EFFECTS OF COMMON GENETIC VARIATION ON FASTING AND POSTPRANDIAL LIPID METABOLISM AND ATHEROSCLEROSIS

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A thesis submitted in accordance with the regulations of the
University of London for the
Degree of Doctor of Philosophy

June 1994

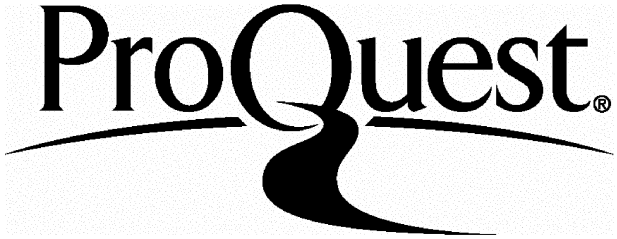
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ABSTRACT

Fasting triglyceride (TG) levels are a strong determinant of postprandial lipaemia, which is thought to influence the development of coronary artery disease (CAD) because of the delayed clearance or prolonged presence of atherogenic remnant lipoproteins. Associations were thus sought between common genetic variation in apolipoprotein (apo) B, AI, CIII and E and lipoprotein lipase (LPL) and both levels of the TG-rich lipoproteins and severity of atherosclerosis. The role of the LPL gene was emphasized because of the known metabolic relationship between LPL activity and lipid metabolism. In Swedish healthy individuals, the C_{-265} allele of the C to T polymorphism in the apoB promoter was associated with higher apoB levels, the X+ allele of the apoB-XbaI polymorphism with higher low density cholesterol (LDL) cholesterol levels, the common allele (V+) of the apoCIII-PvuII polymorphism with higher LDL-TG levels, and both the T allele of the apoCIII C_{1100} to T polymorphism and the common allele (H+) of the LPL-HindIII polymorphism with higher TG levels.

In Swedish myocardial infarction survivors, fasting lipids and lipoproteins were associated with variation at the apoE gene but not at the apoB, apoCIII or LPL genes. However homozygosity for the insertion allele of the apoB signal peptide polymorphism, homozygosity for the C allele of the apoCIII C_{1100} to T polymorphism and the presence of the rare alleles of the HindIII and Serine-Stop447 polymorphisms in the LPL gene were associated with worse baseline severity of atherosclerosis in the MI survivors. When genotypes associated with severe atherosclerosis were considered together, individuals with all three genotypes had a 6-fold greater severity of atherosclerosis compared to those with none. In a British patient sample with CAD, variation at the apoB and apoAI-CIII-AIV genes were associated with baseline severity and progression, respectively, of atherosclerosis; homozygosity for the X- allele of the apoB-XbaI polymorphism and homozygosity for the G allele of the apoAI-G_{-75} to A polymorphism being associated with worse atherosclerosis. In neither of these patient samples, did the genotype effect on atherosclerosis appear to be mediated through genotype-associated differences in fasting lipid and lipoprotein traits.

To test the hypothesis that genetic variation was influencing atherosclerosis severity

via modulation of postprandial lipaemia rather than through fasting lipids, the effects of common genetic variation on levels of postprandial TG-rich lipoproteins and their atherogenic remnants were examined. The apoB signal peptide variants influenced production of postprandial lipoproteins of both intestinal and hepatic origin. Polymorphisms in the apoCIII, LPL and apoB genes exerted their largest effect on lipolysis or catabolism of postprandial lipoproteins, and the H+ allele of the LPL-HindIII polymorphism was associated with higher postprandial LPL activity and levels of free fatty acids. Using Single Strand Conformation Polymorphism analysis and sequencing, an A_{1127} to G substitution that changes Asparagine-291 to Serine was found in exon 6. The frequency of the Serine-291 variant, found on the haplotype defined by the common alleles (presence of cutting site) of the PvuII and HindIII polymorphisms, was approximately 0.1 in two independent samples. The effects of this substitution on fasting and postprandial lipids and lipoproteins, LPL activity, interrelationships between plasma traits and influence on apoE phenotype-mediated effects have been investigated, but do not explain all the effects associated with genotypes of the LPL-HindIII polymorphism.

Common variation in the genes for apoB, apoCIII and LPL influences fasting serum lipid and lipoprotein levels, with LPL having the largest effect on TGs, and modulating postprandial lipaemia and interrelationships between lipid traits; these may explain associations seen between genetic variation and atherosclerosis. Additive effects occur between common genetic polymorphisms, modulated by environmental factors, with implications for future population studies.

ACKNOWLEDGEMENTS

Many thanks are due to Professor Steve Humphries and Dr Philippa Talmud, who have supervised me and for all their patience, encouragement, advice, ideas and our enjoyable discussions over the past four and half years. Thank you to Alison Dunning, Rachel Fisher, Vilmunder Gudnason, Robyn Rennick and Philippa for most helpful discussions and suggestions during the writing of this thesis; also to Rachel for proof-reading.

Special thanks to the following;
Alison, France Mailly, Anne Temple and Fanli Xu for their friendship, encouragement and teaching me laboratory techniques.
Anne Temple for keeping the laboratory running smoothly.
Fiona Green and Adraino Henney for help with computing.
The rest of the group, past and present, for their friendship and for giving that bit extra to ensure I was included, and helping me overcome the problems of my profound deafness to complete the Ph.D.
Collaborators Dr Anders Hamsten and Dr Fredrik Karpe from Stockholm, Sweden, for access to the Swedish population sample, which forms the bulk of this thesis and Dr Gerald Watts from St. Thomas' Hospital, London, for access to the 'STARS' Study on which Chapter 4 is based.

And finally I would not have completed this course but for the constant encouragement and support from my parents and David Heron.

Financial support for this work has come through grants from the British Heart Foundation (RG5 and RG16) and a studentship from the British Medical Research Council.

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Abbreviations used in this thesis

A	Adenine
Ag	Antigen Group
apo	apolipoprotein
ASO	Allele specific oligonucleotides
BMI	Body mass index
C	Cytosine
CAD	coronary artery disease
CE	cholesteryl ester
CETP	cholesterol ester transfer protein
CHD	coronary heart disease
FC	free cholesterol
FCHL	familial combined hyperlipidaemia
FCR	fractional catabolic rate
FDB	familial defective apoB
FDH	familial dyslipidaemic hypertension
FH	familial hypercholesterolaemia
G	Guanine
HDL	high density lipoprotein
HL	hepatic lipase
HVR	hypervariable region
IDL	intermediate density lipoprotein
TGRL	triglyceride-rich lipoprotein
LPL	lipoprotein lipase
LRP	LDL-receptor-related protein
mRNA	messenger RNA
MTTP	microsomal triglyceride transfer protein
oligo	oligonucleotide
PCR	Polymerase chain reaction
RFLP	restriction fragment length polymorphism
SP	signal peptide
T	Thymine
TG	triglyceride
VLDL	very low density lipoprotein

Section 1.1 Mechanisms underlying development of atherosclerosis and Risk Factors

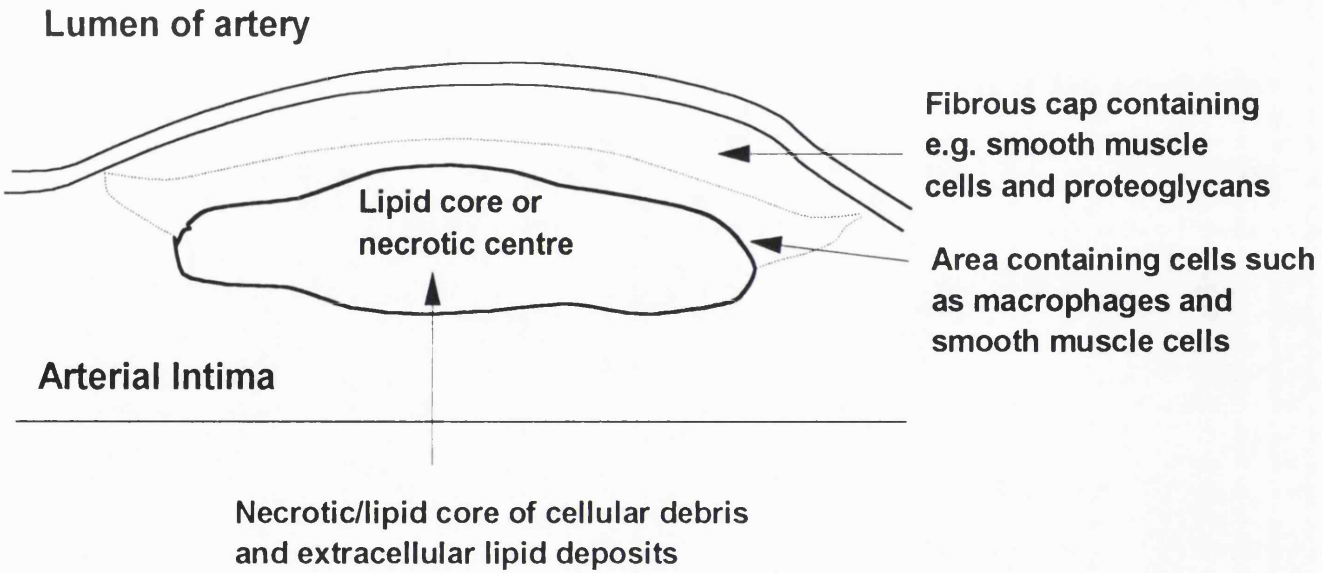
Atherosclerotic cardiovascular diseases are the leading causes of death in modern Western society, responsible for 50% of all mortality in the USA, Europe and Japan. Atherosclerosis is not only a disease in its own right, it is also a process that is a principal contributor to the pathogenesis of myocardial and cerebral infarction, gangrene and loss of function in the extremities (reviewed in Ross 1993). The earliest stage of atherosclerosis is the 'fatty streak', which are lesions in the artery wall that can be identified, even in very young individuals. Fatty streaks are formed by circulating monocytes adhering to and their subsequent penetration of the arterial endothelium , possibly in response to endothelial injury (the process of atherogenesis is reviewed by Ross 1986, 1993). The hypothesis that an injury to the endothelium might precipitate the atherosclerotic process was advanced in 1973 (Ross & Glosmet 1973). One manifestation of this injury is increased trapping of lipoproteins (protein and lipid complexes - described in Section 1.2) in the artery wall (Mora et al. 1987). These monocytes migrate through the endothelium into the arterial wall, where they accumulate and take up cholesterol to become foam cells. The 'fatty streak' stage of atherogenesis is thought to be reversible.

As the plaque grows, other cells, such as smooth muscle cells, are stimulated to proliferate and these cells also become fat-loaded - this is the 'intermediate lesion' stage. Loss of the arterial endothelium may also occur. In time, these intermediate lesions develop into the more advanced, complex, occlusive lesions called fibrous plaques. The fibrous plaques increase in size and, by projecting into the lumen of the artery, may impede the flow of blood. Fibrous plaques are covered by a dense cap of connective tissue with embedded smooth muscle cells. This cap overlays necrotic areas containing dead cells and cholesterol deposits. The fibrous plaque contains monocyte-dervived macrophages, smooth muscle cells and activated lymphocytes. The complex, multi-step process leading to formation of the fibrous plaque takes place over many years (reviewed in Ross 1993). Figure 1.1 is a diagram of a cross-section through an advanced fibrous (atherosclerotic) plaque.

Studies of animals with artifically induced hypercholesterolaemia (reviewed in Ross 1993) confirm that three processes contribute to the formation of atherosclerotic lesions: (1) proliferation of cells such as smooth muscle cells and macrophages (2) formation of

Figure 1.1
Diagram of a cross section through an advanced atherosclerotic plaque

(courtesy of Anne Lane)



connective tissue matrix by smooth muscle cells, this matrix contains proteins, collagens and proteoglycans and (3) accumulation of lipid (mainly free and esterified cholesterol) in cells and surrounding matrix of the plaque.

Post-mortem examination of coronary arteries of patients who had atherosclerosis or coronary artery disease (CAD) reveals multiple atherosclerotic plaques. When a plaque reaches a size which causes almost total occulsion of the artery lumen, angina pectoris is caused. Certain atherosclerotic plaques become fragile and rupture, initiating the clotting cascade and causing myocardial infarction (MI) if the resultant thrombus occludes the artery. It has been shown that most of the sudden deaths from MI are due to ruptures or fissures, particularly around the edge of the fibrous cap where there are more macrophages; this results in haemorrhage into the plaque, thrombosis and plugging of the arterial lumen (Davies & Thomas 1984).

Many risk factors have been recognized that contribute to the development of atherosclerosis i.e. the causes of atherosclerosis are mulifactorial and polygenetic. These risk factors include male gender, increasing age, obesity (measured as body mass index), Type A personality, smoking, high blood pressure (hypertension), high levels of some blood clotting factors (e.g. fibrinogen and Factor VII), positive family history for coronary artery disease (CAD), high dietary saturated fat intake and abnormal plasma lipid values (Nora et al. 1980, Crouse 1984, Grudy 1986, reviewed in Lowe 1993). Hypertension is directly related to blood viscosity; 50-80% of inter-individual variability in blood viscosity is accounted for by the cell volume fraction of blood (indicated by haematocrit) (reviewed in Lowe 1993). That the incidence of cardiovascular diseases is higher in men than in women, especially before menopause, may be partly explained by the higher haematocrit in men than women from puberty onwards (Lowe et al. 1992). Post-menopausal increases in haematocrit, plasma viscosity and fibrinogen, reduce the sex difference in whole blood viscosity, in parallel with the reduction of the sex differences in incidence of cardiovascular disease (Lee et al. 1993). The three main traditional cardiovascular risk factors, smoking, blood pressure and serum cholesterol levels, together predict only about one-third of coronary disease events (Heller et al. 1984).

Some of the above risk factors are chronic sources of injury to the arterial endothelium, such as hypercholesterolaemia, hypertension and smoking (reviewed in Ross 1993). Increasing plasma cholesterol levels are correlated with risk of CAD, however this relationship is not linear. Grüdy (1986) pooled the results from three studies, the Framingham Heart Study, the Pooling Project and the Israeli Prospective Study, and showed that above a plasma cholesterol concentration of 5.17mM, risk begins to rise. This threshold effect was not found in the Multiple Risk Factor Intervention Trail (Stamler et al. 1986), which studied 350,000 men. However these workers found a similar relationship; if the relative risk of CAD is considered to be 1 at 5.17mM, it doubles at a cholesterol concentration of 6.47mM and quadruples at 7.76mM. The age of onset of CAD in an individual is reduced by the interaction of high plasma cholesterol levels with other risk factors such as smoking, hypertension, diabetes, in an additive or possibly multiplicative manner (Grüdy 1986).

Individuals with high plasma cholesterol levels (above the 95th percentile of the normal distribution) due to heterozygosity for a genetic defect in a key receptor that mediates the uptake of cholesterol from the circulation (the low density lipoprotein (LDL) receptor) are particularly suspectible to early CAD, and begin to suffer MIs from their fourth and fifth decades, even in the absence of other risk factors. These individuals have hypercholesterolaemia due to only having half-normal levels of the LDL-receptor. Homozygosity for defects in the LDL-receptor leads to cholesterol levels that are 6 to 10 times higher than normal and death usually occurs in adolescence or early adulthood (Brown & Goldstein 1986).

Fasting plasma triglycerides (TG) are also associated with the risk of CAD. However, the issue of whether raised plasma TG is an independent risk factor for CAD has been controversial. This is due to the elimination of TG as a risk factor in most studies involving multivariate analysis which include other lipid paramters (Austin 1991). Both case-control and prospective studies have shown a significant association between elevated TGs and CAD on univariate analysis, however when multivariate analysis was used (adjusting TG for other lipid measurements, in particular HDL-cholesterol), no significant association between CAD and TG was observed in most studies. One factor to consider here is that

intraindividual variation in TG levels is considerably larger than the variation in plasma cholesterol levels (Jacobs & Barrett-Connor 1982) particularly at levels of cholesterol or TG > 6.4mM. This implies that misclassification of study subjects with regard to hypertriglyceridaemia (HTG) may occur since most large-scale epidemiological studies only sample individual subjects once. If this missclassification bias is the same for both disease and control groups, this will attenuate measures of association between fasting TGs and disease (Kelsey et al. 1986, Austin 1991). In addition, in a large sample of middle aged men, the interindividual variation in TG levels (as measured by standard deviation) was considerably greater than that in cholesterol levels (reviewed by Austin 1991). This is also likely to reduce the ability to detect associations with CAD, especially in comparsion with other less variable lipid measurements (Austin 1991). Recently, in a case-control study of postprandial lipaemia, postprandial TG levels predicted CAD, even in multivariate analysis including HDL-cholesterol (Patsch et al. 1992). These workers concluded that TGs are an independent predictor of CAD on multivariate analyses provided that a challenge test of TG metabolism such as postprandial lipaemia is used (discussed later in Section 1.8.3).

Elevated plasma levels of LDL-cholesterol and apolipoprotein B, the protein constituent of LDL are postive risk factors for CAD (discussed in Section 1.3.2) while levels of high density (HDL) cholesterol and apolipoprotein AI (the major protein in HDL) are inversely related to risk for CAD (discussed in Sections 1.5.1 and 1.7.4).

Thus, atherosclerosis is multifactorial, a result of interaction between genetic and enviromental factors, but lipoprotein metabolism clearly has a pivotal role in atherogenesis. Many proteins are involved in plasma lipid transport alone and the phenotype of plasma lipoproteins alone is very complex, involving many different genes, including those coding for apolipoproteins, lipoprotein receptors, lipid transfer proteins and enzymes involved in all aspects of lipid synthesis, metabolism and catabolism. Atherosclerosis probably represents an even more complex phenotype, involving other factors in addition such as the clotting cascade and the metabolism of cells in the arterial wall such as macrophages and smooth muscle cells (Lusis 1988). One illustration of this is the highly variable severity of atherosclerosis amongst individuals with homozygous FH caused by the same defect in the LDL-receptor (Hobbs et al. 1987).

Section 1.2 Overview of Lipid Metabolism

Cholesterol and triglycerides (TG), along with other lipids such as phospholipids, are transported in the blood in protein-lipid complexes called lipoproteins. As shown in Table 1.2.1, lipoproteins can be distinguished by their buoyant density, apolipoprotein composition and floation rates. Table 1.2.2 summarizes the major characterised apolipoproteins, the protein constituent of lipoproteins. These proteins have three main roles; binding of lipids thus making them soluble in the aqueous environment of the plasma, maintaining structural intergrity of the lipoprotein particle and acting as co-factors for enzymes and acting as ligands for lipoprotein receptors. Therefore apolipoproteins are the mediators of lipid metabolism and catabolism in plasma. Specific domains within the apolipoproteins mediate their functions. Most of the characterised apolipoproteins have amphipathic helices and transmembrane spanning structures which bind and solubilise lipid within lipoprotein particles. The amphipathic helices tend to lie at the lipid/aqueous interface of the lipoprotein particles. ApoB-100 and apoE have domains consisting of charged residues which are exposed on the surface of the lipoprotein particle; these domains are able to interact with residues of opposite charge in the ligand-binding domains of lipoprotein receptors. This enables cells to obtain lipids for their many metabolic processes. Apolipoproteins, which act as cofactors for enzymes, bind to the enzyme and alter enzyme conformation, enabling substrate to bind to the active site of the enzyme. Examples are apoCII which acts as a co-factor for lipoprotein lipase (LPL) and apoAI which acts as a co-factor for lecthin-cholesterol-acyltransferase (LCAT).

The classification of lipoprotein fractions is a general problem in the field of lipoprotein research. Chylomicrons are synthesized in the intestine and contain apoB-48, while very low density lipoproteins (VLDL) are produced in the liver and contain apoB-100. A cut-off point of either a density of 0.96g/cm or a floation rate, Sf of 400 is used during ultracentrifugal isolation of lipoproteins, above which lipoproteins are classified as chylomicrons and below which they are classified as VLDL. However the intestine does produce lipoproteins with a Sf less than 400 and there are some particles of hepatic origin with a Sf greater than 400. Hogg et al. (1990) observed that the human intestine is capable of synthesizing apoB-100 as well as apoB-48. This complicates the issue still further, as there may be lipoproteins with apoB-100 that contain exogenous, rather than endogenous,

Table 1.2.1 Characteristics of the Major Classes of Plasma Lipoproteins

Lipoprotein Class	Density (g/ml)	Flotation Rate *	Apolipo-proteins	Percentage Composition					Electrophoretic Mobility	Particle diameter (nm)
				protein	FC	CE	TG	Phospho-lipid		
Chylo-microns	<0.95	>400	AI, AIV, B-48, CI, CII, CIII, E	2	2	4	85-90	8	origin	100 - 1000
VLDL	<1.006	20 - 400	B-100, CI, CII, CIII, E	5-10	7	12	50-65	15-20	pre-β	30 - 90
IDL	1.006 - 1.019	12 - 20	B-100, E	15-20	8.5	22	20-30	20	'broad-β'	25 - 30
LDL	1.019 - 1.063	0 - 12	B-100	20-25	8.5	38	7-10	15-20	β	20 - 25
HDL	1.063 - 1.21	0 - 9	AI, AII, AIV, CI, CII, CIII, E	40-55	4	11	2-5	20-35	α	8 - 20

* Flotation Rates for lipoproteins of density < 1.063g/ml are in Svedbergs (Sf , 10^{-13} cm s^{-1} $dyne^{-1}$ g^{-1}) and are measured at a density of 1.063g/ml. For lipoproteins of density > 1.063g/ml, flotation rates are measured at a density of 1.20g/ml.
Data from Myant (1990). FC = free cholesterol, CE = cholesteryl ester, TG = triglycerides

Table 1.2.2 Major apolipoproteins in plasma

Apolipoprotein	Molecular Weight (kD)	Plasma concentration (mg/dl)	Function
ApoAI	28	60 - 160	Structural protein of HDL Activator of LCAT Role in peripherial mobilization of cholesterol? Antioxidative properties
ApoAII	17.4	25 - 55	Structural protein of HDL Phospholipid-binding property
ApoAIV	44.5	15 - 37	Required for intestinal fat absorption? Modulator of LPL activity? Roles in LCAT activation and cholesterol efflux from cells?
ApoB-100	550	60 - 160	Structural protein of VLDL and remnants, IDL and LDL Ligand for the LDL-receptor
ApoB-48	264	0 - 2	Structural protein of chylomicrons and chylomicron remnants
ApoCI	6.6	3 - 10	Unknown Modulator of apoE mediated uptake of remnant lipoproteins?
ApoCII	8.9	1 - 6	Activator of LPL
ApoCIII	8.8	4 - 20	Inhibits LPL? Modulator of apoE mediated uptake of remnant lipoproteins
ApoE	34.2	2 - 6	Ligand for the LDL-receptor and LRP

Data from Bhatnagar & Durrington (1993)

lipids. The VLDL subfraction is subdivided into $VLDL_1$ (Sf 60-400) and $VLDL_2$ (Sf 20-60) in a number of studies investigating lipoprotein metabolism (e.g. Fisher et al. 1993, Karpe et al. 1993a); within the Sf interval 20-400, lipoproteins are traditionally called VLDL but may be contiminated with lipoprotein particles of intestinal origin.

