tissue-defined sites. This will also be a useful system in which to compare the functional effects of the S447X variant compared to wildtype.

2. The studies on the LPL S447X SNP have also raised questions as to the role of LPL on endothelial cell biology, which warrant further investigation. Does the LPL protein attached to the vascular endothelium, or its trafficking through the endothelium, influence endothelial cell signaling? Is this effect confined to the S447X variant, or does normal LPL have this function? What is the role of LPL monomer in this process?

This will require additional studies on LPL biology. Specifically, little is understood regarding the roles of the LPL monomer in lipoprotein metabolism. How is it produced and catabolized? What factors regulate dimer stability and its dissociation into monomers? Does it bind both lipoproteins and heparin? Can it aid in lipoprotein uptake? Is LPL secreted initially as a dimer or as monomers, or both? Does increased monomeric LPL account for the effects of the S447X, and if so, how? Additional studies are first needed to confirm the role of the S447X in blood pressure.

In general, future studies on LPL should focus on its many potential roles in atherosclerosis. These include the role of LPL in selective uptake, as suggested in the mouse and cat data, and the influence of LPL on the vascular endothelium and on blood pressure, and on the effects of the LPL protein within the vessel wall. These studies in atherosclerosis should be designed to study the early atherosclerotic process, or should follow the process along the entire length of the vessel, over time. Measures such as intima-media thickness determinations could be adapted for larger animal models, and may be especially useful for following the progression of atherosclerosis in studies such as those examining efficacy of new therapies. Studies in cats should use increased numbers, if possible, and the lower percentage cholesterol diet. Further verification of the roles of LPL in atherosclerosis should also be repeated including CETP, or in an animal model with endogenous CETP such as rabbits.

3. Good animal models of altered ABCA1 expression (both increased and decreased) are needed. There is still much to be learned about ABCA1, including its substrates and exact transporter function, and most biochemical aspects of its regulation. These processes can be studied either in the intact animal, or in tissue samples from such animals, comparing the effects of altered ABCA1 expression levels. These animals will also allow one to directly test the effects of alterations in this gene on the development of atherosclerosis. Such studies may need to include CETP, to fully understand the role of ABCA1 in lipid, specifically HDL, metabolism. A comparison between the two states (with and without CETP) may also be useful, to further validate the RCT hypothesis by either increasing HDL-C without necessarily increasing its return to the liver (as is the case in the absence of CETP), or by also increasing cholesterol delivery to the liver (as is the case with CETP). Additional questions regarding other genes that may be coordinately regulated with *ABCA1* can be studied in the models. For example, if peripheral

ABCA1 activity is increased, does this also lead to increased SRBI expression in the liver to take up this excess cholesterol? Are there any feedback or feedforward signaling pathways between the various proteins?

The identification of *ABCA1* opened the door to the "black box" controlling the efflux of cholesterol from peripheral cells. Clearly this process is highly regulated and involves a large number of signaling molecules. As such, identifying proteins interacting with ABCA1 will be important, and should shed much light on understanding how the pathway of intracellular cholesterol trafficking is regulated *in vivo*. Such interactions can then be tested within this animal model system.

4. To fully understand the role of genetic variation in *ABCA1* on the risk of CAD, a detailed examination of *ABCA1* cSNPs in a general population sample is required. The sample should be free of selection for plasma lipid levels and coronary disease, and include larger numbers for a better examination of the rare variants. Validation of the findings presented in this thesis in other populations is required. The remainder of the SNPs, particularly those that might be involved in the regulation of the gene, should also be studied. Furthermore, potential interactions between these SNPs and environmental factors such as body mass, exercise, or glucose tolerance should be examined, to fully understand their influences on lipid metabolism and atherosclerosis. Recreating these SNPs *in vitro* will be useful to characterize their isolated effects on protein synthesis, stability and function.