Figure 1.2 summarizes the major steps in lipoprotein metabolism. Following the digestion of dietary fat, the intestine secretes large TG-rich particles called chylomicrons which enter the circulation via the lymphatic system. Lipoprotein lipase (LPL) hydrolyzes the core TGs in chylomicrons to free fatty acids which are taken up by peripherial tissues. During this process chylomicrons are converted into remnants which are taken up by hepatic receptors. Redundant surface materials e.g. phospholipid, cholesterol and apolipoproteins are transferred to high density lipoproteins (HDL). The liver also produces TG-rich VLDLs, which are acted upon in the same way as chylomicrons, to produce VLDL remnants. A proportion of the particles (IDL) that are left are removed by the liver via interaction of apoE with specific receptors. A proportion of the VLDL remnants are further metabolized to LDL which have apoB-100 as their sole protein; these LDL particles are taken up by LDL-receptors in peripherial tissues and the liver. Circulating high density lipoproteins (HDL) are of intestinal and hepatic origin, and function in 'reverse cholesterol transport', accepting cholesterol from extrahepatic cells or from other lipoproteins. This cholesterol is esterified by lecthin-cholesterol-acyltransferase (LCAT), which requires apoAI as cofactor. The mature HDL particle, enriched in cholesteryl esters (CE), is taken up by the liver or participates in heteroexchange of lipids with TG-rich lipoproteins, with channelling of cholesterol from HDL to other lipoproteins; this process is mediated by cholesterolesteryl ester transfer protein (CETP) (Brown & Goldstein 1986). As a result of this CETP-mediated heteroexchange, the HDL increases in size and decreases in density; these HDLs are acted upon by hepatic lipase to regenerate smaller HDL particles which participate in CETP-mediated lipid heteroexchange (discusssed in more detail in Section 1.7.2).

Figure 1.2 Schematic Diagram of lipoprotein metabolism

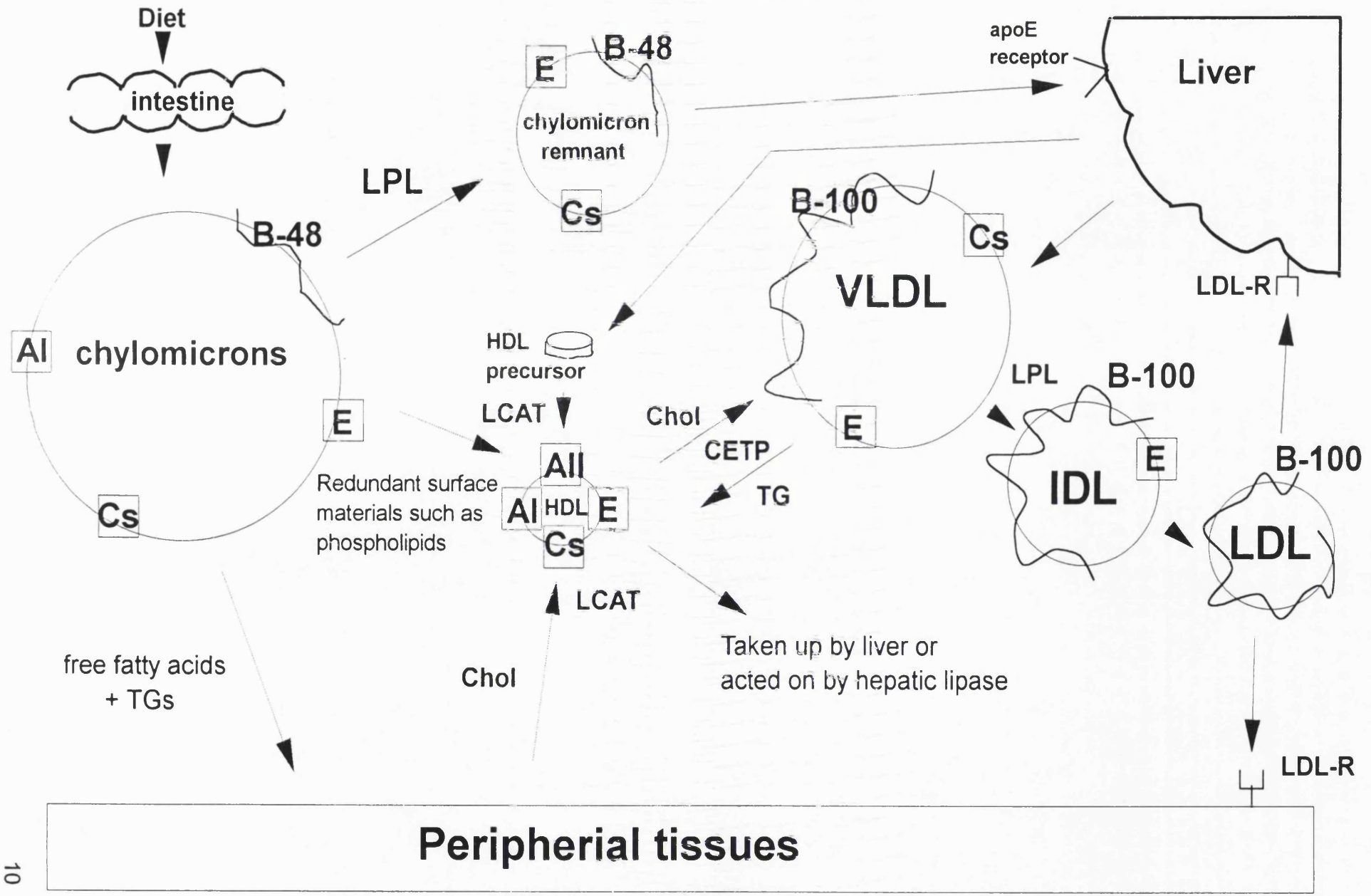


Figure - courtesy of Fanli Xu and Alison Dunning

Section 1.3 Apolipoprotein (apo) B

Section 1.3.1 ApoB protein structure

There are two different forms of apoB, apoB-100 and apoB-48 which are secreted by the liver and intestine, respectively, in the human. Different length variants of apoB are classified using the percentile scale, with full length apoB being apoB-100. Full length apoB is comprised of 4536 amino acid residues plus a 27 or 24 residue signal peptide and has a molecular weight of 550kDa. It is secreted by hepatocytes as a constituent of VLDL (Demmer et al. 1986). ApoB-48 is secreted by the intestine as part of the chylomicron particle, and comprises of 2153 residues. Studies using monoclonal antibodies have shown that apoB-48 is identical to the N-terminal half of apoB-100 (Olofsson et al. 1986, Hardman et al. 1987). ApoB has numerous internal repeats which show homology to each other, but the apoB molecule has very little homology with other proteins or apolipoproteins (Knott et al. 1986, Boguski et al. 1986). ApoB-100 has five long (52 residue) and eight shorter hydrophobic, proline-rich consensus sequences which are postulated to be lipid-binding domains, and may form amphipathic β-sheets (DeLoof et al. 1987a). ApoB-100 also contains nine amphipathic α-helical regions, which are clustered particularly around the middle and at the carboxyl-terminal end of the protein. These α-helic es share consensus sequences and have hydrophobic (lipid-soluble) residues on one side and hydrophilic residues on the other side; these helic es are thought to seek the lipid/aqueous interface of the lipoprotein particles (DeLoof et al. 1987b). Additionally, there are 39 short (approximately 7 residues long) hydrophobic sequences which are not long enough to tra-verse the phospholipid monolayer of the lipoprotein particle, but are believed to anchor the apoB molecule to the lipoprotein particle by dipping into its non-polar phase (Olofsson et al. 1987). There are also eight regions of basic residues, seven of these regions are heparin-binding sites (Weisgraber & Rall 1987 and discussed in more detail in Section 1.3.3).

There are 25 cysteine residues in the apoB-100 molecule, 16 of these participate in disulphide binding (Yang et al. 1990). These 16 cysteines are clustered in the amino terminal region which consequently has a more globular structure than the rest of apoB. Six out of the eight disulphide bridges in apoB-100 are between adjacent cysteine residues, hence the effect of disulphide binding on the tertiary structure of apoB is not large. Sixteen asparagine residues are glycosylated, giving apoB a total carbohydrate content of 10% of the

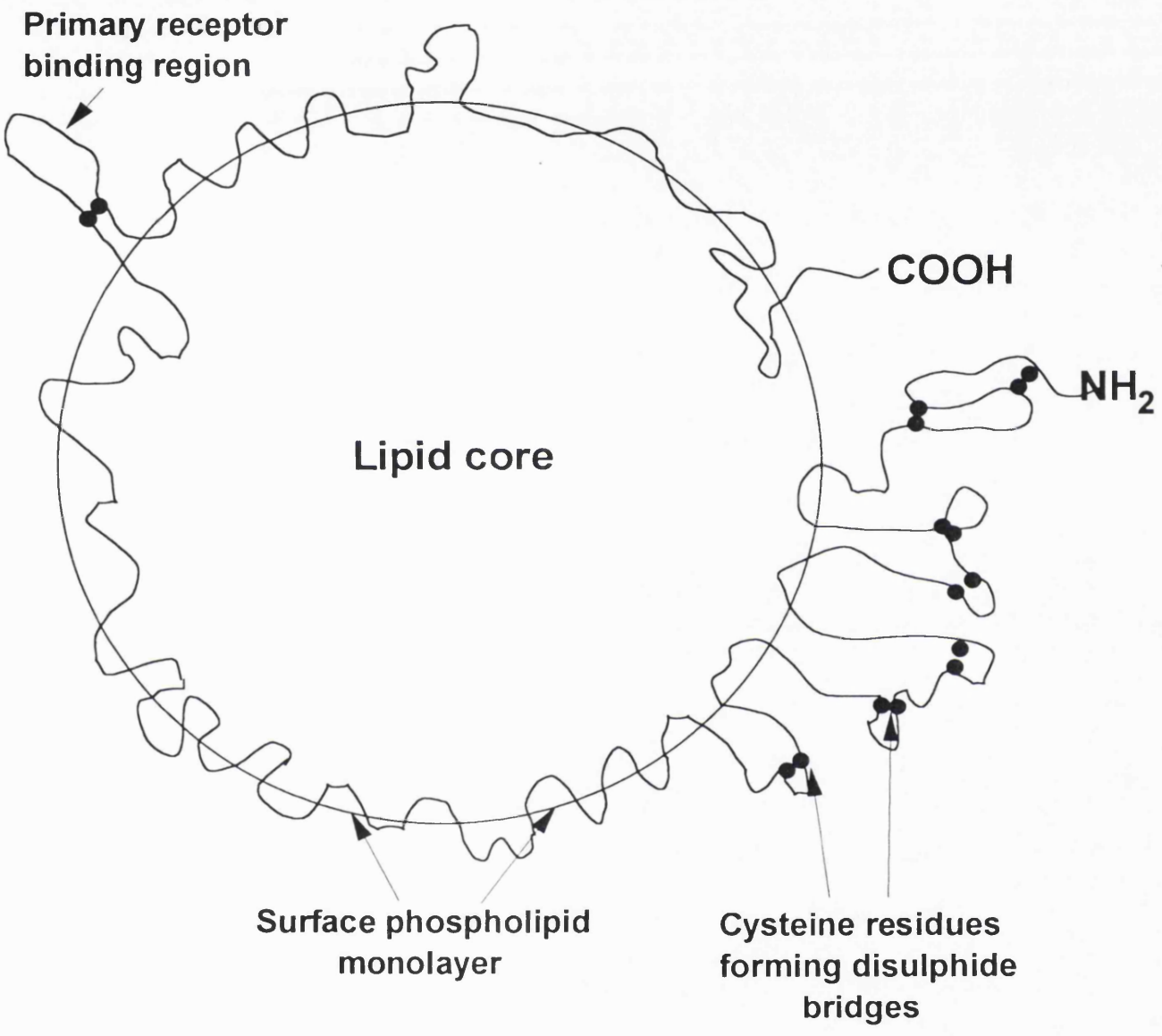
total molecular weight (Yang et al. 1989). Phosphorylation of apoB also occurs at serine and tyrosine residues (Davis et al. 1984) and apoB is also acylated (Hoeg et al. 1986). However the functions of these two modifications of apoB are unknown. Figure 1.3.1 is a representation of a cartoon of the structure of apoB within the LDL particle.

Two of the eight above-mentioned basic regions, which are particularly rich in the positively charged amino acid residues, arginine and lysine, are involved in forming the primary LDL-receptor binding domain, present in apoB-100 but not apoB-48. This LDL-receptor binding domain of apoB is conserved across different mammalian species although the overall positive charge in this region shows inter-specific variability (Law & Scott 1990). Cysteines 3167 and 3297 form a disulphide bridge in humans but not in other species, thus this disulphide bridge is not likely to be important in modulating binding to the LDL-receptor. Several approaches have been used to define the primary receptor-binding region of apoB-100, which is considered to span residues 3000 to 3700. Firstly, residues 3359 to 3367 of apoB, predicted to form an α-helix (Law & Scott 1990), has sequence homology to the clearly defined LDL-receptor binding domain of apoE (the other ligand for the LDL-receptor) which spans residues 142 to 150 (Knott et al. 1986). Secondly, monoclonal antibodies which bind to epitopes of apoB between residues 3000 and 3700, are able to block the binding of apoB to the LDL-receptor (Milne et al. 1989). Finally, apoE-depleted lipoprotein particles containing synthetic peptides spanning apoB residues 3345 - 3381 are able to bind to the LDL-receptor (Yang et al. 1986).

Section 1.3.2 Role of apoB in lipid metabolism

ApoB-100 and apoB-48 have central roles in the exogenous and endogenous lipid pathways, respectively. The exogenous lipid pathway is discussed in more detail with respect to postprandial lipid metabolism in Section 1.8.1. Briefly apoB-48 is synthesized in the intestine and secreted into the lymph in chylomicrons. These lipoproteins carry TGs and other lipids of dietary origin into the circulation. The TGs are hydrolysed by lipases, lipoprotein lipase (LPL) in peripherial capillaries (discussed in more detail in Section 1.6) and by hepatic lipase (HL) in the liver. The resultant free fatty acids are taken up by peripherial tissues, for immediate use as an energy supply or for storage in adipose tissue. The action of LPL generates smaller chylomicron remnant particles which are cleared by the

Figure 1.3.1 Representation of the structure of apoB in LDL (adapted from Yang et al. 1989) by courtesy of Alison Dunning



liver via apoE binding to receptors, probably the LDL-receptor related protein [LRP] (Herz et al. 1988). ApoB-48, which lacks the LDL-receptor binding domain (Yang et al. 1989) is not a ligand for receptor-mediated uptake, however the lipid binding domains in apoB-48 are thought to be important in the maintenance of the structural integrity of the chylomicron and later its remnant.

ApoB-100 is synthesized in the liver and is secreted in VLDL. These lipoprotein particles, which also contain the liver-synthesized apolipoproteins E, CI, CII and CIII, transport cholesteryl esters and TGs of endogenous origin and are acted upon by LPL in the same manner as chylomicrons (discussed in more detail in Sections 1.6 and 1.8.1). Approximately 60% of VLDL-remnants (this proportion is dependent on apoE phenotype) are cleared directly through apoE binding to receptors (Demant et al. 1991 - this is discussed in Section 1.4.6). The density of the remaining VLDL-remnants increases, with a corresponding decrease in diameter, to generate first IDL and then LDL, as more TGs are removed by lipase action. Some IDL particles can be directly cleared via apoE binding to the LDL-receptor (Packard et al. 1985). At the same time that apolipoproteins are being transferred from IDL to HDL, cholesterol ester transfer protein (CETP) mediates the heteroexchange of lipids between lipoproteins, further increasing the relative proportion of cholesterolesteryl esters in IDL and then LDL. ApoB-100 is the sole protein in LDL, the principal carrier of cholesteryl esters, and maintains the structural intergrity of these lipoprotein particles (Yang et al. 1986, 1989).

The receptor-binding domain of apoB-100 only becomes available to the LDL-receptor once the VLDL particle has been metabolized to LDL (Chappell et al. 1991). Since the majority of cholesteryl ester in the circulation is transported as LDL, the role of apoB-100, as ligand for clearance of cholesteryl ester-rich lipoproteins, is critical to cholesterol metabolism (Brown & Goldstein 1986). LDL clearance takes 36 hours to 3 days, in constrast to a few minutes for chylomicrons. The positively charged residues of the apoB-100 receptor-binding domain are thought to interact with the negatively charged aspartate and glutamate residues in the seven ligand-binding domains of the LDL-receptor (Brown & Goldstein 1986). These ligand-binding domains of the LDL-receptor are each capable of binding one apoE molecule, but co-operate to bind apoB. Thus the LDL-receptor can bind

several apoE molecules stimultaneously, dependent on the spatical arrangement of these apoEs on the lipoprotein surface, but only a single apoB molecule. This is reflected in the fact that VLDLs have 4 to 6 apoE molecules, but only one apoB molecule, per lipoprotein particle (Russell et al. 1989). Ligand-receptor interaction is also affected by their relative sizes; the LDL particle being 20 times larger than the LDL-receptor. Most LDL-receptors are clustered within clathrin-coated pits which occupy less than 2% of the cell surface, and in-vitro studies have shown that crowding of receptor-bound lipoproteins within individual pits (demonstrated using electron microscopy) sterically hinder further lipoproteins from binding to the receptors (Chappell et al. 1991). These authors also demonstrated that LDL-receptors can clear fewer large LDL than small LDL particles, due to blocking access to adjacent LDL-receptors within the pit. These large LDL particles, which still carry apoE in addition to apoB-100, are able to bind to two LDL-receptors at the same time, with apoE binding to one and apoB binding to the other. VLDL particles are able to interact with the LDL-receptor in multiple orientations, due to presence of multiple apoE molecules on each VLDL; this probably speeds up receptor-mediated clearance of VLDLs, while LDL containing only apoB-100 can only interact in one orientation.

Many aspects of apoB production and VLDL synthesis are still controversial. Briefly, what has been confirmed is that the apoB gene is transcribed constitutively, with the half-life of mRNA in HepG2 cells being more than 16 hours (Pullinger et al. 1989). Translation of the mRNA on the ribosome takes approximately ten minutes (Borchardt & Davis 1987) and the nascent protein is co-translationally attached to the inner membrane of the endoplasmic reticulum (ER) (Knott et al. 1986, Olofsson et al. 1987). On the inner membrane or lumen of the ER, apoB associates with phospholipids, TGs and unesterified and esterified cholesterol. If insufficient lipid is available, the apoB is degraded within the ER, or possibly the Golgi apparatus (Borchardt & Davis 1987, White et al. 1992). If enough lipid is available, apoB buds off to form a nascent lipoprotein (Olofsson et al. 1987). This passes through the smooth ER and into the Golgi apparatus where more lipid is added (Janero & Lane 1983, Higgins 1988) before the mature lipoprotein is secreted. Thus, control of apoB-containing lipoprotein production is at the post-translational level, not at the transcriptional level. This ensures that there is always sufficient intestinal and hepatic apoB available to efficiently package all dietary lipid input and hepatic lipid output and export it as

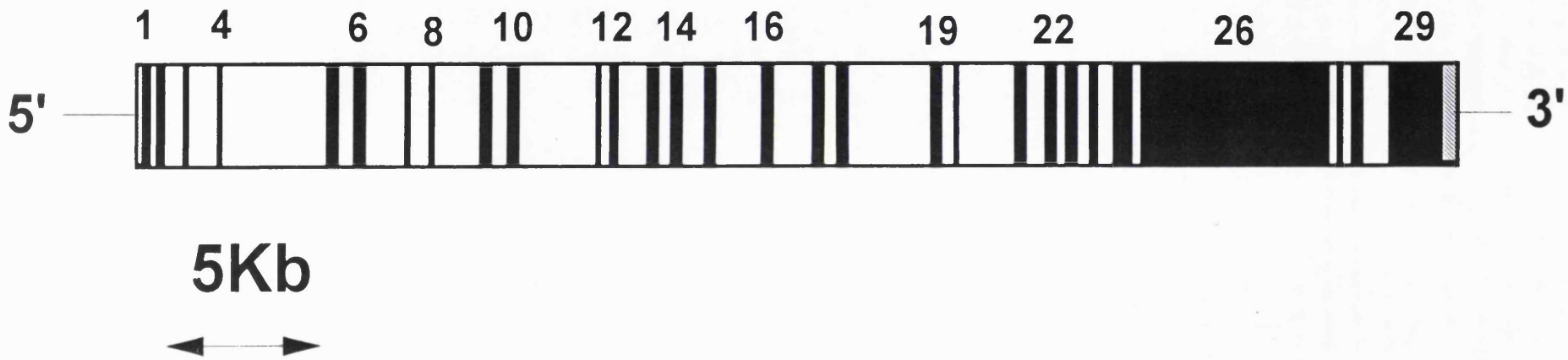
chylomicrons or VLDLs.

Section 1.3.3 The apoB gene

In the human, both apoB-100 and apoB-48 are encoded by a single gene on the short arm of chromosome 2 (Knott et al. 1985, Hardman et al. 1987). The full length gene, which spans 47.5kb, was first cloned in 1986 (Blackhart et al. 1986, Chen et al. 1986, Cladaras et al. 1986, Law et al. 1986, Knott et al. 1986, Ludwig et al. 1987). The gene has 29 exons and 28 introns (Figure 1.3.3) and the mRNA is 14kb in length. The region encoding the amino-terminal 26% of apoB is built up of normal-sized exons (size of exons range from 39 bp to 238bp) separated by introns, while the 10,149bp region encoding the carboxyl-terminal 74% of apoB is divided into only 5 exons (exons 25 to 29) (Blackhart et al. 1986). Exon 26, spanning 7.5kb, is by far the largest known mammalian exon and exon 29, at 1.9kb is also notably long. The reasons for this unusual intron/exon structure of the apoB gene are not known. No signs of duplications of a smaller unit are obvious in the nucleotide sequence. Introns could have been selectively lost from an ancestral gene, or reverse transcription could have occured from a mRNA species followed by reinsertion of the cDNA into the genome. Intron 28 contains a poly-T sequence which might be the residue of a poly-A tail from a reverse-transcribed precursor of exon 29. The gene for apoB shares very little homology to any other gene.

ApoB-48 is identical to the amino-terminal 48% of apoB-100, comprising the first 2152 residues of the full-length polypeptide (Hardman et al. 1987, Innerarity et al. 1987). In the human, apoB-48 is produced as a result of the editing of the mRNA transcript in the intestine (Powell et al. 1987). Codon 2153 (CAA) which codes for Glutamine in apoB-100 is converted to UAA - the stop codon for apoB-48. This C_{6666} to U change in the mRNA transcript has a minimum sequence requirement which spans nucleotides 6649 to 6703 (Driscoll et al. 1989). This mRNA editing requires enzymatic action (Driscoll et al. 1989); the cDNA encoding this enzyme in rat small intestine, was recently cloned and characterized (Teng et al. 1993a). This protein, called RNA editing protein (REPR) is essential for apoB mRNA editing, is 299 residues long, and has consensus phosphorylation sites and leucine zipper domains (Teng et al. 1993a). The catalytic subunit of REPR is a zinc-containing cytidine deaminase of molecular weight 27KDa (Teng et al. 1993b).

Figure 1.3.3 Schematic representation of the apoB gene



The transcribed portion of the apoB gene is represented by the rectangle. Within this rectangle, exons and introns are represented by filled and open boxes. Exons are numbered 1 to 29. Untranslated regions of exons 1 and 29 are shown by shaded boxes (adapted from Blackhart et al. 1986).

Section 1.3.4 Protein and DNA polymorphisms of apoB

Since the cloning of the apoB gene, at least 20 polymorphisms have been reported within the gene and its flanking regions, and some of these are shown in Table 1.3.4. The first polymorphisms reported were those which were detectable by the creation or destruction of a variable restriction endonuclease site. Later, with the advent of techniques for detecting single base changes in genes, polymorphisms that do not alter restriction endonuclease sites have been reported. Six of these DNA polymorphisms result in single amino acid substitutions, and thus alter the primary structure of apoB. Additionally, over 70 potential sequence variants have been revealed by comparsion of the eight published cDNA sequences of the apoB gene (Ludwig et al. 1987, Yang et al. 1989). However these probably include many sequencing artifacts, as well as rare DNA variants and true DNA polymorphisms.

In 1961, protein polymorphisms of apoB were first detected using antisera from multiply-transfused patients (Allison & Blumberg 1961). These workers reported an Antigen Group (Ag) system of variants that are caused by different antigens (epitopes) on LDL, of which there are ten. Butler and Brunner (1974) found that these ten epitopes are arranged into five tightly linked loci, each with two alleles. These epitope pairs are called Ag (c/g), Ag (a1/d), Ag (h/i), Ag (t/z) and Ag (x/y) (reviewed in Breguet et al. 1990). The underlying nucleotide substitutions that are the likely causes of the molecular basis of the Antigenic (Ag) epitopes of apoB are C_{293} to T for Ag (c/g) (Ma et al. 1987, 1989, Young et al. 1989a), C_{1891} to T for Ag (a1/d) (Wang et al. 1988), C_{8344} to T and A_{13141} to G for Ag (x/y) (Wu et al. 1991), G_{11040} to A underlying Ag (h/i) (Huang et al. 1988, Xu et al. 1989) and G_{12669} to A underlying Ag (t/z) (Ma et al. 1987, Dunning et al. 1988), confirming that the antigens are generated by protein polymorphisms of apoB expressed on LDL particles.

Strong linkage disequilibrium and allelic association have been reported between many of the polymorphic sites in the apoB gene. Berg et al. (1986), who first reported linkage between the Ag system and the apoB gene (LOD = 4.5, θ = 0) also noted the allelic association of the X- allele of the XbaI polymorphism with the Ag(x) epitope. There have also been reports of linkage disequilibrium between the XbaI polymorphism and the following sites: Ag(c/g), Ag(x/y), Ag(t/z) and the signal peptide length polymorphism (Berg et al. 1986, Ma et al. 1987, Dunning et al. 1988, Myant et al. 1989, Xu et al. 1990a, Renges et

Table 1.3.4
Selected polymorphisms at the human apoB gene locus

Position	Reference	Base(s) affected	Restriction enzyme	Amino acid change
5' flanking HVR	Ludwig & Carthy 1990	$(TG)_n$ 3256bp 5' to start	-	-
5' flanking	Huang et al. 1990a	≈ 4Kb 5' to start	AvaII	-
Promoter	Jones et al. 1989	C to T at -265bp	MspI	-
Exon 1	Boerwinkle & Chan 1989	9bp deletion	-	Leu-Ala-Leu deletion from signal peptide
Intron 3	Huang et al. 1990a	G to T 92bp 3' of exon 3	-	-
Exon 4	Young et al. 1989a	C_{293} to T	ApaLI	Thr71 to Ile Ag(g) to (c)
Intron 4	Darnfors et al. 1986, Huang et al. 1990a	A to C 171bp 3' of exon 4	HincII	-
Exon 14	Wang et al. 1988	C_{1891} to T	AluI	Ala591 to Val Ag (d) to (a1)
Intron 20	Huang et al. 1990a	G to A 146bp 5' of exon 21	BalI	-
Intron 20	Zuliani & Hobbs 1990	$(TTTA)_n$	-	-
Exon 26	Priestley et al. 1985, Talmud et al. 1985	C_{7673} to T	XbaI	-
Exon 26	Huang et al. 1990b	C_{8344} to T	-	Pro2712 to Leu Ag (y) to (x)
Exon 26	Huang et al. 1988	G_{11040} to A	MspI	Arg3611 to Gln Ag (i) to (h)
Exon 29	Priestly et al. 1985, Shoulders et al. 1985	G_{12669} to A	EcoRI	Glu4154 to Lys Ag (t) to (z)
Exon 29	Navajas et al. 1991, Dunning et al. 1992	A_{13141} to G	-	Asn4311 to Ser Ag (y) to (x)
3' Flanking VNTR	Berg et al. 1986, Boerwinkle et al. 1989	11-16bp AT-rich tandem repeat 181bp 3' to exon 29	MspI, BamHI, EcoRV	-

al. 1992), the PvuII polymorphism with Ag(a1/d) (Dunning et al. 1988), and Ag(t/z) with the 3' VNTR polymorphism (Hegele et al. 1986, Renges et al. 1992). These were indications that there is strong linkage disequilibrium and allelic association between polymorphic sites across the entire apoB gene, which is evidence of an evolutionary relationship between these alleles, but which does not necessarily depend on the physical distance between the polymorphic sites (Dunning et al. 1993).

Section 1.3.5 Associations of apoB polymorphisms with lipids and lipoproteins

In 1976, Berg et al. reported that the Ag(x-) genotype was associated with higher cholesterol and TG levels. Later Berg et al. (1986) found a high degree of linkage disequilibrium between the Ag(x) variation and the XbaI polymorphism. These workers demonstrated that the Ag(x+) frequency was nearly 60% in the X-X- group but less than 10% in the X+X+ group. In a study from Sweden, presence of the Ag(x) was associated with a modest lowering effect on total and LDL-cholesterol, TGs and apoB and significantly raised levels of HDL-cholesterol (Dunning et al. 1991); the effect of Ag(x) on TGs was to lower by 16%, of the same order of magnitude as the original findings of Berg et al. (1976). This effect has recently been confirmed in samples of male MI survivors and matched healthy controls from three centres in Europe (Moreel et al. 1992). Strong linkage disequilibrium between the Ag(x/y) and XbaI polymorphisms, with the allele encoding Ag(x) being nearly always found on the same chromosome as the X- allele, was reported (Dunning et al. 1992, 1993).

Population studies have shown that variation at a polymorphic XbaI site within the apoB gene is associated with differences in lipids, with the strongest and most consistent effects seen on total or LDL-cholesterol levels; the X+ allele (presence of the XbaI cutting site) being associated with raised levels (Table 1.3.5 and reviewed in Humphries et al. 1992), at least in Caucasian populations. The X+ allele has also been associated with significantly lower levels of HDL-cholesterol and apoAI (Myant et al. 1989, Saha et al. 1992) - one explanation for this is that variation in apoB structure, in linkage disequilibrium with the X+ allele, determines, in part, the rate of transfer of lipid components from VLDL and its remnants to HDL (Saha et al. 1992). Studies using small samples may have failed to detect this association, if confounding variables such as gender, age, body mass index were not

taken into account, or due to the inclusion of individuals in the sample who are more severely hyperlipidaemic. Studies on non-Caucasian populations, Japanese (Aburatani et al. 1988) and Chinese (Saha et al. 1992, Tong et al. 1992) report that the X+ allele is associated with lower total and LDL-cholesterol levels, contrary to Caucasian samples (see Table 1.3.5). This might be explained by the very low X+ frequency in the Chinese (Saha et al. 1992) or a differential expression of association of genotypes of the XbaI polymorphism with serum lipids due to a difference in environmental factors such as diet and socioeconomic factors (Tan et al. 1984), or the X+ allele being in linkage disequilibrium with a different causative mutation in these non-Caucasian populations. The size of the effect associated with this polymorphism is modest, explaining 3 - 8% of the variance in cholesterol levels in samples of healthy, normolipidaemic Caucasian individuals, and is of the same order of magnitude as is seen with variation at the apo E gene.

To date, the molecular basis of this association is not completely understood. The base change that creates the XbaI site does not alter an amino acid, as it is in the third (wobble) position of a codon for threonine 2488 (Carlsson et al. 1986), and so cannot be the direct cause of the effects seen. It is thus likely that there is additional sequence variation elsewhere in the gene, and in linkage disequilibrium with the XbaI polymorphism, that leads to altered serum lipid levels. Such sequence differences could occur in the promoter region of the apo B gene where the change may alter transcription of the gene and thus affect production of the protein from the intestine or liver. Alternatively, as with apo E2 (Davignon et al. 1988) and the apo B3500 mutation [see later - Section 1.3.7] (Innerarity et al. 1990), a sequence change may cause an amino acid substitution in the protein and reduce the affinity of LDL for its receptor. Evidence in favour of the latter mechanism has come from two in-vivo studies that have shown that, compared with the X- allele, the X+ allele is associated with a reduced fractional catabolic rate (FCR) of autologous LDL in a group of patients with moderate hyperlipidaemia (Demant et al. 1988) and a group of healthy men (Houlston et al. 1988). X+X- heterozygotes had intermediate LDL catabolic rates (Demnant et al. 1988). These data therefore predict that LDL from individuals with the genotype X-X- would bind to the LDL-receptor with increased affinity compared to LDL from individuals with the genotype X+X+.

Table 1.3.5 - Associations observed with the X+ allele of the XbaI polymorphism in the apolipoproteinB gene in healthy individuals.

Reference	Sample (age in years; ranges or means ± SD given)	Total Cholesterol	LDL-cholesterol	ApoB	Triglycerides
Berg 1986	56MF (20-29)	↑ p=0.036	not given	↑ p=0.004	↑ NS
Hegele et al 1986	84MF (58 ± 10)	↑ NS	↑ NS	↑ NS	↑ NS
Law et al 1986	83M (60 ± 7)	↑ p=0.03	not given	↑ NS	↑ p=0.02
Talmud et al 1987	62MF (20-65)	↑ p<0.025	↑ NS	not given	↑ p<0.05
Aalto-Setala et al 1988	176MF (20-66)	↑ p=0.01	↑ NS	↑ NS	↑ NS
Aburatani et al 1988 ⊘	54MF (19-62) **	↓ NS	not given	↓ NS	↓ NS
Houltson et al 1988	22M (35-49)	↑ NS	↑ NS	not given	not given
Jenner et al 1988	22MF (25-40)	↑ NS	not given	not given	↑ p=0.05
Rayput-Williams et al 1988	290M (49-65) *	none	not given	not given	none
Darnfors et al 1989	187MF (27-65)	↑ NS	↑ NS	none	↓ NS
Myant et al 1989	102M (25-60)	↓ NS	none	↓ NS	↓ NS
Genest et al 1990	122M (55 ± 5)	none	↑ NS	↓ NS	↓ NS
Myklebost et al 1990	105M (43)	↑ NS	not given	not given	↑ NS
Paulweber et al 1990	118M (50 ± 5)	↑ NS	↑ NS	↓ NS	↓ NS
Renges et al 1991 ⊘	153M (40-69)	↑ p<0.1	not given	not given	↑ NS
Deeb et al 1991	86MF (<65)	↑ p<0.05	↑ NS	↑ NS	not given
Saha et al 1992 ⊘	196MF (25-60)	↓ NS	↓ NS	↓ NS	↑ NS
Tong et al 1992 ⊘	154MF (44 ± 1.0)	↓ NS	↓ NS	↑ NS	↑ NS
Vilella et al 1992	228M (19-65)	↑ p<0.005	↑ p<0.005	↑ p<0.025	not given
Evans et al. 1993 ⊘	143M (33-67)	↑ NS	↑ NS	↑ NS	↓ NS
Friedlander et al 1993a + **X**	37MF (33 ± 5)	none	none	↑ p=0.08	↑ NS
Friedlander et al 1993b +	525MF (29 ± 6)	↑ p=0.02	↑ p=0.02	↑ p=0.03	↑ NS
Hansen et al 1993	464M (40)	↑ p=0.017	not given	↑ p=0.043	none

M = men; F = women * random sample ** normo- and hypertriglyceridaemic individuals analyzed together
⊘ = non-Caucasian population used + Israeli population used **X** effect on baseline lipids prior to dietary intervention trial.

Support for this hypothesis has been obtained using an in-vitro competitive binding and internalisation assay of radiolabelled LDL to normal fibroblasts, where "X+" LDL competed poorly compared to "X-" LDL, as expected from the turnover data (Series et al. 1990). LDL from X-X- individuals was catabolized faster than LDL from X+X+ individuals; this was most pronounced in older normocholesterolaemic and in hypercholesterolaemic individuals. The effect of XbaI genotypes may be stronger when the LDL-cholesterol level is higher. LDL-cholesterol levels and composition of LDL are strongly modulated by age (Austin & Krauss 1986). Series et al. (1989) concluded that with increasing age, X+ LDL becomes less recognisable to the LDL-receptor and results in decreased catabolism of the lipoprotein. This is compatible with the hypothesis that a common amino acid change in the apo B protein in linkage disequilibrium with the XbaI polymorphism, may affect the affinity of LDL-apoB for the LDL receptor. More recently, Vilella et al. (1992) observed a paradoxically elevated in-vitro binding and degradation of LDL from X+X+ individuals compared to X-X- individuals, with X+X- individuals being intermediate and all three genotype groups were matched for age and body mass index (BMI). These workers suggest that the increased LDL concentrations in individuals with the X+X+ genotype result from a phenomenon other than a defective ligand-receptor interaction directly related to the X+ allele.

It is possible that a certain genotype may predispose to an elevated level of plasma lipids only in the presence of a particular dietary environment. Such knowledge would be useful in counselling certain individuals, in order that they may avoid such environments. Several recent studies have provided evidence that the response of plasma lipid levels to dietary modification is under genetic influence. Studies in humans have demonstrated the existence of hypo- and hyper-responders to dietary change, and that these traits are stable over several years (Katan et al 1986). The magnitude of dietary effects on total and LDL-cholesterol seems to vary between individuals (Katan et al. 1988). However, the genes responsible for these differences are unknown. Several studies have examined the possibility that the common protein polymorphisms of apoE are associated with such differences (discussed in Section 1.4.5).

The effect of polymorphisms at the apoB locus in determining differences in

variability in response to dietary changes have been investigated in fewer studies, and no consistent picture has emerged yet. In the North Karelia Dietary Intervention study (Ehnholm et al 1984), significant differences associated with apoB genotype were found, with associations between the signal peptide polymorphism and levels of triglycerides (Xu et al 1990a), and the XbaI polymorphism and levels of apoAI and HDL-C (Tikkanen et al 1990, Xu et al 1990b). The signal peptide polymorphism is caused by the deletion of 9bp, coding for the three amino acids leucine-alanine-leucine in the hydrophobic core sequence of the 27 amino acid signal peptide. On changing from a high fat to a low fat diet the mean serum triglyceride levels of the whole sample fell by 2.7%, but individuals who were homozygous for the common 27 amino acid allele had a 10% mean reduction in plasma triglycerides (0.11 mmol/l) while individuals with one or more deletion allele showed a slight mean increase in plasma triglycerides (0.07 mmol/l, $p = 0.03$). A similar effect has been noted in a smaller study from the US (Boerwinkle et al 1991), where the reduction in plasma triglycerides induced by the low-cholesterol diet in those homozygous for the SP-27 isoform was twice that seen in those with the SP-24 isoform (3.9 mg/dl compared to 1.5 mg/dl) although these differences did not reach statistical significance.

For the XbaI polymorphism, several studies have reported associations with dietary-induced changes in levels of apoAI and HDL-C. In the North Karelia study the mean reduction in apoAI levels in response to the dietary change in individuals with the genotype X-X- was less than half that seen in those with one or more X+ allele (reduction of 4.1mg/dl compared to 9.5mg/dl $p < 0.05$). In a small study from Israel (Friedlander et al 1993a) those with one or more X+ alleles showed a larger fall in apoAI when changing from a high cholesterol to a low cholesterol diet compared to those with the genotype X-X-, who showed an increase in apoAI levels (-9.8mg/dl compared to +4.3mg/dl), although this difference was not significant between genotypes. This same dietary manipulation caused an average decrease of 0.47mM in total cholesterol and 0.41mM in LDL-cholesterol in X-X- individuals, compared to only 0.25mM and 0.12mM, respectively, in carriers of the X+ allele (not significant). By contrast, in a study of children from Italy who were recommended to consume a low fat diet (Xu et al 1992) those with the genotype X-X- showed the greatest reduction in levels of apoAI (18 mg/dl compared to 9 mg/dl, $p < 0.05$), and no association with XbaI and dietary response was seen in a study of 51 subjects from Australia (Abbey et

al 1991). The major problems with interpreting these studies are the different ages, and genetic and cultural (dietary) backgrounds of the participants, the different dietary protocols used and the difficulty of ensuring compliance. Taken together, the data suggest that the effects associated with apoB genotype (but not apoE genotype) are likely to be making a small contribution, with genetic variation interacting with baseline levels to determine individual dietary responses. However, it is clear that the genes involved in determining the strong genetic effects that have been observed on dietary response have yet to be identified, with the apoB XbaI locus not being a major determinant of inter-individual differences in dietary responses.

Section 1.3.6 Associations of apoB polymorphisms with dyslipidaemias and atherosclerosis

A number of case-control studies have reported an altered frequency of alleles of the XbaI and/or EcoRI polymorphisms in patients with dyslipidaemias or atherosclerosis. The frequency of the X+ allele (associated with higher total and LDL-cholesterol levels in most studies discussed in Section 1.3.4) was higher in Type III hyperlipoproteinaemia (HLP) (Talmud et al. 1987) but was significantly lower in MI survivors (Hegele et al. 1986) and in patients with peripherial arterial disease (Monsalve et al. 1988) or with CAD (Myant et al. 1989). This suggests that genetic variation, in linkage disequilibruim with the X- allele, that predisposes to CAD, is distinct from that in linkage disequilibrium with the X+ allele that predisposes to high LDL-cholesterol levels. The X- allele was an independent discriminator for MI on multivariate analysis (independent of lipid and lipoprotein levels), being the third most important predictor of atherosclerosis after HDL_3-cholesterol and VLDL-TGs, by step-wise multivariate analysis (Tybjaerg-Hansen et al. 1991). These workers suggest that variation associated with the XbaI polymorphism may determine suspectibility to CAD independently of other conventional risk factors i.e. that some attribute of apoB other than its ability to bind to the LDL-receptor may determine suspectibility to CAD. Studies on Japanese (Aburatani et al. 1988) and Chinese (Saha et al. 1992) samples failed to detect case-control differences probably because of the much lower frequency of the X+ allele in these populations compared to Caucasian samples.

More consistent results have been obtained with the EcoRI polymorphism. In several

studies, the frequency of the rare allele (R-) is increased in patients with peripherial or coronary artery disease (Deeb et al. 1986, Monsalve et al. 1988, Myant et al. 1989, Genest et al. 1990, Paulweber et al. 1990, Talmud & Humphries 1992) and in Type IV and V HLP (Jenner et al. 1988). Myant et al. (1989) suggested that in caucasian men the R- and X- alleles are in linkage disequilibrium with a nearby allele that is causally related to CAD, e.g. via variability of risk factors. Berg (1987, 1989) suggested that genetic variation may contribute to the variability of a risk factor e.g. plasma cholesterol, with the R- allele being associated with higher within-pair differences in apoB levels in monozygotic twins. In an early study (Groover et al. 1960), the 16 cases of MI observed, over a 5 year period, in a sample of 177 individuals, occured in the group showing the greatest fluctuations in cholesterol levels over time. More recently, Monsalve et al. (1991) showed that genotypes of both the XbaI and EcoRI polymorphisms are involved in determining the size of the within-individual variation in serum cholesterol levels over time, with X+X- or R+R- genotypes being associated with more variation than homozygosity for the X+ or R+ alleles.

Studies have implicated the involvement of the 3' end of the apoB gene in the regulation of VLDL metabolism (Paulweber et al. 1990, Tybjaerg-Hansen et al. 1991). The R- allele of the EcoRI polymorphism was associated with higher levels, in the VLDL fraction, of apoB, cholesterol and TG in patients with CHD; this may reflect a casual relationship between VLDL levels and an increased risk for CHD. The R- allele was associated with higher total and VLDL-TG and VLDL-cholesterol levels both in patients with atherosclerosis and in controls, but did not predict MI or peripherial atherosclerosis independently, suggesting that the R- allele predisposes to atherosclerosis by co-association with other risk factors such as lipoprotein levels (Tybjaerg-Hansen et al. 1991).

A model to explain these findings based on evolutionary history has been proposed (Talmud & Humphries 1992). In the original population, a mutation predisposing to development of atherosclerosis, occured on the apoB gene. By chance, the mutation (ATH) occured on a chromosome carrying the rare allele (R-) of the EcoRI polymorphism. There were now three different types of chromosome; R+ (normal), R- (normal) and R- (ATH). As a result, many generations later, due to this historical association, many people with premature atherosclerosis would have the R- allele, but this allele would also occur in healthy

people. Patients with the genotype R+R+ would also be found, explained either by recombination or the occurrence of an independent mutation. In general, the rate of recombination between two loci is dependent on the genetic distance between these two loci. For the apoB gene, the small genetic distance across the gene means that it will take many hundreds of generations for recombination to lead to the loss of association between ATH and a particular allele of a DNA polymorphism. However this ATH locus has not yet been identified and the mechanism of action of this locus is not yet known. However it is not mediated via differences in plasma lipid levels.

The EcoRI polymorphism is caused by a G to A base change at nucleotide 12669, which also causes a Glu to Lys substitution at residue 4154 of the mature protein and corresponds to the Ag (t/z) polymorphism (Ma et al. 1987). This alters the primary sequence of apoB, but due to the enormous size of the protein, it has not been detected as an isoelectric variant. The possible functional significance of this Glu (R+) to Lys (R-) substitution has not yet been eludicated, and it is not known if it has any direct effect on apoB structure or function. Gallagher & Myant (1992) did not find any significant difference in the LDL-receptor binding affinity of apoB in individuals homozygous for the R+ or R- alleles, although mean affinity of apoB for the LDL-receptor was 29% higher for R+R+ homozygotes than for those homozygous for the R- allele, with mean concentration of unlabelled competitor IDL required for 50% competition being 14% higher for R+R+ homozygotes. This suggests that this Glu to Lys substitution does not have a major influence on the function of the LDL-receptor binding domain of apoB, and that an association between CAD and the EcoRI polymorphism is not mediated via levels of LDL-cholesterol. This is in line with the absence of association reported between cholesterol levels and genotypes of the EcoRI polymorphism (Hegele et al. 1986, Myant et al. 1989).