Ultimately, one will need to examine interaction of SNPs between genes. Gene-gene interaction and haplotypes in polymorphism analysis will be important for the identification of the cluster of SNPs that are most informative (best predictive powers, the least number of SNPs needed to genotype) and that have biggest effects. This will be required for identification of risk factor clustering or genetic profiling. Studies should also be performed in multiple ethnic groups. Better methods of deriving haplotypes from individual (not family) data are needed. For this study, one may need to examine flanking CA repeat markers to be able to develop unambiguous haplotypes. This suggests that mapping based solely on SNPs may be difficult unless using family data, or that it may not be good for situations where there is likely more than one disease-bearing haplotype.

9.3 Significance

LPL is clearly a multifunctional protein, involved in many different aspects of lipid metabolism and the development of atherosclerosis, several of which have been suggested by studies described in this thesis. LPL influences HDL in many ways, not just in the classical mechanism through surface remnant generation. Multiple mechanisms and the role of LPL in all its metabolic processes thus need to be considered when examining the influence of alterations in LPL on plasma lipid levels and atherosclerosis. Furthermore, there are likely additional mechanisms by which LPL may influence the development of atherosclerosis, such as the uptake of lipoproteins in the liver, a potential role in selective uptake of CE from lipoproteins, or perhaps a direct influence on the vascular endothelium and blood pressure, which are not well understood. Further examination of these aspects of LPL biology may shed light on yet other ways in which the LPL protein may alter the risk of CAD.

The finding that the role of LPL in atherosclerosis may be different depending on its actions in plasma compared to those in the tissues, has important implications for the development of therapeutics. Whereas increasing plasma LPL activity is beneficial, increased macrophage expression leads to increased lesion formation. New therapies thus must be designed to increase plasma activities, independent of macrophage expression. Understanding the functions of LPL at all levels seems of critical importance, before new therapies may be designed.

The identification of the *ABCA1* gene has clearly had a significant impact on our understanding of HDL metabolism and reverse cholesterol transport. While much work remains to understand the biological function of this protein, it is clear that increasing ABCA1 activity is likely to have significant therapeutic benefit. The studies presented in this thesis have provided proof of principle of this concept.

9.4 Conclusions

Several genes are involved in lipoprotein metabolism and atherosclerosis. Genes influencing TG and HDL-C are important risk factors for atherosclerosis. *LPL* and *ABCA1* are two such genes. Small changes within these genes may have significant functional effects, and variants associated with even a small change in risk may have a large effect on the population if they are present at high frequencies. LPL and ABCA1 are both good targets for therapeutic

development in the prevention of dyslipidemia and atherosclerosis. For LPL, therapies will have to avoid increasing its presence in the vessel wall, whereas the opposite effect will likely be desired for ABCA1.

We are entering an exciting new age of genetic medicine. Examination of the functional effects of SNPs in known genes is the first step in this process. Characterization of their frequencies and phenotypic effects will ultimately allow us to be able to build up a constellation of the SNPs which are the most informative, have the largest effects, and are the best predictors of disease outcome (good or bad). Screening these SNPs will ultimately allow us to identify which drugs are likely to be most useful in any given individual (pharmacogenomics), and which individuals are most at risk or are best advised to modify their environment (smoking, exercise, fat intake, etc.) to influence their risk of disease. For late-onset disorders such as diabetes (type II) or atherosclerosis, a risk-profile can be determined based upon the constellation of SNPs present in at risk individuals, and therapies or lifestyle interventions can be commenced prior to the onset of clinical symptoms. Furthermore, understanding the molecular etiology of these diseases in any given individual will allow the appropriate drug to be identified, maximizing the therapeutic benefit and minimizing side-effects. SNPs are being rapidly discovered by the human genome project. As of February 12, 2001 > 1.6 million SNPs have been catalogued and may be used for such studies. The challenge will be to identify their functional effects and the underlying mechanism, to sort out which are truly functional.

With the near completion of the Human Genome Project[485,486], undoubtedly many additional genes involved in lipid metabolism and atherosclerosis will be identified. Furthermore, global analyses such as expression profiling and proteomics, as well as large-scale identification of interactions between proteins, will provide much insight into the true functioning of these genes *in vivo*. Thus, the end of this thesis marks what is clearly only the beginning of an exciting era in human biology.

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