It is possible that the Glu to Lys substitution at residue 4154 might favour the formation of oxidized species of LDL in-vivo (Gallagher & Myant 1992). This is supported by an earlier observation that the mechanism of LDL-oxidation appears to involve the covalent linkage of decomposition products of fatty acids to Lys residues in apoB (Steinbrecker 1987). Alternatively, this Glu to Lys change might increase the 'atherogenecity' of the LDL particle by increasing the binding of apoB to proteoglycans in

the artery wall or to the cellular scavenger receptor (Gallagher & Myant 1992, Talmud & Humphries 1992). In support of this, Camejo et al. (1988) demonstrated in-vitro that binding of LDL to proteoglycans extracted from the human aorta, is mediated by interaction of basic residues (e.g. Lys) in apoB with acidic groups in the polysaccharide moiety of the proteoglycan. These workers also showed that a peptide comprising residues 4230 to 4254 of apoB associates strongly with aortic proteoglycans in-vitro.

Studies have examined the apoB signal peptide (SP) polymorphism in relation to atherosclerosis. In the Pathological Determinants of Atherosclerosis in Youth (PDAY) Study (Hixson et al. 1992), in Afro-carribeans, homozygotes for the SP-27 isoform had the least involvement of the thoracic and abdominal aorta with lesions, homozygotes for the SP-24 isoform had the greatest involvement, with heterozygotes being intermediate. No differences were noted in Caucasians. In Afro-carribeans, the SP-24 isoform was also associated with higher levels of atherogenic apoB-containing lipoproteins. The association of signal peptide genotypes with lesions in young Afro-carribeans may be one explanation for the excess of fatty streaks observed in young Afro-carribeans compared to young Caucasians (PDAY report 1990). More recently, a significantly higher frequency of the SP-24 isoform was observed in MI survivors compared to controls from Belfast (Visvikis et al. 1993) and the same trend, though weaker, was observed in samples from Strasbourg and Toulouse. In all three populations, homozygotes for the SP-24 alleles had the highest levels of lipoprotein (a). Lipoprotein (a) is a significant and independent predictor of CHD (Sandkamp et al. 1990). Visvikis et al. (1993) propose that their reported association of the signal peptide polymorphism with lipoprotein (a) levels may be due to a coincidental relationship between two independent risk factors for atherosclerosis; lipoprotein (a) and LDL. Saha et al. (1992) detected a slightly higher frequency of the SP-24 isoform in Chinese patients with CAD compared to controls, but this difference was not statistically significant. This may be due to the significantly lower frequency of the SP-24 isoform in the Chinese population compared to Caucasian populations.

Taking the available data overall, these observations suggest that variation in the apoB gene locus is involved in the development of atherosclerosis and some forms of hyperlipidaemia.

Section 1.3.7 ApoB, LDL and atherosclerosis

Variation at the apoB gene locus is an obvious candidate for being one of the factors affecting between-individual differences in plasma cholesterol levels, and hence the development of CAD, by virtue of its role in lipid metabolism and the positive correlation between plasma apoB levels and LDL-cholesterol levels. Elevated levels of LDL and of apoB are correlated with increased risk of premature atherosclerosis in epidemiological studies as well as with the severity and rate of progression of coronary atherosclerosis assessed by angiography (reviewed in Lusis 1988, Hamsten et al. 1989). Some studies have shown that apoB levels discriminate better between patients with CAD and healthy control subjects, than levels of lipids or lipoproteins (Avagaro et al. 1979, Whayne et al. 1981). These findings are probably explained by the pronounced variations in lipid compositions of lipoproteins due to considerable migration of lipids between lipoproteins and between lipoproteins and tissues, while there is less movement of apolipoproteins, particularly apoB. Path analysis and twin studies have shown that plasma LDL-cholesterol and apoB levels have a high heritability of between 0.5 and 0.6 (Hamsten et al. 1986a, Berg et al. 1987).

As discussed futher in Section 1.8.3, LDL is involved in all stages in the process of atherogenesis by increasing permeability and turnover of cells in the arterial endothelium (Aviram & Brook 1982) and by increasing smooth muscle proliferation (Fless et al. 1982). Oxidised LDL (oxLDL) is particularly important in atherogenesis. Macrophages internalize oxLDL through both scavenger receptors (Goldstein et al. 1979) and a putative oxLDL receptor and are able to oxidize LDL through several pathways, including lipoxygenase enzymes (Parthasarathy et al. 1989). LDL is subject to free radical damage and oxidation, which particularly alters the positively charged lysine residues on the LDL surface (important in interaction with the LDL-receptor - discussed in Sections 1.3.1 and 1.3.2) (Steinbrecker et al. 1989), thus destroying the normal interaction of apoB with the LDL-receptor. In-vitro oxidation can also result in the apparent aggreg ation of LDL particles (Gandjini et al. 1991); this might destroy normal ligand-receptor interaction. Mechanisms by which in-vivo oxidation of LDL takes place are not fully understood, but cells within the arterial wall may mediate these reactions. LDL are oxidised in-vitro by incubation with macrophages, endothelial cells and smooth muscle cells (Parthasarathy & Steinberg 1992); these are all cells known to participate in the formation of the atherosclerotic plaque (see Section 1.1).

Additionally phospholipase A2 has a role in the oxidative modification of LDL, and there is evidence that apoB has an intrinsic phospholipase A2 activity (Parthasarthy & Barnett 1990). This indicates that some of the damage to LDL may be mediated by the apoB protein itself.

Every LDL particle has one molecule of apoB, but the amount of cholesterol per LDL particle can vary (Teng et al. 1983). Small dense LDL have a lower unesterified cholesterol to apoB ratio than normal and these small dense LDL have an increased susceptibility to oxidation (Tribble et al. 1992). This may be due to greater accessibility of susceptible surface components to oxidising agents. A preponderance of small LDL particles have been specifically linked to increased risk of MI (Austin et al. 1988) and the presence of CAD (Campos et al. 1992). Austin et al. (1988) identified two distinct phenotypes, 'A' and 'B', based on analysis of LDL subclasses, with phenotype 'A' corresponding with a major peak representing LDL particles with diameters of > 25.5nm, while phenotype 'B' corresponds to a major peak representing LDL particles with diameters of 25.5nm or less. This LDL particle size distribution has been shown to be under strong genetic influence (Austin & Krauss 1986) and phenotype 'B' is associated with increases in plasma levels of TG and apoB and a more atherogenic lipoprotein phenotype (Austin et al. 1990a). In the general population, the frequency of the allele for LDL phenotype 'B' has been estimated to be in the range of 0.19 [in healthy families] to 0.3 [in kindreds with familial combined hyperlipidaemia, FCHL] (reviewed in Austin 1993). Thus, the allele for LDL phenotype 'B' appears to be common in the general population and is not unique to FCHL (Austin 1993). A dense and protein-rich LDL (defined as having a density of between 1.04 and 1.063g/ml) is a characteristic of patients with manifest CAD (Sniderman et al. 1982, Swinkels et al. 1989).

An increased number of small dense LDL particles is the common feature of FCHL and hyperapoB. FCHL, first described by Goldstein et al. (1973), is a disease of hepatic apoB overproduction and high plasma apoB levels due to VLDL overproduction (Sniderman et al. 1982). It has an estimated frequency of 0.3% to 2% in the general population and is likely to be a major contributor to the development of early-onset CAD (Grundy et al. 1987, Babirak et al. 1992). FCHL is an frequently occuring dyslipidaemia, typically phenotypes IIa (↑ LDL), IIb (↑ LDL and VLDL) or IV (↑ VLDL) combined with a high incidence of

atherosclerosis (Rose et al. 1973, Goldstein et al. 1973). FCHL is an important clinical entity, associated with premature CAD, variable hyperlipidaemia, small dense VLDL and LDL and elevated apoB levels (Babirak et al. 1992) and lower HDL levels (Sniderman et al. 1982). FCHL is very heterogenous phenotypically, the lipid phenotype varies among affected relatives within the family and may change with time in affected individuals (Brunzell et al. 1983), and is suggested to be due to the expression of several loci (a 'double hit' hypothesis) ie. combinations of alleles that affect apoB synthesis and induce HTG (Kwiterovich et al. 1993). HyperapoB was originally defined as a high ratio of apoB to cholesterol in LDL (Sniderman et al. 1980). Grundy et al. (1987) proposed that the characteristic common to all family members with FCHL is apoB-100 overproduction and increased secretion of apoB-containing lipoproteins by the liver. Later FCHL and hyperapoB were proposed to be overlapping syndromes (Sniderman et al. 1992). LDL phenotype 'B' (discussed above) is thought to modulate the severity of FCHL in affected family members; those affected individuals with LDL phenotype 'B' have higher serum levels of apoB and TGs than those with LDL phenotype 'A' (Austin et al. 1990b). The fact that FCHL individuals can have either LDL phenotype 'A' or 'B' indicates that LDL phenotype 'B' is not the direct cause of FCHL. Secondary hyperlipidaemias, e.g. those associated with diabetes mellitus or chronic renal failure are also associated with apoB overproduction (Grundy et al. 1987).

One mechanism for controlling the rate of hepatic apoB secretion, and hence LDL density, may be acting through the level of fatty acid uptake by peripherial tissues. Sniderman and coworkers developed the hypothesis that reduced fatty acid uptake by peripherial tissues may result in an augumented supply of hepatic TGs, as partially hydrolyzed chylomicrons and fatty acids, returning to the liver. The resultant raised levels of fatty acids generated in the liver would lead to increased hepatic secretion of apoB-containing lipoproteins (Sniderman et al. 1992). In support of this theory, it has been demonstrated that, after an oral fat load, even normotriglyceridaemic (NTG) hyperapoB individuals clear TGs more slowly than normal individuals (Genest et al. 1986), and that reduced fatty acid uptake by peripherial tissues results in slower TG synthesis in adipocytes of NTG hyperapoB individuals (Teng et al. 1986).

A genetic disorder called Familial Defective apoB-100 (FDB) that is associated with premature atherosclerosis (Tybjaerg-Hansen et al. 1990, Schuster et al. 1990) has been reported. The prevalence of FDB is about 1 in 700 in Caucasian populations from Europe and the USA (Innerarity et al. 1990). FDB is caused by an Arg3500 to Gln substitution in apoB-100 which interferes with normal binding of apoB to the LDL-receptor, causing moderate (Soria et al. 1989) or severe (Tybaerg-Hansen & Humphries 1992) hypercholesterolaemia. LDL particles containing this mutant apoB-100 have 3-5% of the receptor-binding activity of LDL containing normal apoB-100 (Innerarity et al. 1988). Arg3500 is within the primary LDL-receptor binding domain of apoB-100 (discussed in Section 1.3.1) and replacement by Gln alters the microenviroment of about six lysines on LDL-associated apoB-100, raising their pK values from 8.9 to 10.5, so that these lysines can no longer interact effectively with negatively charged residues in the LDL-receptor (Innerarity et al. 1990). Lysines are known to be involved in apoB binding to the LDL-receptor (Weisgraber et al. 1978). Individuals with FDB probably exhibit normal IDL clearance, as this process is mediated by apoE, and hepatic overproduction does not occur in FDB (Rauh et al. 1992).

Another mechanism by which apoB-containing lipoproteins might initiate atherogenesis is through the interaction of apoB with proteoglycans such as heparan sulphate and chondroitin sulphate found on the arterial wall. Seven regions of basic amino acids in apoB have heparin-binding ability (Weisgraber & Rall 1987) and at least three regions of apoB can interact with chondroitin sulphate-rich aortic proteoglycans (Camejo et al. 1988). Heparin is able to displace LDL from the LDL-receptor (Goldstein et al. 1976). The interaction between apoB and heparin is believed to be mediated by electrostatic forces between positively charged residues (Arg and Lys) on apoB and negatively charged residues on heparin. This interaction may contribute to atherogenesis (Hollander 1976). The fact that LDL have a higher affinity than TG-rich VLDL for arterial proteoglycans (Camejo et al. 1988) indicates that the heparin-binding regions in apoB only become exposed when VLDL has been acted upon by LPL.

LDL complexed with apo(a) is called lipoprotein (a) [Lp(a)], and was first identified by Berg (1963). High serum levels of Lp(a) are an independent risk factor for development

of CAD (Kostner et al. 1976, Armstrong et al. 1986). The apoB-100 in LDL is complexed with one molecule of apo(a) via a covalent disulphide bridge (Coleman et al. 1990). Levels and size of Lp(a) vary markedly between individuals due to the large variation in apo(a) size (between 400 and 700kDa) (Lackner et al. 1991) and the strong inverse relation between serum levels of apo(a) and apo(a) size (Utermann et al. 1987). Over 90% of the variation in levels of Lp(a) are due to variation in the apo(a) gene itself (Boerwinkle et al. 1992). Apo(a) isoforms associated with high plasma Lp(a) concentrations were more frequent in CHD subjects than in controls, regardless of ethnic group (Sandholzer et al. 1992). The effects of the apo(a) size polymorphism on Lp(a) levels were similar in these CHD subjects and control subjects, leading these authors to conclude that alleles at the apo(a) gene locus determine the risk for CHD through their effects on Lp(a) concentrations (Sandholzer et al. 1992). Native Lp(a) particles have low affinity for the LDL-receptor (Knight et al. 1991) and are not significantly taken up by the macrophage scavenger receptor (Snyder et al. 1992). However, Lp(a) particles are more suspectible to oxidation than LDL (Naruszewicz et al. 1992a, 1992b), and once oxidised they are cleared by the scavenger receptor (Haberland et al. 1992), hence Lp(a) particles are important in cholesterol accumulation within macrophages in the atherosclerotic plaques. Lp(a) may also have an adverse effect on the clotting cascade, Harpel et al. (1989) showed that Lp(a) competes with plasminogen (the zymogen of plasmin, a protease which digests fibrin clots) for binding to fibrin and fibrinogen, thus Lp(a) may inhibit the normal degradation of blood clots. This competition is assumed to be due to the high degree of homology in the primary sequences of apo(a) and plasminogen (McLean et al. 1987), the gene for apo(a) having been derived from the plasminogen gene by duplication (Malgaretti et al. 1992). That Lp(a) is directly implicated in atherogenesis is further supported by observations that apo(a) is present in human atherosclerotic plaques but not in normal aorta (Cushing et al. 1989, Rath et al. 1989) and that transgenic mice, expressing human apo(a) and on a high fat diet, develop apo(a)-containing atherosclerotic plaques (Lawn et al. 1992).

Section 1.3.8 ApoB deficiency syndromes

Two rare genetic apoB deficiency disorders have been characterised, these are abetalipoproteinaemia and familial hypobetalipoproteinaemia (Kane & Havel 1990). In both disorders, there is failure of the liver and intestine to manufacture apoB-containing

lipoproteins: VLDL, LDL and chylomicrons, but premature atherosclerosis does not occur. The main resultant problems are malabsorption of dietary fat and fat-soluble vitamins. If untreated, the vitamin deficiencies cause neural damage such as spinocellular degeneration and retinitis pigmentosa. Very low serum cholesterol levels are a characteristic of apoB deficiency, but do not themselves appear to be a major problem - steroid hormone manufacture is normal, except at times of very high demand and even then women with the diseases can maintain pregancies (Biemer et al. 1975). However acanthocytosis is a serious problem in apoB deficiency - this is the phenomenon of misshapen fragile erythrocytes, due to abnormal membrane fluidity arising from low cholesterol in the plasma membrane (Singer et al. 1952).

Abetalipoproteinaemia is autosomal recessive in inheritance, and probands have no detectable apoB-containing lipoproteins in their circulation despite normal levels in their carrier parents. In these patients apoB mRNA can be detected in the liver and intestine, and the apoB protein is synthesized but not secreted, indicating that the defect occurs at a post-translational step (Lackner et al. 1986). In two affected families, abetalipoproteinaemia did not co-segregrate with haplotypes of the apoB gene, indicating that mutations in the apoB gene itself are not involved in abetalipoproteinaemia (Talmud et al. 1988). Recently, mutations in the gene for microsomal triglyceride transfer protein (MTTP) generating premature stop codons and truncated MTTP variants of 78 and 594 residues, respectively, was demonstrated to be the cause of abetalipoproteinaemia in at least some families (Sharp et al. 1993). These workers also cloned the MTTP gene - this gene has no homology to any other known gene and encodes a protein of 894 amino acids including the signal peptide. These probands with abetalipoproteinaemia had no detectable MTTP activity (Sharp et al. 1993). From these studies on abetalipoproteinaemia, the role of MTTP in the assembly of apoB-containing lipoproteins has been deduced. MTTP tips the balance between apoB degradation and secretion in favour of the latter by adding lipid to nascent lipoproteins early in assembly within the ER. MTTP also continues to add lipid to the growing lipoprotein particle up to the point of secetion, hence MTTP may have a pivotal role in controlling the size and density of apoB-containing lipoproteins produced by the liver and intestine.

Familial hypobetalipoproteinaemia (FHB) has a co-dominant pattern of inheritance,

and some cases of FHB have been shown to be due to mutations in the apoB gene (Young & Linton 1991). The heterozygote frequency in Western populations is estimated to be between 1/500 and 1/1000 (Linton et al. 1993). Heterozygotes are asymptomatic but have 5-50% of normal plasma apoB levels. Homozygotes have no detectable apoB containing lipoproteins in their plasma, and may be as severely affected as patients with abetalipoproteinaemia. Co-segregration of FHB with the apoB gene has been demonstrated (Young et al. 1987a, Leppert et al. 1988) and at least 25 different mutations causing FHB have now been reported (Gabelli 1992, Linton et al. 1993). Nearly all of these mutations generate premature stop codons via deletions or substitutions of nucleotides, although two splicing mutations have been reported (Huang et al. 1991, Talmud et al. 1994a). The lipoprotein phenotype in FHB varies, depending on the size of the truncated apoB variant. Null alleles with no detectable apoB protein in plasma in FHB homozygotes are the usual result of premature stop codons generated 5' of exon 26. Secreted apoB variants that are less than 37% the length of apoB-100 are found in lipoproteins in the HDL density range, this is presumably because these truncated apoB variants cannot bind enough lipid to reduce the density to below the HDL range. Only premature nonsense mutations generating proteins larger than apoB-37 result in the secretion of lipoprotein particles in the VLDL/LDL density range. Among subjects who are heterozygous for truncated apoB variants, it has not been possible to discern a relationship between the length of the truncated apoB variant and LDL-cholesterol levels (Linton et al. 1993), with apoB-32 carriers and apoB-46 carriers having mean levels of 0.75mM and 1.92mM respectively, but apoB-75 carriers have a mean level of only 1.25mM. ApoB-37 and apoB-86 heterozygotes have the same mean LDL-cholesterol level (reviewed in Linton et al. 1993). These FHB heterozygotes typically have cholesterol levels of 2.34 to 3.64mM. The low cholesterol levels in FHB heterozygotes are present from birth, and the diagnosis of FHB can be made from umbilical cord blood (Stein 1977, Andersen et al. 1979). One female individual who is heterozygous for both FHB and defects in the LDL-receptor gene has a normal lipid profile (she has a LDL-cholesterol level of 3.25mM and a TG level of 0.87mM), presumably due to the opposing effects of two different genetic mutations that cancel out one another (Emi et al. 1991).

The low serum levels of apoB and cholesterol in FHB heterozygotes might be due to underproduction or accelerated catabolism of apoB-containing lipoproteins; both are probably

involved, the relative importance of each depending on the particular type of truncated apoB variant. The shortest apoB species (smaller than apoB-31) are not detected in plasma lipoproteins, presumably because of insufficient binding of lipid to apoB prior to secretion; in these cases underproduction of apoB-containing lipoproteins is more important. However, recently a truncated apoB, with an estimated length of 27.6% of apoB-100 and thought to arise from differential RNA splicing, was reported to be detectable in the plasma of both FHB heterozygotes and homozygotes (Talmud et al. 1994a). A patient heterozygous for apoB-46 did not secrete apoB-46-containing chylomicrons in response to dietary fat intake, although production of chylomicrons containing apoB-48 was normal (Young et al. 1989). This suggests that there is a region of approximately 95 residues between where apoB-46 ends and where apoB-48 ends, which is critical for chylomicron secretion.

Several groups have investigated the clearance of lipoproteins containing apoB truncated variants. ApoB-37 does not bind to the LDL-receptor, as would be expected since it does not contain the LDL-receptor binding region (Young et al. 1987c). ApoB-75 only has part of the LDL-receptor binding region but binds more avidly to the LDL-receptor than does apoB-100 (Krul et al. 1992). Two other truncated variants, apoB-87 and apoB-89, containing the whole of the primary LDL-receptor-binding region, also have higher affinity for the LDL-receptor (Krul et al. 1989, Gabelli et al. 1989), indicating that the carboxyl-terminus of apoB has an inhibiting effect on LDL catabolism and might act to prevent premature hepatic uptake of VLDL, before it is catabolized to IDL and LDL. This is supported by the fact that a higher proportion of VLDL containing apoB-75 and apoB-89 are cleared directly from plasma (Krul et al. 1989, Krul et al. 1992). Lipoproteins containing truncated variants of apoB might be enriched in apoE, compared to apoB-100-containing lipoproteins due to reduced steric constraints. Accelerated apoE-mediated clearance of these lipoproteins might result; this is supported by the observation that a patient homozygous for apoB-50 had apoE-rich VLDL which were cleared directly and rapidly (Hardman et al. 1991). Finally, two complicating factors in the metabolism of lipoproteins containing truncated apoB variants, are firstly that the length of apoB has a large effect on the density of secreted lipoproteins (Gabelli 1992, Linton et al. 1993), which, in turn will determine the metabolic fate of these lipoproteins (e.g. Demant et al. 1991) and secondly apoE phenotype has an profound influence on metabolism of apoB-containing lipoproteins (Demant et al. 1991

and discussed in more detail in Section 1.4.6).

Section 1.4. Apolipoprotein E

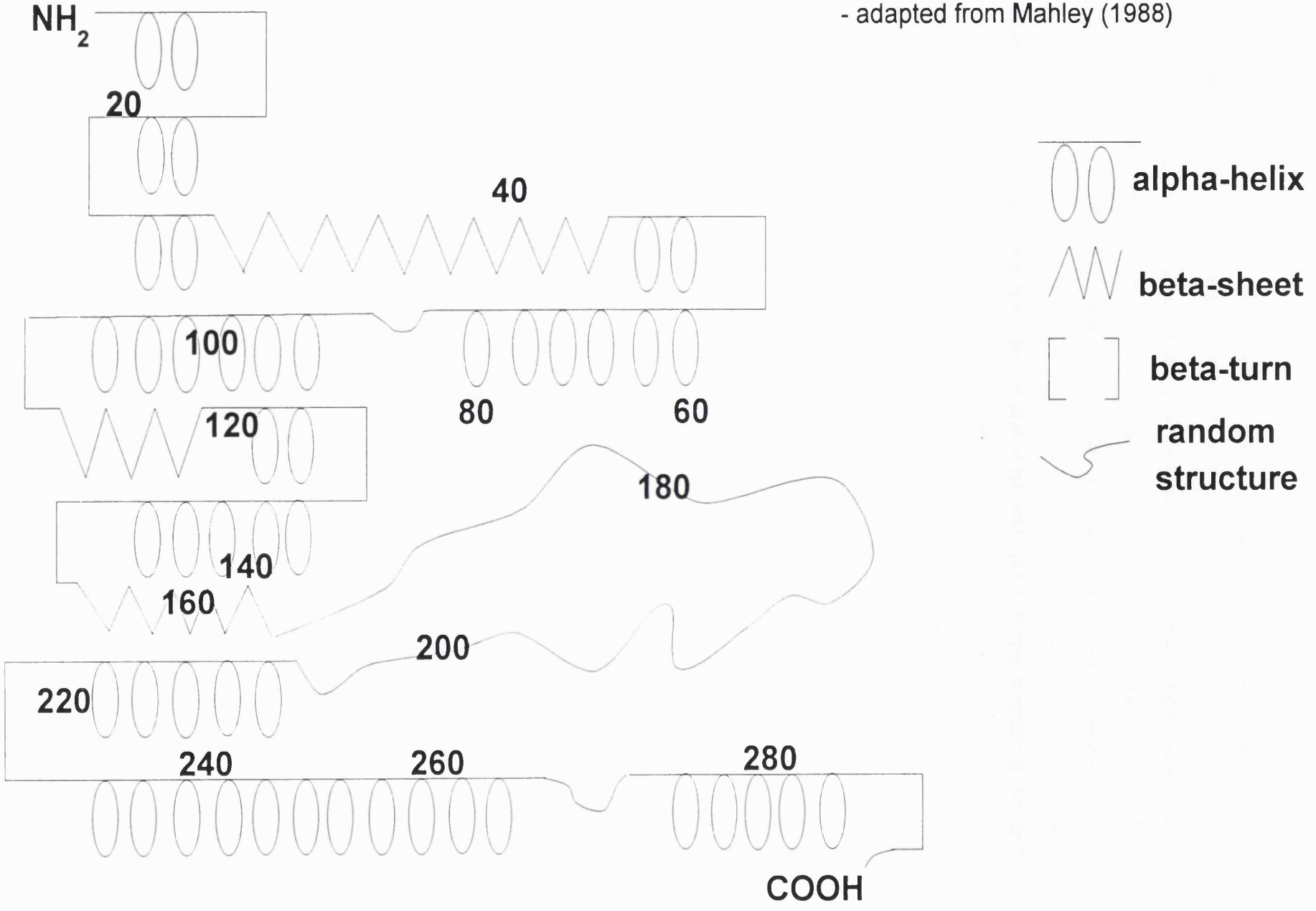
1.4.1. Synthesis and Structure of apolipoprotein E

Apolipoprotein (apo) E is a single polypeptide of 299 amino acids with a molecular weight of 34.2kD. Shore & Shore (1973) first identified apoE as an apolipoprotein constituent of VLDL, and it was initially termed the 'arginine-rich' apolipoprotein. ApoE is synthesized in most organs, including brain, kidney and adrenal glands but the primary site of synthesis is the liver (mainly hepatic parenchymal cells), which probably accounts for 2/3 to 3/4 of the total circulating plasma apoE (reviewed in Mahley 1988). The brain is the second major site of synthesis, astrocytes producing most of brain apoE and the amount of apoE produced is about 1/3 that from the liver (Boyles et al. 1985, 1989). Newly synthesized apoE has 317 amino acids and cleavage of the 18 amino acid hydrophobic signal peptide occurs co-translationally followed by post-translational glycosylation (reviewed in Rall & Mahley 1992). ApoE in the circulation cannot penetrate the 'blood-brain' barrier so there are two well separated pools of apoE in the body of hepatic and brain origins (Kraft et al. 1989). The tissue-specific synthesis of apoE is controlled by an array of tissue-specific elements scattered throughout the apoE-CI-CII gene cluster on chromosome 19 (reviewed in Mahley et al. 1990). This gene cluster and its regulation will be discussed in Section 1.4.4. Normal plasma concentrations of apoE are in the range 30-70μg/ml (Mahley & Innerarity 1983).

ApoE consists of two independently-folded structural domains, an amino-terminal domain (amino acids 1 to 164) and a carboxy-terminal domain (residues 201-299). A region of random structure (residues 165 to 200) separates these two domains, as shown in Figure 1.4.1.1. There is evidence that these domains, which are structurally distinct, represent functional domains (Wetterau et al. 1988, Aggerbeck et al. 1988). The 22kD amino terminal domain contains the receptor-binding domain of apoE, mapped to amino acids 134-160, one of four α-helices in apoE (Wilson et al. 1991), which mediates the high affinity interaction of apoE with the low-density lipoprotein (LDL) receptor. Nine of these amino acids are postively charged and these amino acids are believed to interact with the negatively charged amino acid residues present in the ligand-binding regions of the LDL receptor (Mahley et al.

Figure 1.4.1 Predicted secondary structure of apoE

- adapted from Mahley (1988)



1990). The 10kD carboxyl-terminal domain, which is strongly α-helical and the major lipid-binding region of apoE, mediates the binding of apoE to the surface of lipoproteins but does not bind to the LDL receptor (Rall et al. 1982, Gianturco et al. 1983, Aggerbeck et al. 1988). This carboxyl-terminal domain has at least one heparin binding region, probably between residues 214-236, which is thought to play an important role in the interaction of apoE with proteoglycans. This region of apoE may anchor the lipoprotein particle to the endothelial layer enabling lipoprotein lipase-catalyzed hydrolysis of triglycerides to occur (Weisgraber et al. 1986). Residues 230 to 265 consists of a long stretch of α-helices, thought to be involved in lipid binding (Weisgraber et al. 1986). In the absence of lipid, the carboxyl-terminal domain mediates the self association of apoE into tetramers (Aggerbeck et al. 1988).

1.4.2 Roles of apolipoprotein E

A major function of apoE is in lipid transport, involving the three lipoprotein metabolic pathways; exogenous, endogenous and reverse cholesterol. ApoE is a constituent of liver-synthesized VLDLs that distribute triglycerides from the liver to peripheral tissues, and a subclass of HDL (HDL_1) which participates in cholesterol redistribution from peripheral tissues to the liver, the so-called reverse cholesterol transport pathway (Mahley et al. 1984, Mahley 1988 and discussed in more detail in Section 1.7.4). The major role of apoE is to act as a ligand, mediating catabolism of cholesterol-enriched remnant lipoproteins via both the LDL-receptor (reviewed in Davignon et al. 1988) and the LDL-receptor related protein (LRP) (Beisiegel et al. 1989). The existence of LRP was suggested by the normal clearance of chylomicron remnants in patients with homozygous familial hypercholesterolaemia (FH) (Fredrickson et al. 1978) and in Watanabe Heritable hyperlipidaemic (WHHL) rabbits (Kita et al. 1982), both of which have reduced or absent LDL-receptor activity. In the exogenous lipid pathway, chylomicron remnants are rapidly taken up via both the LRP (Hussain et al. 1991) and the LDL-receptor (Schneider et al. 1981, Weisgraber et al. 1982, Mahley et al. 1984). Premature uptake of remnant lipoproteins is prevented by apoC molecules, particularly apoCI (Windler et al. 1985, Kowal et al. 1990, Weisgraber et al. 1990, Sehayek & Eisenberg 1991). The mechanism of this inhibition reflects association of apoC's to lipoproteins and specific concentration-dependent competition effects on apoE at the lipoprotein surface (Sehayek & Eisenberg 1991). This

is discussed further in Sections 1.5.2 and 1.8.1).

ApoE has also been suggested to play a role in the normal conversion of VLDL to LDL, by modulating the lipolytic action of LPL and/or HL. Addition of 'normal' apoE (apoE3) to plasma containing β-VLDLs results in lipolysis of β-VLDLs of hepatic origin to LDLs (Ehnholm et al. 1984). β-VLDLs are abnormal VLDLs that are less susceptible to lipolysis than normal VLDL (Chait et al. 1978) and have β electrophoretic mobility and a higher ratio of cholesteryl ester relative to triglycerides, the hallmark of Type III hyperlipidaemia (reviewed in Brown et al. 1983 and discussed in Section 1.4.7). The conversion of hepatic β-VLDLs requires the addition of lipoprotein lipase (LPL), apoCII (activator of LPL - see section 1.6) and apoE3 suggesting that remodelling of the VLDL particle is necessary, possibly through a reduction in CE or phospholipid content (Ehnholm et al. 1984). ApoE may also act as an enhancer of the CE and triglyceride transfer between VLDL and HDL via cholesteryl ester transfer protein (CETP) (Yoon et al. 1991). This effect was mediated via enhanced affinity of CETP for VLDL (Kinoshita et al. 1993), with the amounts of CE transferred to VLDL with apoE being significantly greater than to VLDL without apoE.

Additionally, apoE has a number of roles which are not directly involved in plasma lipid metabolism (reviewed in Mahley 1988). In nerve regeneration, apoE is important in lipid transport (Mahley 1988), suggested by the correlation between the high levels of apoE produced by macrophages at the regenerating perpherial nerve and expression of LDL-receptors on the growing tips of the neurites and the Schwann cells (Boyles et al. 1986, 1987). In normal peripheral nerves, apoE levels are low; a denervating crush injury leads to apoE secretion by macrophages and to its accumulation in the nerve to very high concentrations (Ignatius et al. 1986, Snipes et al. 1986, Boyles et al. 1989, 1990). Most of this apoE is associated with lipid and forms lipoprotein particles (Boyles et al. 1989) but some of the apoE is also present as free protein (Boyles et al. 1990). ApoE-lipid complexes capable of interacting with LDL-receptors of neurites, have been isolated from injured nerve segments (Ignatius et al. 1987). In-vivo experiments have shown that the LDL-receptor, which recognizes apoE-containing lipoproteins, is present at high concentrations in regenerating nerve tips (Boyles et al. 1989). These two observations have led to the

hypothesis that accumulation of apoE-containing lipoproteins and the expression of lipoprotein receptors by regenerating axons promote their rapid elongation and regeneration, through the delivery of cholesterol and other lipids needed for membrane synthesis. Direct evidence that apoE and apoE-containing lipoproteins can modulate the outgrowth of neuronal processes has come from a recent in-vitro study on rabbit dorsal root ganglion neurons (Handelmann et al. 1992). These workers proposed that apoE may play two roles in neurite outgrowth, the stimulation of neurite growth by the receptor-mediated delivery of cholesterol and perhaps other components and the modulation of neurite interaction with the extracellular matrix; both roles could facilitate rapid, target-directed axon growth in-vivo.

Roles unrelated to lipid transport are more speculative. ApoE might act as a neurotrophic or neurotropic factor in nerve survival and repair (Mahley 1988). Neurons use heparin-binding sites in matrix proteins for adhesion and axon extension (Edgar et al. 1984, Akeson & Warren 1986, Schubert et al. 1987). ApoE, which binds to heparin (Mahley et al. 1979, Weisgraber et al. 1986) might promote cell-matrix interactions and stimulate axon extension via formation of apoE-heparin-like complexes (Mahley 1988). ApoE might also alter the interaction of various growth factors that bind to the matrix, thus modulating their mitogenic activity (Mahley 1988). ApoE could enhance or interfere with interactions between smooth muscle cells (SMCs) and the extracellular matrix in the arterial wall, due to the ability of apoE to bind to heparin (Mahley et al. 1979, Weisgraber et al. 1986) and to heparin-like glycosaminoglycans (Mahley 1988). The arterial extracellular matrix contains proteoglycans; accumulation of these proteoglycans is one of the characteristic features of atherosclerosis (e.g. Wight 1985). Proteoglycans present in the vessel wall, which include heparan sulphate proteoglycans, are synthesized by endothelial cells and SMCs (e.g. Marcum et al. 1986, Kinsella & Wight 1988). Proteoglycans may regulate the proliferation of SMCs (Clowes & Karnovsky 1977); this proliferation of SMCs is one important feature of atherosclerosis (reviewed in Ross 1986). Heparin has been shown to inhibit proliferation of SMCs both in-vivo (Clowes & Clowes 1985) and in-vitro (e.g. Campbell & Campbell 1985). Heparin and heparin-like glycosaminoglycans are also potent inhibitors of SMC mobility (Majack & Clowes 1984). Enhanced apoE synthesis by growth-arrested SMCs has been demonstrated (Mahley 1988); growth arrest of SMCs was shown to promote differentiation of these cells (Owens et al. 1986).

Lastly apoE may have roles in immunoregulation; LDLs and apoE-containing lipoproteins (one class of macromolecules with immunoregulatory activity) inhibit or stimulate antigen or mitogen-induced T-lymphocyte activation and proliferation (Mahley and Innerarity 1983, Mahley et al. 1984), depending on whether binding of lipoproteins is to the LDL-receptor, expressed on lymphocytes (Ho et al. 1976, 1977), or the immunosuppressive receptor (Curtiss & Edgington 1976). It appears that the inhibitory effects of the lipoproteins on lymphocyte activation and proliferation are independent of the LDL-receptor (Cuthbert & Lipsky 1984). Finally, apoE synthesized and secreted by macrophages (Basu et al. 1981, 1983), may have a role in atherogensis. At the molecular level it has been shown not only that apoE-bearing lipoprotein particles are taken up by macrophages, but also that macrophages secrete apoE associated with phospholipids, which may be involved in cholesterol efflux from cells (Basu et al. 1982, Getz et al. 1988). Accumulation of lipid-laden macrophages is one step in the progression of atherosclerotic lesions (reviewed in Ross 1993).

1.4.3 Common alleles and frequencies in population samples.

ApoE exists as three major alleles, apoE2, apoE3 and apoE4, detectable by isoelectric focusing (Utterman et al. 1975, 1977). These authors were the first to establish the polymorphic nature of apoE. Zannis & Breslow (1981) further elucidated this polymorphism and showed that it is of two types, a genetically determined polymorphism and a second type caused by post-translational glycosylation. Attachment of a carbohydrate moiety at Thr-194 in apoE is the molecular basis for the post-translational glycosylation polymorphism, and gives rise to the minor, more acidic alleles via variable sialylation (Zannis & Breslow 1981, Wernette-Hammond et al. 1989). The genetic polymorphism is caused by single base substitutions which give rise to the three common alleles of apoE, each differing by a single amino acid substitution (Rall et al. 1982). ApoE3 (reviewed in Davignon et al. 1988), has Cys at residue 112 and Arg at residue 158. ApoE2 and apoE4 each differ from apoE3 by a single amino acid substitution; in apoE2 Cys replaces Arg at residue 158 and in apoE4 Arg replaces Cys at residue 112. Thus, apoE2 has two cysteine residues and apoE4 completely lacks cysteine. Three homozygous phenotypes (apoE2/2, apoE3/3 and apoE4/4) and three heterozygous phenotypes (apoE3/2, apoE4/3 and apoE4/2) arise from the expression of any two of the three alleles, which correspond to different alleles of the apoE gene, inherited in

a Mendelian manner (Zannis & Breslow 1981, Mahley et al. 1990). There are also numerous rare variants of apoE, frequencies of which are below 0.001 (reviewed in deKniff 1992); these will not be discussed in this thesis. This polymorphic nature of apoE is unique for human beings; in 10 other mammalian species studied so far, apoE is found as a monomorphic protein (Chan & Li 1991). ApoE3 has been suggested to be the ancestral form due to its high frequency (Mahley 1988). However Larsen et al. (1993) suggest that apoE4 is the parent form on the basis that the likely direction of mutation is CpG (Arg) to TpG (Cys) (Sved & Bird 1990) and that the sequenced apoE genes in at least six mammalian species studied, including baboon and cynomologus monkey, is apoE4-like and is apoE3-like in only two mammalian species (reviewed in Larsen et al. 1993).

There is a large body of data on the relative frequencies of the three common apoE alleles in healthy population samples (reviewed in Davignon et al. 1988, Hallman et al. 1991, Gerdes et al. 1992) and summarized in Table 1.4.3.1.

Table 1.4.3.1 Frequencies (weighted averages) for different ethnic groups
(adapted from Davignon et al. 1988 and Gerdes et al. 1992)

Ethnic group	Total number of individuals	Common apoE allele		
		E2	E3	E4
Amerindians	95	0	0.82	0.18
Chinese	196	0.08	0.85	0.06
Japanese	880	0.04	0.85	0.11
Caucasians	5805	0.08	0.77	0.15
Afro-Carribeans	357	0.03	0.67	0.30
New Guineans	110	0.15	0.49	0.37

The apoE3 allele is the most common in all populations studied but there is significant heterogenity in allele frequency among populations of different ethnic origins, and also among Caucasian populations (noted by Davignon et al. 1988), with, for example, apoE allele frequencies estimated in New Zealand (E2=0.12 and E4=0.16), France (E2=0.13 and

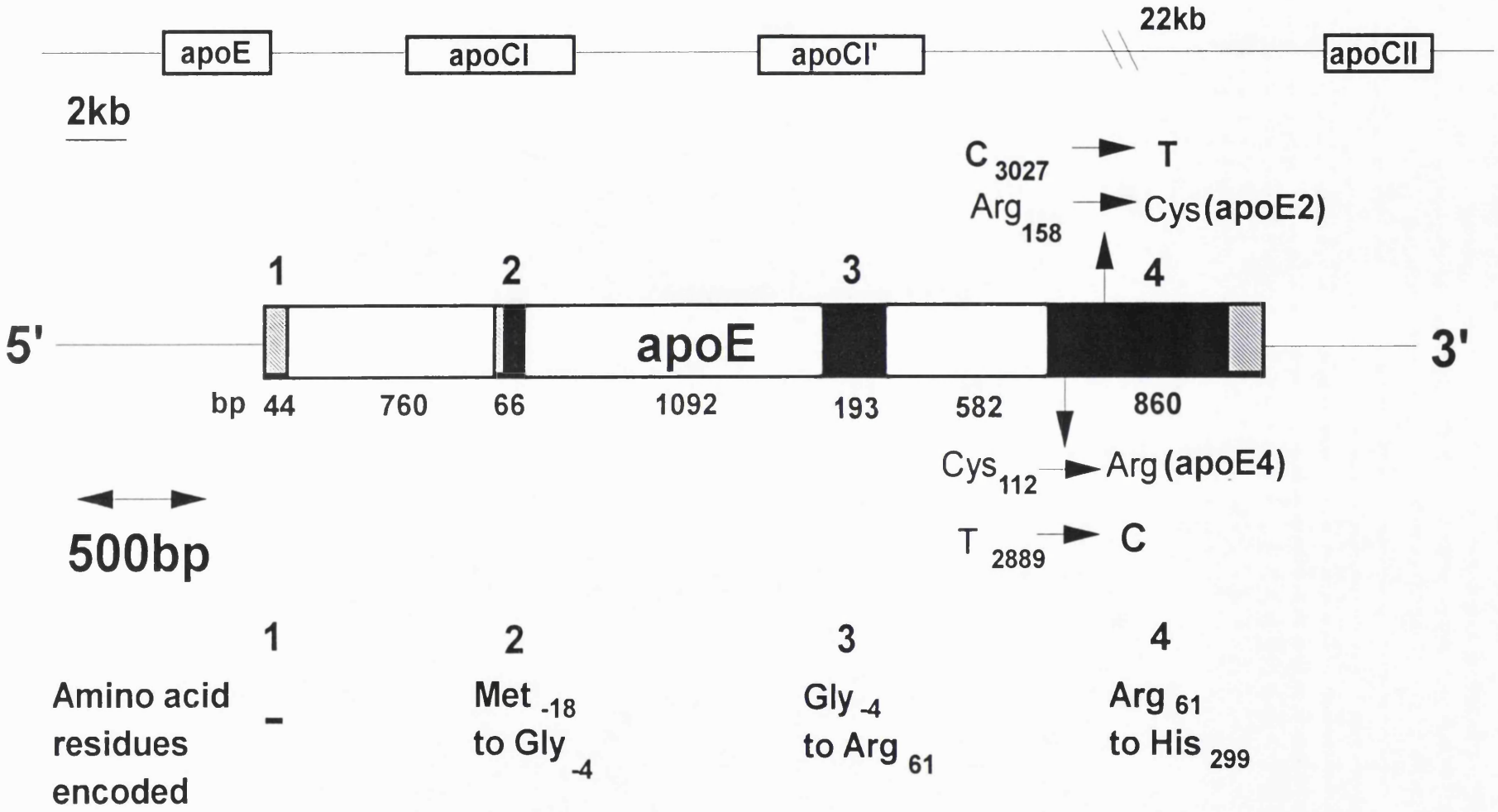
E4=0.13) and Finland (E2=0.04 and E4=0.23) differing significantly from those in the six other Caucasian population samples. There are differences in apoE allele frequencies between Caucasians, Japanese and Chinese populations (Davignon et al. 1988), with the relative frequency of apoE3 being less than 0.79 in every Caucasian sample (average = 0.77), whereas in Chinese and Japanese samples it is greater than 0.82 (average = 0.85, which is significantly greater than the Causcasian average). The major apoE genotypes in all populations were E2/3 (frequency ranges from 7.0% in Indians to 16.9% in Malays), E3/3 (frequency ranges from 39.8% in Sudanese to 72.1% in Japanese) and E4/3 (frequency ranges from 11.3% in Japanese to 35.9% in Sudanese) (Gerdes et al. 1992). Populations in the southern regions of Europe have similar apoE allele frequencies (0.10 to 0.12) to those found in Japanese populations, whereas the Caucasian populations in the Northern regions of Europe, in Canada and in the USA, consistently have a 1.5 to 2 times higher frequency (0.14 to 0.27) of the apoE4 allele (Gerdes et al. 1992).

The range of apoE allele frequencies across studies (reviewed by Hallman et al. 1991) drawn from populations of the same ethnic origin is wide enough to leave some doubt as to whether sampling variation or population substructure is the cause (Hallman et al. 1991). However an overview analysis by Gerdes et al. (1992) identified nine distinct clusters and found that selection of individuals, from a sample of 466 Danish men, to exclude those with hyperlipidaemia or cardiovascular disease did not change the cluster membership of this sample. These authors concluded that differences in study population sampling designs is not a likely explanation for major inter-study differences in apoE allele frequencies.

Section 1.4.4 The apoE gene and its regulation

The apoE gene is located on the long arm of chromosome 19 (q12-q13.2) (Olaisen et al. 1982) and forms a gene cluster together with the apoCI gene, a pseudogene called apoCI' and the apoCII gene; the whole gene cluster spans 48Kb (Humphries et al. 1984, Smit et al. 1988). RFLP analysis demonstrated strong linkage disequilibrium between the apoE and apoCI genes (Humphries 1984). The gene for apoE is 3597 nucleotides long and consists of 4 exons and 3 introns (Paik et al. 1985), as shown in Figure 1.4.4. This exon/intron structure is very similar to that of other apolipoprotein genes (ie. apoAI, AII, AIV, CI, CII and CIII), suggesting a common ancestral origin for this family of genes (Paik et al. 1985).

Figure 1.4.4 Structure of apoE gene - adapted from Paik et al. (1985)



The apoE mRNA is 1163 bp in length (McLean et al. 1984, Zannis et al. 1984) and the primary translation product is 317 amino acids long (Paik et al. 1985).

A number of regulatory elements have been found in and around the apoE gene that control transcription of apoE, and studies have revealed an extremely complex regulatory mechanism, where multiple elements control the expression of apoE in different tissues. Numbers refer to nucleotide positions relative to the transcriptional start site. Eight regions within the apoE promoter region and the first intron have a non-tissue specific positive effect on apoE expression (Smith et al. 1988). These regions are -360 to -314, -277 to -254, -243 to -227, -175 to -163, -163 to -125, -113 to -80, -80 to -35 and +75 to +87. Additionally, three negative elements, within regions -254 to -243, -207 to -186 and -125 to -113, were found to suppress transcription of the apoE gene in both HeLa and HepG2 cell lines (Smith et al. 1988). The 12bp 5' proximal negative element in the apoE gene (within region -125 to -113) shares partial homology with the 16bp repeat 3 of the sterol regulating element found in the promoter of the LDL-receptor gene (Sudhoff et al. 1987). Elements within three regions specifically suppress intestinal apoE expression; these regions are; -1000 to -506, -227 to -207 and +87 to +349 and an element within the region -186 to -175 is required for hepatic apoE expression (Smith et al. 1988). Three elements (5' nucleotides are -184, -161 and -54) interact with a positive transcription factor Sp1 used in a number of different promoters; at each of these three elements, Sp1 has a different effect (Chang et al. 1990). When Sp1 binds at -161 there is a marked stimulation of transcription, this effect is moderate when Sp1 binds at -54 while Sp1 binding to -184 has no effect.

Various cis-acting regulatory elements, required for tissue-specific expression of apoE are found at some distance from the apoE gene promoter. For example, expression of apoE in the kidney requires only -650 nucleotides of the 5' and 72 nucleotides of the 3' region of the apoE gene (reviewed in Smith et al. 1990). However longer stretches are needed for hepatic expression. A hepatic control region in the intergenic region between apoCI and apoCI' is required for hepatic expression of both apoE and apoCI in transgenic mice (Simonet et al. 1990, 1991); more recently this enhancer, which may function as a general hepatocyte-specific enhancer for the apoE-CI-CII gene locus, was localized to a 154bp fragment in the intergenic region between the apoCI and apoCI' genes, approximately 2.29kb

5' of apoCI' (Shachter et al. 1993). This enhancer element contains two footprints (DNA regions protected from DNase I digestion, due to protein binding to the DNA, during incubation of DNA fragment with nuclear extract), identified in a DNaseI protection assay and containing the sequence TGACCT (Shachter et al. 1993). This sequence has been found in transcriptionally active regions of numerous hepatically expressed genes (Ramji et al. 1991) such as acyl-CoA oxidase (Osumi et al. 1991) and genes coding for apolipoproteins that are produced in the liver (Leff et al. 1989). Several transcription factors, members of the steroid receptor superfamily (Kliewer et al. 1992), interact specifically with the sequence TGACCT, regulating apolipoprotein gene expression according to lipid availability (reviewed in Zannis et al. 1992).

A 3' CpG island in exon 4 of the apoE gene was reported (Larsen et al. 1992). This CpG island is methylated in DNA from all tissues analyzed except sperm. This methylation does not repress apoE transcription (Larsen et al. 1993), establishing a functional difference between 5' and 3' CpG islands (Larsen et al. 1993) as methylation of 5' CpG islands invariably leads to repression of transcription (Larsen et al. 1992). Thus, multiple elements direct expression of apoE in different tissues. Smith et al. (1988) hypothesized that such a complex system regulating transcription was required to allow different cell types either to increase or decrease apoE mRNA synthesis in response to their cholesterol status.

Section 1.4.5 Association of apoE alleles with lipids and lipoproteins

During the past 15 years, a large number of studies have demonstrated a relationship between apoE phenotype and plasma lipid levels (reviewed in Davignon et al. 1988, Dallongville et al. 1992). The consistency of this relationship is greater for total and LDL cholesterol levels than for HDL cholesterol and TG levels. Utermann et al. (1979, 1985) observed that in the normal population, individuals carrying the apoE2 allele had lower levels of total and LDL-cholesterol than individuals homozygous for the apoE3 allele. The first studies on normolipidaemic individuals to show that carriers of the apoE4 allele had higher levels of total and LDL-cholesterol than did those homozygous for the apoE3 allele were carried out by Bouthillier et al. (1983). Subsequent studies (reviewed in Davignon et al. 1988) demonstrated that, regardless of the mean cholesterol level for the population sample studied, individuals with the apoE2/2 genotype have the lowest, while individuals carrying

the apoE4 allele have the highest average level. The genotype means of cholesterol show an increase from genotype E2/2 to E4/4 in this order; $E2/2 < E2/3 < E3/3 \leq E4/2 < E4/3 < E4/4$ in most studies, implying that the quantitative impact of an individual's apoE genotype on the level of cholesterol is independent of the average cholesterol level for the population (Davignon et al. 1988). This trend is even seen in children of 3 years old (Lehtimaki et al. 1990).

However, according to Utermann (1987) this relationship between cholesterol levels and apoE genotypes is not independent from cultural and ethnic background. The association of the apoE4 allele with high cholesterol is strong in Finns (Ehnholm et al. 1986), moderate in Germans (Boerwinkle & Utermann 1988) but less significant or absent in some Asian populations (Ordovas et al. 1987). One possibility is that these populations differ significantly from each other in dietary habits such as fat intake and that individuals with the apoE4 allele acquire elevated cholesterol levels only when on a high fat diet (Utermann 1987). This is contrary to suggestions put forward that any difference in risk attributable to apoE phenotype between populations of different ethnic origins, is due to differences in apoE allele frequencies and not to modulation of impact of apoE phenotype on plasma lipids, by other genetic or environmental factors (Hallman et al. 1991, Luc et al. 1993). As much as 16% of genetic variance and 8.3% of total variance in LDL-cholesterol is attributable to apoE phenotype, and no other gene has been identified that contributes as much to normal cholesterol variability (Sing & Davignon 1985). Thus, the apoE polymorphism plays an important role in determining interindividual differences in cholesterol levels in every population studied, but is modulated by other factors such as dietary intake (see later in this section).

Recently Dallongville et al. (1992) analyzed the combined data from 45 published population studies (number of subjects ranging from 44 to 2018) from 17 countries, therefore populations of different ethnic origins were considered. This meta-analysis, a powerful statistical method that increases the power to detect statistically significant associations, confirmed that the genotype means for cholesterol rank: $E2/2 < E3/2 < E3/3 < E4/3 < E4/4$. The results of the meta-analysis also indicated a consistent relationship between TG levels and apoE phenotype among different populations. TG concentrations were significantly

higher in apoE2/2, E3/2, E4/3 and E4/2 groups than in apoE3/3 group, with genotype means for TGs ranking: E3/3 < E4/3 < E2/3 < E4/2 < E2/2. In the apoE4/4 group, probably because of small numbers, mean TG levels were higher in some populations but lower in other populations, than in the apoE3/3 group. This trend was found in obese individuals, those with diabetes or hyperlipidaemia, as well as normolipidaemic samples, indicating that this relationship is ubiquitous. Conflicting results reported in the literature could be due either to a lack of power of some studies to detect small effects, or to the great variability of TG levels among and within individuals (Austin 1990), masking a clear effect of apoE phenotype on TG levels. There was no clear association between HDL-cholesterol levels and apoE phenotype in the apoE2/2, apoE2/3 and apoE4/2 groups. However average HDL-cholesterol levels were lower in the apoE4/3 group and higher in the apoE4/4 group in most of the population samples surveyed (Dallongeville et al. 1992).

Individuals with the apoE2 allele and those with the apoE4 allele have the highest and lowest levels of apoE in plasma, respectively (Utermann et al. 1985, Smit et al. 1988). Sing and Davignon (1985) compared mean apoE and apoB levels across the three commonest genotypes, E2/3, E3/3 and E4/3. For both men and women, ranking for mean apoE levels was E2/3 > E3/3 > E4/3. Conversely, apoE2 and apoE4 carriers have, respectively, the lowest and highest levels of apoB in plasma (Sing & Davignon 1985, Utermann et al. 1985, Smit et al. 1988).

Differences in intestinal cholesterol absorption efficiency were related to apoE genotypes in a random sample of Finnish men (Kesaniemi et al. 1987), a population characterised by an unique distribution of apoE alleles, with amongst the highest apoE4 frequencies in the world (Ehnholm et al. 1986), high serum cholesterol levels, and a high incidence of CHD (Keys 1970). Subjects carrying the apoE2 allele absorbed less and synthesized more cholesterol than those with the apoE4/3 or apoE4/4 genotypes, with the values for the apoE3/3 group falling in-between (Kesaniemi et al. 1987). Individuals carrying the apoE4 allele exhibit greater responsiveness of plasma cholesterol levels to dietary change (Miettinen et al. 1988, Tikkanen et al. 1990, Manttari et al. 1991, Miettinen et al. 1992) with a greater reduction in total and LDL-cholesterol levels when switching to a low-fat intervention diet, compared to those without this allele. These studies suggest that the effect

of apoE genotype on plasma cholesterol is modulated by dietary fat and cholesterol intake (Tikkanen et al. 1990). However, other studies failed to detect any special susceptibility of serum cholesterol levels to diet related to apoE polymorphism in young German normolipidaemic subjects (Beil et al. 1991) or healthy European normolipidaemic men and women (Glatz et al. 1991). The most probable reasons are the low numbers of individuals homozygous for apoE4 and/or the different 'baseline' diet, which was low in fat in a Finnish study (Miettinen et al. 1988) and high in saturated fat in another study; complicated by the fact that the only E2/2 homozygote in this study (Glatz et al. 1991) was a consistent hyperresponder.

Section 1.4.6 Possible Mechanisms underlying the effect of common apoE alleles on lipids and lipoproteins

An explanation for the observed effects of apoE phenotype on lipid and lipoprotein levels was given by Utermann (1985). Due to the delayed clearance of apoE2-containing lipoproteins relative to lipoproteins containing apoE3 or apoE4, transport of cholesterol to the liver occurs at a lower rate and the rate of hepatic VLDL formation is decreased. This causes an up-regulation of LDL receptor activity, manifested as an increase in number of receptors, overall this leads to relatively elevated levels of remnant lipoproteins while LDL levels are low. ApoE4-containing chylomicron and VLDL remnants are cleared at a higher rate than apoE3-containing remnants, delivering more cholesterol and triglycerides to the liver, leading to a down-regulation of hepatic LDL receptor activity and thus to higher LDL-cholesterol levels. In agreement with this, Gregg et al. (1986) demonstrated in E4/E4 and E3/E3 homozygotes that apoE4 is metabolised faster than apoE3 in-vivo. Variation in efficiency of intestinal absorption of dietary cholesterol, associated with apoE genotypes, may contribute to variation in plasma and LDL-cholesterol levels (Kesaniemi et al. 1987).

ApoE4 and apoE3 distribute differentially among ultracentrifugally isolated lipoprotein fractions, with apoE4 being more abundant in VLDLs and less so in HDL, relative to apoE3 (Steinmetz et al. 1989). Since apoE3 and apoE4 bind with equal efficiency to the LDL-receptor (Weisgraber et al. 1982), their differential distribution among lipoprotein fractions is thought to be a key to understanding the mechanism underlying the different effects of apoE3 and apoE4 on plasma cholesterol and LDL-cholesterol levels. Increased association

of apoE4 with chylomicrons was thought to underlie the faster removal of alimentary lipoproteins from the circulation which results in the down-regulation of hepatic LDL receptors and its attendant elevations in LDL cholesterol (Weintraub et al. 1987). An increased apoE content of TG-rich lipoproteins may allow endocytosis of these particles at an earlier stage of delipidation, therefore increasing hepatic fatty acid concentration, which is the driving force of VLDL synthesis and secretion. A common mechanism i.e. hepatic uptake of lipid-rich remnants, may therefore underlie the associations of the apoE4 allele with elevated cholesterol and TGs (Surguchov et al. 1994). Weisgraber et al. (1990) demonstrated that the cysteine-arginine interchange at position 112, in the amino terminal region, determines the lipoprotein class to which the carboxy terminal of apoE will bind i.e. there is an interaction between the two structural domains of apoE that determines the apoE distribution. ApoE3 and apoE2 exist in the plasma as apoE-AII, a disulphide-linked heterodimer which mainly exists in the HDL fraction (Weisgraber et al. 1978). As a result of this heterodimer formation, not possible with apoE4, apoE3 distributes preferentially to HDL, thus decreasing the relative number of ligands available on VLDLs for binding to hepatic apoE receptors. In addition, these apoE-AII complexes have been shown to block binding to the LDL receptor (Innerarity et al. 1978, Weisgraber et al. 1991).

In constrast, the differences in lipid metabolism between individuals with apoE2 and with the other common alleles can clearly be traced to the grossly diminished LDL-receptor-binding ability of apoE2 relative to either apoE3 or apoE4 (Schneider et al. 1981, Weisgraber et al. 1982), probably by altering the conformation of the apoE molecule (Utermann et al. 1983). ApoE2 has less than 2% of normal binding, as assessed in an in-vitro competition assay (Weisgraber et al. 1982). The Arg to Cys substitution at residue 158 is the only difference between apoE2 and apoE3, therefore it is probably responsible for the grossly diminished receptor-binding ability of apoE2 (Weisgraber et al. 1982). ApoE2 also shows impaired binding to the LRP (Kowal et al. 1990) with delayed removal of chylomicrons and their remnants in normolipidaemic individuals homozygous for apoE2 (Brenninkmeijer et al. 1993). The resulting failure to catabolise cholesterol-enriched remnants of triglyceride-rich lipoproteins in E2/E2 homozygotes is probably responsible for the accumulation of these remnants (Havel et al. 1980, Gregg et al. 1981). The receptor-binding difference associated with apoE2 and the lipoprotein association difference exhibited by apoE4 could be properties

that contribute to the effect of apoE alleles on normal plasma lipid variation discussed in the previous section (Rall & Mahley 1992).

Recent in-vivo turnover studies have shown that apoE phenotype influences metabolism of apoB-100-containing lipoproteins along the $VLDL_1 \rightarrow VLDL_2 \rightarrow LDL$ cascade, confirming the profound impact of apoE alleles on apoB metabolism throughout the spectrum of lipoproteins of hepatic origin (Demant et al. 1991). Individuals homozygous for apoE3 converted about 50% of $VLDL_2$ particles to LDL, the remainder being lost by direct catabolism. ApoE2 homozygotes produced less $VLDL_1$ than did apoE3 homozygotes but converted proportionally more of their $VLDL_1$ to $VLDL_2$ with most of this $VLDL_2$ being catabolized before it could be converted to LDL. ApoE4 homozygotes converted most of their $VLDL_2$ particles to LDL. Demant and co-workers concluded that these changes in the $VLDL_1 \rightarrow VLDL_2 \rightarrow LDL$ cascade are partly explained by alterations in LDL-receptor activity, with apoE2 and apoE4 homozygotes having higher and lower activities, respectively, compared to apoE3 homozygotes. The LDL-receptor does not appear to be responsible for catabolism of $VLDL_1$-apoB since $VLDL_1$ are removed from the circulation normally in patients with homozygous FH (James et al. 1989).

IDL to LDL conversion is impaired in apoE2 homozygotes, balanced by increased IDL catabolism, probably via apoB and LDL-receptors, but the mechanism for this is unknown. LDL from apoE2 homozygotes is cleared more slowly than autologous LDL in normal subjects (Gregg et al. 1984, Gabelli et al. 1986) (unexpected given that LDL-receptor activity is upregulated in apoE2 homozygotes) and normal LDL was cleared rapidly in an apoE-deficient patient (Gabelli et al. 1986). These data support the hypothesis that apoB in LDL of apoE2 homozygotes has a conformation that is unable to recognize receptors efficiently (Demant et al. 1991). The concentration and distribution of apoB-containing lipoproteins in apoE4 homozygotes seems to be due to suppressed receptor-mediated catabolism of $VLDL_2$, IDL and LDL (Demant et al. 1991). As discussed above, efficient lipolysis of VLDL to LDL by lipoprotein lipase is poorly supported by apoE2 (Ehnholm et al. 1984), hence retarded delipidation would provide an additional mechanism for the formation of $VLDL_2$ remnants and the very low conversion of IDL to LDL in apoE2 homozygotes (Demant et al. 1991). These conclusions are strengthened by one study

demonstrating that pure apoE activated purified LPL and that an antibody to apoE inhibited LPL (Yamada & Murase 1980), and other studies demonstrating that apoE modulates both LPL and hepatic lipase activities (Wang et al. 1985, McConathy et al. 1989).

Higher TG levels in subjects carrying the apoE2 allele can be explained by their slower plasma clearance of chylomicron and VLDL remnants (Gregg et al. 1982, Brenninkmejer et al. 1987, Weintraub et al. 1987), and possibly also by an alteration in the lipolytic process contributing to TG accumulation in these subjects (Chung & Segrest 1983, Ehnholm et al. 1984, Dallongeville et al. 1992). The higher TG levels in individuals with the apoE4/3 phenotype could be explained by delayed catabolism of TG rich lipoproteins and it is possible that the apoE4 allele, due to its preferential association with VLDLs, interferes with plasma lipase activities and/or with the TG removal system resulting in delayed lipolysis and/or plasma TGs (Dallongeville et al. 1992). This hypothesis is supported by in-vitro experiments that demonstrate a modulating effect of apoE on LPL (Yamada et al. 1980, Wang et al. 1985, McConathy & Wang 1989). Individuals homozygous for apoE4 are reported to have similar TG levels to those homozygous for apoE3 (Dallongeville et al. 1992), this could be due, in part, to the small numbers of individuals studied, who are homozygous for apoE4. Alternatively, as proposed by Dallongeville and coworkers, a mild putative defect in lipolysis observed in apoE4/3 individuals might be compensated for by the known faster clearance of VLDL remnants in individuals homozygous for apoE4 (Brenninkmeijer et al. 1987, Weintraub et al. 1987). Lower mean HDL-cholesterol levels in apoE4/3 subsets compared to apoE3/3 subsets could therefore be explained by the possible alteration in TG metabolism in these individuals (Dallongeville et al. 1992).

Section 1.4.7 ApoE2 and Type III hyperlipoproteinaemia

Homozygosity for apoE2 plus presence of another gene or genes with lipid raising effects or environmental factors (Mahley 1982, Utermann 1982, Mahley & Angelin 1984) can overrule the cholesterol-lowering effect normally associated with the apoE2 allele. In this situation, Type III hyperlipoproteinaemia (HLP) becomes manifest. Type III HLP is defined as the plasma accumulation of remnants of both chylomicrons and VLDLs in patients who have elevated plasma concentrations of both cholesterol (8-20mM) and TGs (3.5-8mM) and develop xanthomas (yellow-orange discolouration of skin creases) of the palmar creases

and tuberous xanthomas, particularly on elbows and knees (Mahley & Rall 1989). These skin creases are the first manifestations of Type III HLP (Borrie 1969). The principal abnormal lipoproteins occuring in Type III HLP are β-VLDLs which differ from normal VLDLs in their apolipoprotein composition, β-electrophoretic mobility and higher content of cholesteryl esters per particle relative to TGs (Mahley & Angelin 1984). The estimated frequency of Type III HLP is 4/10,000 to 1/100,000 in the population (Cladaras et al. 1986). Type III HLP is associated with increased risk of atherosclerosis, but the precise population risk is difficult to gauge because of the rarity of this HLP, and the only presymptomatic individuals will be those presenting themselves to dermatologists because of xanthomas. Only four out of fourteen male Type III HLP patients, referred from dermatologists, who were investigated had cardiovascular disease; duration of Type III HLP ranging from 7 to 27 years before onset of CAD (Borrie 1969). Upon treatment, the eruptive xanthomas clear and the hyperlipoproteinaemia is resolved, with total plasma lipid levels returning to near normal; however the characteristic β-VLDL distribution of Type III HLP remains (Borrie 1969).

Approximately 95% of Type III patients are E2/2 homozygotes (Utermann et al. 1979) but less than 5% of E2/2 homozygotes develop Type III HLP (Davignon et al. 1988). All apoE2/2 homozygotes have dysbetalipoproteinaemia, which is due to slow clearance of remnant lipoproteins. In about 5% of these individuals, secondary genetic or environmental risk factors cause them to develop overt Type III HLP (Mahley & Rall 1989). Reported secondary genetic factors are mutations in the gene for the LDL-receptor (Hopkins et al. 1991), in the gene for LPL (Ma et al 1993a) and variation in the apoB gene (Talmud et al. 1987). The other risk factors are diet, drugs, increasing age, obesity and an often postulated second genetic lipid abnormality such as familial combined hyperlipidaemia. Examples of risk factors are hypothyroidism, diabetes and other hormonal factors. Such risk factors could lead to overproduction of VLDL, influence lipase processing of VLDL to LDL or decrease the number of LDL receptors (Utermann 1985). In addition to homozygosity for apoE2, some rare variants of apoE which are also defective in receptor binding, have been found to cause Type III HLP. Unlike apoE2, however, some of these rare variants generate dominantly inherited disease (Rall & Mahley 1992), and recently transgenic mice expressing a rare apoE variant (Arg-112, Cys-142) having Type III HLP were created (Fazio et al.

1993). The aetiology of Type III HLP is a good illustration of the multifactorial and polygenetic nature of hyperlipidaemias in general.

Section 1.4.8 ApoE deficiency

ApoE deficiency is rare in humans and has been described in five patients in three families (Ghiselli et al. 1981, Schaefer et al. 1986, Cladaras et al. 1987, Mabuchi et al. 1989, Lohse et al. 1992). All apoE-deficient patients were diagnosed with severe Type III HLP, including the presence of β-VLDL, premature cardiovascular disease and xanthomas. In general, plasma TG levels are decreased and VLDL is more enriched in cholesterol in these apoE-deficient patients compared to 'typical' Type III HLP patients who are homozygous for apoE2. ApoE-deficient mice strains were recently created by homologous recombination, and found to exhibit spontaneous hypercholesterolaemia, primarily due to elevated levels of VLDL and IDL, particularly when fed a high-fat Western type diet (Plump et al. 1992, Zhang et al. 1992). These mice also developed atherosclerotic lesions in the aorta and coronary and pulmonary arteries. This would seem to indicate an important protective role for functional apoE against atherogenesis, at least in mice. However, extrapolating the situation in mice to that in humans, to give insights into human lipid metabolism, is fraught with uncertainity. This is due to differences in lipoprotein metabolism between humans and mice. HDL is the primary transporter of plasma cholesterol in mice (reviewed in Ishida & Paigen 1989) and in rodents, unlike humans, hepatic apoB mRNA is edited, producing truncated apoB-48 that does not have the LDL-receptor binding region. Thus, hepatic clearance of lipoproteins in the mouse is almost entirely dependent on apoE, which would explain the much more severe hypercholesterolaemia phenotype in apoE-deficient mice compared to their human counterparts (Plump et al. 1992). An additional difference between humans and mice is that mice are usually resistant to development of atherosclerosis even when challenged with an atherosclerotic diet, though certain highly inbred (susceptible) strains do exhibit fatty lesions of the aorta in response to being fed a diet rich in saturated fat and cholesterol (Paigen et al. 1985). These fatty lesions exhibit a histopathology similar to human atheromatous plaques (Stewart-Phillips & Lough 1991).

Section 1.4.9 Associations of common apoE alleles with presence and severity of atherosclerosis

The impact of allelic variation at the apoE locus on plasma lipid and lipoprotein levels (discussed in Section 1.4.5) led to the early suggestion that the apoE polymorphism may play a part in determining susceptibility to atherosclerosis in man (Cumming & Robertson 1984). The apoE2 allele could be cardio-protective via its cholesterol-lowering effect, however this effect can be disrupted by the presence of exacerbating factors (either genetic or environmental) which would raise the level of potentially atherogenic cholesterol-enriched β-VLDLs and lipoprotein remnants, and therefore predispose to hyperlipoproteinaemia. ApoE4 could favour the development of atherosclerosis via its raising effect on LDL-cholesterol. Several studies have examined these possibilies. The apoE2/3 genotype was significantly less frequent in patients with CAD compared to patients without CAD (11.2% and 16.4% respectively); this difference was even more pronounced in males (19% vs. 11%); and in favour of the lipid hypothesis, those apoE3/2 individuals without CAD had the lowest cholesterol levels (Menzel et al. 1983). These authors suggested that apoE3/2 heterozygosity, through its hypocholesterolaemic effect, may be considered a negative risk factor for CAD. They also suggested that the occurrence of β-VLDLs in individuals homozygous for the apoE2 allele is not necessarily associated with an increased risk for CAD; with only extreme elevation of β-VLDL in plasma, as occurs in Type III HLP, being associated with premature atherosclerosis (Utermann et al. 1975, 1979).

Cumming & Robertson (1984) were the first to show that the presence of apoE4 was significantly associated with an increased risk and prevalence of ischaemic heart disease (IHD); they concluded that the presence of the apoE4 allele might confer an increased risk of MI at an earlier age, at least in men. Since then evidence has accumulated that in the population at large, apoE4 predisposes to and apoE2 protects against development of atherosclerosis. This evidence is of two types, an increased apoE4 allele frequency in individuals with CAD compared to controls and an assocation of the apoE4 allele with greater severity of atherosclerosis.

Table 1.4.9.1 shows the apoE4 and apoE2 allele frequencies in selected case-control studies. The apoE4 allele frequency has been found, in cross-sectional studies, to be elevated

Table 1.4.9.1 ApoE frequencies in selected case-control studies

Study (author and year)	Criteria for case	Age (in years)	Number of:		ApoE4 frequency		Results of χ^2 test (1 degree of freedom) - see footnote
			Cases	Controls	Cases	Controls	
Menzel et al. 1983	Presence of angiographically verified CAD	Mean = 52	469	1000	0.139	0.139	$\chi^2=0.01$ NS
Cumming and Robertson 1984	MI survivors	45-60	239	400	0.207	0.147	$\chi^2=7.54$ $p<0.01$
Utermann et al. 1984	MI survivors	not given	523	1031	0.121	0.150	$\chi^2=4.81$ $p<0.05$
Kuusi et al. 1989	Angiographically verified CAD (≥50% narrowing of lumen in at least one artery	37-60	91	207 †	0.324	0.227	$\chi^2=4.62$ $p<0.05$
Laakso et al. 1991	Type II diabetics	45-64	138	64	0.145	0.109	$\chi^2=5.68$ $p<0.025$
Nieminen et al. 1992	Angiographically verified CAD (≥50% narrowing of lumen in at least one artery	<60	111	46	0.293	0.174	$\chi^2=4.80$ $p<0.05$
van Bockxmeer and Mamotte 1992	Angiographically verified CAD (>50% average obstruction of arterial lumen	30-50	1	172	0.258	0.128	$\chi^2=14.62$ $p<0.001$

† from Ehnholm et al. (1986) - healthy individuals from Finland
NS = not significant
Footnote; the null hypothesis of the χ^2 test was that frequency distributions of apoE4 and (apoE2 & apoE3 combined) were not different between cases and controls.

in MI survivors (Cumming & Robertson 1984), patients with angiographically verified and well defined CAD (Kuusi et al. 1989, Nieminen et al. 1992), in patients with combined hyperlipidaemia (Houlston et al. 1991) and in Type II diabetic patients with CAD (Laakso et al. 1991), compared to controls. The prevalence of definite MI and ischaemic electrocardiographic changes or angina pectoris or both were highest in the diabetic men with the phenotypes E4/4 or E4/3 (Laakso et al. 1991). Only one study showed a significantly lower frequency of the apoE4 allele in patients with CAD (Utermann et al. 1984); in this study type III HLP was over-represented in the MI survivors despite no difference in frequency of the E2/2 genotype between patients and controls. These authors made no reference to the age or sex distribution, or to the prevalence of major risk factors for CAD, such as hyperlipidaemia, amongst their MI survivors, nor were lipids and lipoproteins measured. In another early study (Menzel et al. 1983), the distribution of apoE phenotype was virtually the same in patients and controls despite the well defined CAD in the patients and large sample size; this is most likely to be due to the fact that the patients were considerably older than the controls. Any excess of the apoE4 allele in the patient population might have been removed by early death of apoE4 carriers with particularly severe CAD.

As shown in Tables 1.4.3.1 and 1.4.3.2, apoE2 and apoE4 occur at a reduced frequency in certain Asiatic populations compared to Caucasians, thus the apoE polymorphism may contribute to interpopulation differences with respect to CAD (Davignon et al. 1988, Hallman et al. 1991). These interpopulation differences in the incidence of CAD are well-established (Pyorala et al. 1987). Populations having high apoE4 frequencies also tend to have a high incidence of MI and/or CHD, for example the Finns (Ehnholm et al. 1986, Kuusi et al. 1989, Lehtimaki et al. 1990), where the incidence of MI is the highest (World Health Statistics Annual 1989). Kuusi et al. (1989) suggested that the high apoE4 allele frequency in the Finnish population may be one factor contributing to the Finns' increased suspectibility to CAD. Conversely, southern European countries have a lower incidence of cardiovascular disease, which is usually attributed to the healthier, Mediterranean-type diet (Keys 1970, Pyorala et al. 1987). Recently a significantly lower apoE4 allele frequency (0.094) was found in an Italian population sample (James et al. 1993) compared to the average apoE4 allele frequency (0.150) in Causcasians (Davignon et al. 1988). The apoE4 allele is associated with high triglycerides and low HDL-cholesterol, i.e.

a more atherogenic lipid profile, compared to homozygosity for apoE2 or presence of apoE2. The reduced occurrence of apoE4 in the Italian population sample, may be a contributory factor to the relatively lower incidence of cardiovascular disease in the Italian population (James et al. 1993). Higher and lower frequencies of the apoE4 and apoE2 alleles, respectively, were found among offspring of fathers who survived a premature MI (<55 years of age) compared to age and sex matched controls with no parental history of premature MI (Tiret et al. 1994). ApoE polymorphism was significantly associated with parental history of MI ($p<0.01$) across regions of Europe analyzed (Finland, Great Britain, Northern and middle France), with the exception of Southern France. When Southern France was excluded, the population-adjusted odds ratios (with the ratio for genotype E3/3 being 1.0) were 1.16 and 1.33 for genotypes E4/3 and E4/4, respectively (Tiret et al. 1994). The authors suggest that apoE polymorphism strongly contributes to the development of CHD and is one major factor responsible for the familial predisposition to CHD. As discussed in section 1.4.6, the raising and lowering effects of apoE4 and apoE2, respectively, on cholesterol levels, are evident across many different populations. Thus the potential importance of apoE polymorphism in cardiovascular risk is related to differences in the relative frequencies of apoE alleles (Davignon et al. 1988). Davignon et al. (1988) stress the importance of excluding or controlling for confounding factors, such as age, sex, hyperlipidaemia, differences in definition of CAD and presence of peripherial atherosclerosis in future studies of the relationship between apoE phenotype and atherosclerosis.

The apoE common alleles are also associated with severity of atherosclerosis, manifested either as occurrence of MI at a younger age or greater extent of atherosclerotic lesions. Mean age at the time of the first MI was lower in individuals with the phenotype E4/3 compared to absence of apoE4 (Cumming & Robertson 1984); they concluded that the presence of the apoE4 allele might confer an increased risk of myocardial infarction (MI) at an earlier age, at least in men. Lenzen et al. (1986) reported, in a study of 570 male MI survivors with documented CAD (>50% stenosis in at least one major coronary artery), that MI occurred an average of 4.6 years earlier in apoE4/3 individuals than in apoE2/3 individuals. The prevalence of apoE alleles in patients with early (<50 years) and late (≥50 years) MI was significantly different (Lenzen et al. 1986). Moreover, the prevalence of the apoE4 allele was significantly higher in the group of individuals who had a MI at <50 years

of age than in those who had a late MI (van Bockxmeer & Mamotte 1992). These authors also reported a 16 fold higher prevalence of apoE4 homozygotes in patients with atherosclerosis aged 30-40 years and a 60% higher apoE4 frequency in patients aged 40-50 years compared to healthy controls. They concluded that inheritance of apoE4 seems to confer risk of premature IHD in males with apoE4 homozygotes being particularly at risk. ApoE4 homozygosity is also found in excess in patients with restenosis of coronary arteries occurring within six months of balloon angioplasty (van Bockxmeer et al. 1993). In the Pathological Determinants of Youth (PDAY) study (Hixson et al. 1991), apoE genotypes differed in mean percent surface area involvement with lesions in aorta in both Caucasians and Afro-Carribeans ($p < 0.0001$). Among the common genotypes, apoE2/3 and apoE4/3 had least and greatest, respectively, involvement of both thoracic and abdominal aortas; an effect that may not be mediated entirely by changes in serum cholesterol concentration (Hixson et al. 1991). However apoE genotypes were not associated with angiographic score for severity of atherosclerosis in another study (Reardon et al. 1985).

Finally, presence of apoE4 may predispose older men to exercise-induced silent myocardial ischemia (SI), even in the presence of normal LDL-cholesterol levels (Katzel et al. 1993). The apoE4 allele was present in 44% of men with SI on graded exercise treadmill testing compared with 17% of men of comparable age with normal exercise tests ($p < 0.001$). The relative risk for SI in men with the apoE4 allele is 2.6 fold greater than the risk in those without this allele.

Further evidence that the common apoE alleles have an impact on longevity is in the form of decreased apoE4 frequencies in Canadian octogenarians (Davignon et al. 1988), in French centenarians (Schachter et al. 1994) and in older female diabetic patients (Boemi et al. 1993), compared to younger controls. The apoE4 allele frequency was 0.14 in healthy individuals under 60 years of age but only 0.05 in healthy individuals who were 65 years and over (Chartier-Harlin et al. 1994). A significantly lower apoE4 allele frequency was reported in individuals over 60 years of age compared to younger individuals (Eggertsen et al. 1993). A decrease in apoE4 allele frequency between nonagenarians and centenarians might be explained by a steep negative selection differential of apoE4 carriers due to Alzheimer's disease, discussed in the next section, thus apoE4 appears as a risk factor for

both heart disease and Alzheimer's disease, two major causes of mortality and morbidity at advanced ages (Schachter et al. 1994).

Section 1.4.10 Associations of common apoE alleles with Alzheimer's Disease

Alzheimer's disease (AD) is a devastating neurologic disorder and the most common primary dementing illness affecting 5-10% of the population over the age of 65. Prevalence of AD rises exponentially with age to reach at least 1 in 5 by the eighth decade (Pfeffer et al. 1987). The heritability of AD appears to increase as the age of onset falls (Wright et al. 1991). AD is characterised neuropathologically by the presence in the brain of senile (amyloid) plaques and neurofibrillary tangles. A major constituent of the senile plaque is the β-amyloid peptide, deposition of which is the central event in the pathogenesis of AD (Glenner et al. 1984). β-amyloid peptide is a 42-43 amino acid cleavage product of the larger amyloid precursor protein (APP) (Kang et al. 1987), and is produced in both AD patients and normal individuals, as well as cultured cells (Seubert et al. 1992, Haass et al. 1992). Early onset may be caused by a mutation in the APP gene located on chromosome 21 (Goate et al. 1991) or, more commonly, by an unindentified gene on chromosome 14 (St George-Hyslop et al. 1992). Previous evidence of the involvement of chromosome 19 in late onset AD (Pericak-Vance et al. 1991, Schellenberg et al. 1992) has now been confirmed in a number of studies which demonstrate a strong relationship between the development of AD and presence of the apoE4 allele (Corder et al. 1993, Poirier et al. 1993, Saunders et al. 1993a, Strittmatter et al. 1993a). The frequency of the apoE4 allele (=0.52) was increased in late-onset familial AD (Strittmatter et al. 1993a), and in sporadic AD patients (=0.38-0.40) (Saunders et al. 1993a, Poirier et al. 1993), compared to frequencies of 0.12-0.16 in age-matched controls (Poirier et al. 1993, Saunders et al. 1993b). Recently, the apoE4 allele frequency was reported to be significantly increased in both early-onset (age of onset <60 years, apoE4 frequency = 0.32) and late-onset (age of onset $\geq$ 65 years, apoE4 frequency = 0.24) sporadic AD, compared to age-matched controls (Chartier-Harlin et al. 1994).

The proportion of individuals affected by late onset AD increased with the dose of apoE4 alleles from 20% of subjects with genotypes E2/3 or E3/3, to 47% of subjects with genotypes E4/2 or E4/3 and to 91% of subjects with genotype E4/4 in a study of 234 individuals (Corder et al. 1993). The presence of one apoE4 allele was associated with a

2.8-fold increased risk of AD and homozygosity of apoE4 increased risk of AD by more than 8 times compared with absence of apoE4. At the same time, mean age of onset decreased with increasing number of apoE4 alleles, from 84.3 years (absence of apoE4) through 75.5 years (one apoE4 allele) to 68.4 years (homozygosity for apoE4). These results are consistent with a co-dominant effect of apoE4. ApoE4 gene dose was also related to survival, mean survival decreasing with increasing number of apoE4 alleles (Corder et al. 1993); this was reflected in the significantly decreased apoE4 allele frequency (0.052) in centenarians compared to younger controls (0.112) (Schachter et al. 1994). The finding that those with the apoE4 allele are at increased risk of CAD (section 1.4.7) and AD may explain the reduced apoE4 frequency in older populations.

ApoE is present in the brain as apoE-lipid complexes (Boyles et al. 1989) and as free protein (Boyles et al. 1990), and is implicated in neuronal regeneration. Recent studies implicate the importance of apoE in the pathogenesis of AD with the protein accumulating extracellularly in the senile plaque and congophilic angiopathy of AD and intracellularly in the neurofibrillary tangle (Namba et al. 1991, Wisniewski et al. 1992, Strittmatter et al. 1993a, 1993b). ApoE can interact directly with β-amyloid to form a stable adduct (Wisniewski et al. 1992, Strittmatter et al. 1993a), and is also associated with amyloid plaques in any disease in which amyloid forms, independent of the amyloid-forming protein (Namba et al. 1991, Wisniewski et al. 1992, Strittmatter et al. 1993a). However in amyloid-forming diseases other than AD, apoE4 allele frequency was not increased in cases, indicating a specific association of apoE4 with late-onset AD (Saunders et al. 1993), with apoE4 carriers having more amyloid plaques and denser amyloid immunoreactivity (Schmechel et al. 1993) than individuals without this allele.

Recently Strittmatter et al. (1993b) demonstrated differences in binding of apoE3 and apoE4 alleles to β-amyloid, implicating the importance of the Cys-Arg interchange at amino acid 112. Both apoE alleles bind avidly to β-amyloid peptide in-vitro resisting dissociation by SDS (Strittmatter et al. 1993a). Both apoE3 and apoE4 bind β-amyloid. However, binding of apoE4 occurs much more rapidly and is more pH-sensitive, being prevented at $pH < 6.6$ (whereas apoE3 binds β-amyloid over a larger pH range, 7.6 to 4.6), indicating differences between the apoE3 and apoE4 alleles in complexing with β-amyloid (Strittmatter

et al. 1993b). This binding appears to require both oxidation of apoE and the carboxyl terminus of apoE, with residues 244-272 of apoE being critical for apoE-β-amyloid complex formation (Strittmatter et al. 1993b). Evidence for increased protein oxidation in normal ageing (Smith et al. 1991, Stadtman et al. 1992) and in AD (Smith et al. 1991) has been reported, with over-expression of a cytochrome oxidase gene being demonstrated in AD brain (Parker et al. 1991), however the precise role of apoE oxidation in the pathogenesis of AD has not been eludicated.

The carboxyl-terminus of apoE was previously shown to be important in lipoprotein association ie. lipid-binding (Aggerbeck et al. 1988), and recently residues that are carboxyl-terminal to residue 244 were demonstrated to play a major role in lipoprotein binding (Westerlund & Weisgraber 1993). Since β-amyloid binds to the lipoprotein binding domain, not the receptor binding domain, of apoE, this might allow apoE-mediated targeting of apoE-β-amyloid complexes to neurons via apoE receptors (Strittmatter et al. 1993b), by the same route as apoE-lipid complexes, uptake of which occurs during nerve regeneration after injury (Handelmann et al. 1992). Internalization of apoE/β-amyloid complexes into neurons would result in incorporation of these complexes into lysosomes and pH-dependent dissociation; for apoE4 this dissociation would occur more readily than for apoE3 (Strittmatter et al. 1993b). The appearance of apoE in neurofibrillary tangles (Namba et al. 1991, Strittmatter et al. 1993a) suggests that apoE is targeted to intracellular loacations in neurons, this mechanism may be critical to the loss of neurons central to AD (Strittmatter et al. 1993b). There is much that still remains speculative about the role of apoE in the pathogenesis of AD.

Section 1.5 ApoAI, apoCIII and apoAIV

Section 1.5.1 ApoAI; synthesis, structure and functions

ApoAI is synthesized and secreted in the liver and small intestine (Green et al. 1978, Schonfeld et al. 1978). ApoAI is secreted as a 249 amino-acid proprotein that is converted by extracellular proteolysis to the mature protein in the plasma (Zannis et al. 1982). Mature apoAI is a single 243 amino-acid polypeptide with a molecular weight of about 28KDa and six tandem 22-amino acid repeats (Brewer et al. 1978). These tandem repeats might mediate lipid binding and activation of LCAT (Kaiser & Kezdy 1984, Anantharamaiah et al. 1990). Following entry of chylomicrons into plasma, almost all of the apoAI particles are rapidly transferred to HDL particles (Koren et al. 1987). Over 90% of apoAI in normal fasting

human plasma is associated with HDL (Cheung & Albers 1977). During their circulating lifetime, apoAI molecules are released and transferred between HDL particles.

ApoAI is the cofactor of LCAT (lecthin:cholesterol acyl transferase) that catalyses the conversion of free cholesterol to cholesteryl esters in HDL (Fielding et al. 1972). The LCAT reaction mediates essentially all of the intravascular cholesterol esterification (Glomset 1972). ApoAI also plays a direct role in reverse cholesterol transport; acting as an acceptor protein mediating cholesterol efflux from cells in-vitro (Eisenberg 1984). The apoAI-mediated cholesterol efflux from cells is coupled with LCAT-catalysed esterification of cholesterol in HDL and transport of cholesteryl esters from HDL to TG-rich lipoproteins, mediated by CETP (reviewed in Reichl & Miller 1989). Recently an immunochemical approach was employed to delinate the specific parts of the apoAI molecule responsible for its LCAT-activating property (Banka et al. 1991). These authors proposed that residues 95-121 of mature apoAI is critical in the activation of LCAT, this region includes the third of eight 22-amino acid sequence repeats (residues 100-121) in the apoAI molecule, which are believed to form amphipathic helices (Segrest et al. 1973). A mutation resulting in an amino acid substitution at residue 107 of apoAI results in decreased LCAT activity (Rall et al. 1984).

ApoAI levels, like HDL-cholesterol concentrations, are inversely correlated with CHD risk (Berg et al. 1976, Albers et al. 1978, Maciejko et al. 1983, Miller 1987 and discussed in Section 1.7.3). ApoAI may act directly within the arterial wall to prevent oxidation (Parthasarathy et al. 1990) or self-aggregation (Khoo et al. 1990) of LDL, or to promote mobilization of cholesterol from arterial-wall cells (DeLamatre et al. 1986, and discussed in more detail in Section 1.7.4). In studies involving transgenic mice expressing human apoAI, plasma HDL-cholesterol levels were directly proportional to human apoAI gene expression (Walsh et al. 1989) and a high plasma concentration of human apoAI has a direct inhibitory effect on the early steps of atherogenesis in these mice i.e. is protective (Rubin et al. 1991). Two important determinants of plasma apoAI levels in individuals with normal plasma lipids are input rates and residence times for apoAI. Normolipidaemic individuals with low HDL and apoAI levels had an apoAI residence time that was shortened by about 30% compared to those with normal HDL and apoAI levels, regardless of concomitant factors such as

smoking, obesity and HTG (Gylling et al. 1992). This confirmed previous observations of a shortened residence time of apoAI in plasma being a primary cause of low apoAI concentrations in normo- or hyperlipidaemic obese subjects (Brinton et al. 1991). Gylling et al. (1992) propose that enhanced clearance of apoAI is an important contributor, under a variety of circumstances, to low HDL levels. Within groups having low or normal HDL levels, input rates for apoAI were highly correlated with plasma concentrations of apoAI (Gylling et al. 1992), indicating that the role of apoAI production should not be dismissed either in causation of low apoAI levels or in the potential for therapeutic modulation of apoAI levels.

Recently, the apoAI primary structure (in human versus mouse) was shown to have a profound influence on HDL particle size distribution and metabolism (Chajek-Shaul et al. 1991). HDL distribution changed from being primarily monodisperse (major peak at 10.2nm) in control mice to polydisperse (major peaks at 11.4, 10.2 and 8.7nm, corresponding to HDL_1, HDL_2 and HDL_3) with a pattern like that of human HDL occuring in the transgenic mice expressing human apoAI (which accounts for about 90% of total apoAI in these mice). In control mice (expressing mouse apoAI), in-vivo turnover studies demonstrated that the fractional catabolic rates (FCR) was significantly greater for HDL-cholesteryl ester (CE) than the FCR for apoAI. However transgenic mice, expressing human apoAI, had identical FCRs for HDL-CE and apoAI (Chajek-Shaul et al. 1991). These results suggest that human apoAI does not allow selective uptake [the proportion of HDL particles taken up by selective uptake is measured by the difference between apoAI FCR and HDL-CE FCR). Selective uptake of HDL-CEs is removal of HDL-CEs without whole particle removal (Glass et al. 1983, 1985). Thus, clearance of HDL-CEs in humans may be divided between the particulate uptake (catabolism of whole HDL particles) and the CETP-mediated pathways (Chajek-Shaul et al. 1991). Another illustration of the importance of apoAI in modulating the HDL subclass distribution is given in a report of a kindred with complete apoAI deficiency due to a stop mutation in the signal peptide region (Ng et al. 1994). Individuals homozygous for this mutation had a single HDL peak diameter of 7.9nm (= very small HDLs containing apoAII only). Heterozygotes had 2 peaks at 7.9 and 8.4nm (=HDL_3) but no peaks corresponding to HDL_2.

Section 1.5.2 ApoCIII; synthesis, structure and functions

ApoCIII is a 79 amino-acid glycoprotein, present both as non-glycosylated and glycosylated forms containing either one or two moles of sialic acid (Brown et al. 1970, Albers & Scanu 1971, Herbert et al. 1982). ApoCIII contains two amphipathic helicies located between residues 11-28 and 35-52, suggested to be involved in lipid binding; evidence for this comes from synthetic peptide studies and computer analysis (Brasseur et al. 1992, Segrest et al. 1992). Three genetic variants of apoCIII, arising from missense mutations have been reported; T74A (Maeda et al. 1987), L58E (von Eckardstein et al. 1991) and A23T (Fanli Xu, personal communication). The T74A mutation prevented O-glycosylation of apoCIII, but did not affect the secretion or lipid binding of apoCIII nor was associated with any dyslipoproteinaemia (Maeda et al. 1987, Roghani et al. 1988), while carriers of Glu58 had decreased apoCIII levels and atypically large HDL particles, compared to unaffected family members (von Eckardstein et al. 1991). The A23T substitution is predicted to disturb the amphipathicity of the first helix, due to the induction of a hydrophilic residue into a hydrophobic domain and thus likely to reduce the affinity of the mutant apoCIII polypeptide for a lipid surface; this would explain the decreased apoCIII levels in carriers of Thr23 (Fanli Xu, personal communication).

ApoCIII is a major protein component of the TG-rich lipoproteins (chylomicrons and VLDLs) and a minor protein component of HDL, accounting for 50% of the protein in VLDL and 2% of the protein in HDL (Catapano 1980, Herbert et al. 1983). However, since total protein mass on VLDL is only about 10mg/dl, and that on HDL is about 150mg/dl, most of the circulating apoCIII is found on HDLs. The transfer of apoCIII between TG-rich lipoproteins and HDL is rapid; apoCIII shifts towards newly synthesized TG-rich lipoproteins from HDL during postprandial lipaemia (Havel et al. 1973) and during lipolysis, apoCIII transfers back to HDL from the remnants of TG-rich lipoproteins (Eisenberg et al. 1979). Tracer kinetic studies have suggested that this exchange may be very rapid and complete (Bilheimer et al. 1972, Eisenberg et al. 1972). However there is a fraction of the apoCIII pool in VLDL as well as HDL that does not participate in this spontaneous exchange process (Bukberg et al. 1985). The rate at which apoC's, including apoCIII, exchange between fasting plasma lipoproteins (eg. HDL) and newly synthesized TG-rich lipoproteins seems to depend on the composition of the circulating lipoproteins, as well as the size of the acceptor

TG-rich lipoproteins. Thus, while transfer of apoCIII to TG-rich lipoproteins may be needed to stabilize these lipoproteins, excessive enrichment of these with apoCIII may not be desirable, with the consequences of reduced lipolysis of TG-rich lipoproteins and accelerated catabolism of HDL (Tornoci et al. 1993).

It is known that plasma apoCIII levels correlate with plasma TG and cholesterol levels (Marz et al. 1987, Le et al. 1988, Shoulders et al. 1991). The precise role of apoCIII in-vivo remains unclear but evidence is accumulating that apoCIII is involved in regulating TG metabolism, by modulating both lipolysis and receptor-mediated uptake of TG-rich lipoproteins. It has been claimed that the LPL-stimulating property of apoCII is counteracted by apoCIII (Brown & Baginsky 1972); however the extent of inhibition is dependent on the experimental conditions employed. LPL in solution can be inhibited but endothelium-bound LPL cannot be inhibited by apoCIII (Kotlar & Borensztajn 1979). ApoCIII was shown, in-vitro, to inhibit the hydrolysis of TGs by LPL (Brown & Baginsky 1972, Wang et al. 1985, Ginsberg et al. 1986) and recently synthetic peptides of apoCIII were shown to inhibit LPL activity (McConathy et al. 1992) - this is also discussed in Section 1.6.3. ApoCIII also inhibits in-vitro hepatic lipase activity (Kinnunen et al. 1983). Recently transgenic mice overexpressing human apoCIII exhibited severe hypertriglyceridaemia (Ito et al. 1990) confirming an important role for apoCIII in TG metabolism in-vivo. ApoCIII was also found to delay uptake of TG emulsions and TG-rich lipoproteins by the perfused rat liver (Shelburne et al. 1980, Windler & Havel 1985) and by hepatocytes (Quarfordt et al. 1982, Windler & Havel 1985). That apoCIII may inhibit LPL is supported by in-vivo observations of rapid VLDL to LDL conversion with combined apoAI and apoCIII deficiency (Ginsberg et al. 1986).

ApoCIII has been demonstrated to reduce clearance of modified lipoprotein particles in cebus monkeys (Stephan et al. 1986). These findings have been explained by the demonstration of reduced binding of apoE to hepatic apoE receptors in the presence of apoCIII (van Berkel et al. 1983). Sehayek & Eisenberg (1991) showed that the C apolipoproteins, including apoCIII, inhibited the apoE-mediated uptake of VLDL subfractions, reconstituted with recombinant apoE3 and the various apoC particles, irrespective of the apoE content of the particles, via the LDL-receptor. This mechanism was

shown to apply to LDL-receptor-related-protein-mediated uptake as well (Kowal et al. 1990). Patients with familial combined apoCIII/apoAI deficiency have very rapid fractional catabolic rates of VLDL-apoB and -TG in-vivo (Ginsberg et al. 1986). Recently two different strains of transgenic mice expressing apoCIII, low and high expressors, were used to study the mechanism for the apoCIII-mediated hypertriglyceridaemia (Aalto-Setala et al. 1992). The high expressor line had higher plasma TG levels and larger and more TG-rich VLDLs with more apoCIII and less apoE per VLDL compared to either the low expressor or control lines. VLDL from transgenic mice was taken up much more slowly than control VLDL; this was not due to reduced LPL activity or increased VLDL-apoB production, indicating that the HTG in transgenic mice was due to delayed uptake of TG-rich lipoproteins, because of reduced apoE on the lipoprotein particle, from the circulation and suggesting one possible mechanism for primary HTG in humans (Aalto-Setala et al. 1992). Both in-vivo and in-vitro observations thus suggest that apoCIII exerts a major influence on apoE-dependent cellular uptake of remnants of TG-rich lipoproteins. In support of this, the content of apoCIII in plasma HDL is correlated with the fractional catabolic rate of HDL-apoAI (Le et al. 1988).

Several studies have indicated a possible role for apoCIII in the development of CAD. ApoCIII levels in apoB-containing lipoproteins (LpB) were significantly higher in men with CAD than in healthy controls, and the amount of apoCIII in LpB was more discriminatory, than total TGs, between cases and controls (Chivot et al. 1990). These authors thus proposed that apoCIII-LpB would provide a more specific indication of the dyslipoproteinaemia observed in coronary bypass populations. TGs were more strongly correlated with total apoCIII and apoCIII in LpB in patients than in controls. Genest et al. (1991) demonstrated a reduced apoCIII level in CAD patients compared to healthy controls, which related to the fact that HDLs are the main carriers of apoCIII (containing nearly 2/3 of the total apoCIII) and that both the cholesterol and protein content in HDL are reduced in CAD. These CAD patients had significantly higher levels of TGs and of particles containing both apoE & apoB, but not of those containing apoB & apoCIII. Genest et al. (1991) therefore made two proposals. Firstly, TG-rich particles rich in apoCIII may not represent added cardiovascular risk, due to decreased formation of atherogenic remnant particles from these lipoproteins, because of inhibition of LPL by apoCIII (see this section and Section 1.6.3). Secondly TG

levels are associated with increased cardiovascular risk when the TG-rich lipoproteins are of a size and composition that allows greater formation of cholesterol-rich remnant lipoproteins; this would be in addition to the effect of TGs on HDL levels (discussed in Section 1.7). In agreement, Blackenhorn et al. (1990) demonstrated, in a group of men with coronary artery bypass grafts, a significantly higher ratio of apoCIII in HDL to apoCIII in non-HDL in those treated with a cholesterol-reducing drug compared to those given placebo. Drug treatment produced no significant change in total apoCIII levels but radically altered the distribution of apoCIII between HDL and non-HDL. On-trial HDL-apoCIII was significantly inversely correlated with the risk of progression of CAD in the treatment group. ApoCIII in VLDL is believed to be associated with denser, smaller and more atherogenic VLDL subclasses (Carlson & Ballantyne 1976, Kuusi et al. 1980, Le et al. 1988). The half-life of these particles would be prolonged due to apoCIII inhibiting lipolysis and receptor-mediated uptake (as discussed above). Transfer of apoCIII from VLDL to HDL would stimultaneously increase the removal of atherogenic VLDLs and decrease the removal of HDL (Blankenhorn et al. 1990), the latter due to apoCIII-mediated inhibition of the action of hepatic lipase on HDL (Kuusi et al. 1980).

In contrast, total apoCIII levels were higher in patients with carotid artery disease, but not in patients with CAD, compared to controls (Wiseman et al. 1991) and these authors suggested that the control of the metabolism of TG-rich lipoproteins by apolipoproteins may predispose to atherosclerosis in specific sites, with high levels of apoCIII selectively promoting carotid artery disease. They proposed that delayed uptake of remnant lipoproteins, secondary to elevated apoCIII levels, would selectively accelerate the deposition of atheroma in the carotid arteries - this was supported by the observation that cholesterol and apoB, which are major constituents of VLDL remnants, were highest in these patients.

Section 1.5.3 ApoAIV; synthesis, structure and functions

In man, apolipoprotein (apo) AIV is primarily synthesized in the enterocytes of the intestine, during absorption of fat, and is secreted into the lymph in association with chylomicrons, but is not present in significant amounts in chylomicron remnants (Green et al. 1980). ApoAIV mRNA production in the liver is regulated by developmental and by nutritional factors (Karathanasis & Yunis 1986). The mature protein contains 376 amino acid

residues (Yang et al. 1989) of molecular weight 46kDa. The deduced amino acid sequence of apoAIV includes a 20 amino acid signal peptide (Karathanasis 1985). ApoAIV is a relatively abundant protein with a plasma concentration of 150-370mg/l (Utermann & Beisiegel 1979, Weinberg & Scanu 1983, Green et al. 1979).

The precise function of apoAIV in lipid metabolism is still unknown; however the observation that the plasma concentration of apoAIV is significantly increased after consumption of fat (Bisgaier et al. 1985) suggests a role for apoAIV in intestinal fat absorption. This is supported by the observation that a deletion of the entire apoAI-CIII-AIV gene cluster, leading to a deficiency of all three proteins, is associated with malabsorption of vitamin K and essential fatty acids, but that patients with familial apoAI and apoCIII deficiency, due to a 6.5 kilobase inversion in the apoAI-CIII-AIV gene cluster, do not have these symptoms (Ordovas et al. 1989). It has been suggested (Scheraldi et al. 1988) that apoAIV is a modulator of apoCII activation of LPL, because of apoAIV-mediated release of apoCII [an essential activator of LPL (LaRosa et al. 1970)] from HDL particles to enable LPL-mediated hydrolysis of TG-rich lipoproteins to take place. Goldberg et al. (1990) demonstrated that apoAIV enhances the transfer of apoCII to chylomicrons. It is thus possible that a defect in or deficiency of apoAIV would be associated with HTG. The rapid dissociation of this protein from the surface of lymph chylomicrons during catabolism of these particles (Dallinga-Thie et al. 1985) and the possible involvement of apoAIV in activation of LCAT (Steinmetz & Utermann 1985), respectively, suggest roles for apoAIV in TG and cholesterol metabolism. ApoAIV might promote the formation of very small HDL particles, thus furthering the stimulation of cholesterol efflux from cells (Lagrost et al. 1991); in support of this, apoAIV can cause efflux of cholesterol from cholesterol-loaded mouse macrophages due to the formation of HDL-like particles (Hara et al. 1992). Cholesterol of cell origin in these small HDL particles is esterified by LCAT; apoAIV has about 1/3 of the efficiency of apoAI in activating LCAT in-vitro (Steinmetz & Utermann 1985).

There is some controversy concerning the in-vivo plasma distribution of apoAIV (Tankanen & Ehnholm 1993) but it is found associated with chylomicrons, HDL and the lipoprotein-free fraction of the plasma (Weisgraber et al. 1978, Green et al. 1979). The biophysical behaviour of apoAIV is distinct from that of other apolipoproteins. ApoAIV is

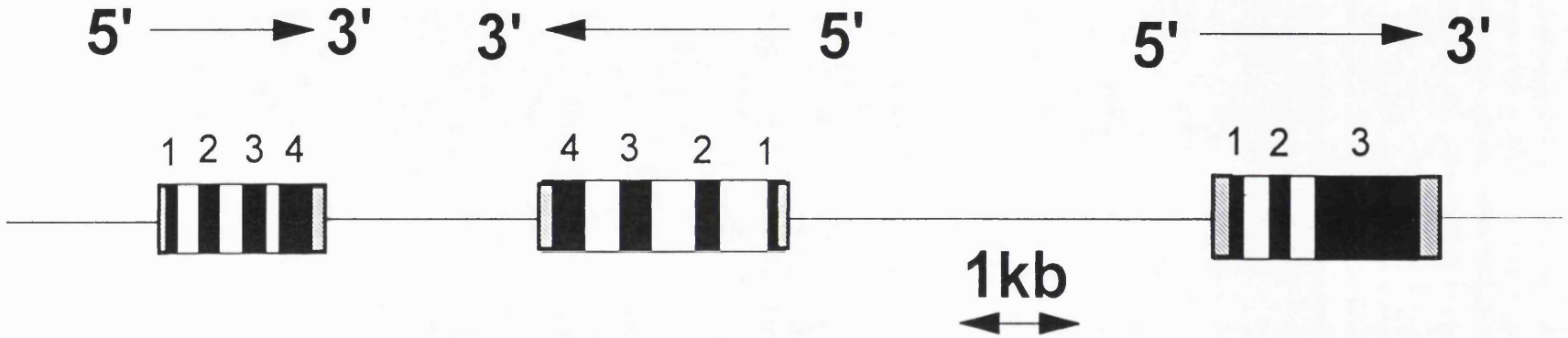
the most hydrophilic of all the known apolipoproteins (Weinberg 1987); its amphipathic α-helices may not be able to penetrate lipid surfaces to the same depth as the helices of other apolipoproteins. The binding of apoAIV to the HDL surface is greatly increased following the action of LCAT on HDL (DeLamatre et al. 1983). This explains why apoAIV only associates with HDL_3 and not HDL_2 (Weinberg & Spector 1986). More recently, Weinberg et al. (1992) calculated that the surface pressure of native HDL_3 is 33 millinewtons (mN) /m, this pressure rapidly decreases upon LCAT action, thus allowing apoAIV to bind when the HDL surface pressure is less than the exclusion pressure of apoAIV. The exclusion pressure of apoAIV (28-29mN/m) is the surface pressure above which apoAIV cannot penetrate lipid monolayers (Weinberg et al. 1992). This may explain why, during gel filtration most of the plasma apoAIV is found associated with HDL, but after ultracentrifugation of lipoproteins, most apoAIV exists in the free form. It may also explain the influence of LCAT and lipid transfer proteins on the association of apoAIV with HDL; the action of LCAT would lower the surface pressure of HDL_3 particles, resulting in association of apoAIV on these particles. CETP- and HL-mediated depletion of the HDL_3 particle would increase the surface pressure and lead to dissociation of apoAIV from these particles. ApoAIV, together with apoAI, may modulate the distribution of apoE among the lipoprotein particles and thus affect their clearance from the circulation (Bisgaier et al. 1989).

ApoAIV is elevated about 3-fold in patients with end-stage renal disease and in these patients the apoAIV accumulates in large HDL, which may reflect impaired reverse cholesterol transport, which would, in turn, provide an explanation for the high prevalence of atherosclerosis in patients with uremia (Dieplinger et al. 1992). Recently a high level of expression of soluble apoAIV protein was achieved in *Escherichia coli* (Duverger et al. 1991), which would be a useful tool for studying the role of apoAIV in reverse cholesterol transport and atherosclerosis.

Section 1.5.4 The apoAI-CIII-AIV gene cluster and its regulation

In man (and in all mammals), the genes coding for apoAI, apoCIII and apoAIV are closely linked, occuring in a tight cluster on chromosome 11 (Karathanasis et al., 1983a, 1985, Cheung et al. 1984, Schamaun et al. 1984). The cluster spans about 15Kb with the apoCIII gene being located 2.6Kb downstream from the apoAI gene and transcribed in the

Figure 1.5.4 ApoAI-CIII-AIV gene cluster



Direction of transcription of genes is shown from 5' to 3'. Boxes represent genes for, from left to right, apoAI, apoCIII and apoAIV. White, hatched and black regions within genes represent, respectively, introns, untranslated and translated regions of exons. Exons are numbered in ascending order in the 5' to 3' direction. Intergenic regions are represented by lines between the boxes.

opposite direction. The apoAIV gene is located 12Kb downstream from the apoAI gene and transcribed in the same direction. A schematic representation of the apoAI-CIII-AIV gene cluster is shown in Figure 1.5.4. The three genes share similar exon/intron structures and sequence homology at both the DNA and protein levels, suggesting that these genes are evolutionally related and arose from a common ancestral gene (Li et al. 1988). The apoAI gene is 1863bp in length and contains 4 exons. The 3 introns occur, respectively, in the 5' untranslated region, the sequence coding for the 18 amino-acid signal peptide and the sequence coding for the mature protein (Karathanasis et al. 1983b). Exon 4 contains six 66bp tandem DNA repeats responsible for the 22 amino acid segments observed in the protein sequence analysis. The apoCIII gene is about 3.6Kb long with 3 introns in positions similar to those of the introns in the apoAI gene (Karathanasis et al. 1984) and multiple tandem repeats in exon 4 (Li et al. 1988). The apoCIII mRNA sequence was eludicated and contains a 20 amino acid signal peptide (Sharpe et al. 1984). The apoAIV gene is 2.7Kb long with two introns interrupting the nucleotide sequence coding for the prepeptide and mature protein, respectively. The only difference between the structure of the apoAIV gene and the genes for apoAI and apoCIII, is the absence of the intron occuring in the 5' non-coding region in the apoAIV gene (Karathanasis et al. 1986).

Tissue-specific repression of apoAI gene expression in non-apoAI producing cells is mediated by two sequences from -2067 to -1476bp and from -199 to -80bp, relative to the transcription start site, in the apoAI promoter. In addition, three distinct regions (-1021 to -691bp, -487 to -413bp and -250 to -199bp) contain sequences required for maximum gene expression (Higuchi et al. 1988). Hepatocyte-specific expression is mediated by elements within the -256 to -41bp DNA region, but these are not sufficent for intestinal expression. Deleting the region from -1021 to -691bp results in a 50% reduction in apoAI promoter/enhancer activity. It includes the sequence 5'-ATCAAAT-3' which is an enhancer element in apoAII (Shelly & Baralle 1987). When two other regions, (-487 to -413bp and -250 to -199bp) were deleted, a dramatic loss of enhancer or promoter activity of the apoAI gene occured. These regions contain nucleotide sequences similar to GC boxes which are present in promoters of many human genes and bind to the Sp1 protein trans-acting factor (Breathnach & Chambon 1981). The -222 to -110 apoAI gene region, functioning as a powerful hepatocyte-specific transcriptional enhancer containing three specific protein-binding

sites, is necessary and sufficient for expression in HepG2 cells. Protein binding to all three sites is necessary for maximal apoAI gene expression (Widom et al. 1991), indicating that the transcriptional activity of the apoAI gene in liver cells is dependent on synergistic interactions between transcription factors bound to its enchancer.

Walsh et al (1989), using transgenic mice, showed that 256bp of 5' flanking DNA was sufficient for liver expression of apoAI, but that as much as 5.5Kb of 5' and 4.0Kb of 3' flanking DNA did not allow intestinal expression of the human apoAI gene. A 10.5Kb fragment, extending from 300bp 5' to the apoAI gene to 2.5Kb 5' to the apoCIII gene (which includes both the apoAI and CIII genes) produced high levels of in-vivo apoAI intestinal expression (Walsh et al. 1993). These in-vivo experiments suggested that the intestinal control region for the apoAI gene is distinct from the liver control region, resides within the apoCIII promoter and behaves as an enhancer element. It is possible that apoAI and apoCIII share an intestinal control region (Walsh et al. 1993). The studies of Walsh et al. (1993) and Reisher et al. (1993) together suggest that a single DNA element (within the region 0.2 to 1.4Kb 5' to the apoCIII gene but not yet identified) in the middle of the apoAI-CIII-AIV gene complex controls intestinal expression of all three genes and that this gene cluster may have stayed tightly linked through evolution to preserve intestinal expression of these three genes. This would be analogous to the sharing of a single liver control region in the apoE-CI-CII gene cluster on chromosome 19, for which evidence has been presented (Simonet et al. 1990, 1991 and discussed in Section 1.4.4).

Gene-specific methylation patterns in the apoAI-CIII-AIV gene cluster indicates that this cluster is not one entity with respect to methylation. Extent of methylation of the apoAI-CIII-AIV gene cluster in adult tissues seems to be determined by an interplay between demethylation and de-novo methylation (Shemer et al. 1991). In tissues that do not express any of the three genes, the cluster is almost entirely methylated, however individual gene regions are unmethylated in the tissue of expression. ApoAI (Shemer et al. 1990), and both apoCIII and apoAIV (Shemer et al. 1990, 1991) have tissue-specific methylation patterns which correlate with their activity. The 5' end of the apoAI gene shows little methylation in the intestine and none in the liver but is completely methylated in tissues that do not express apoAI (Shemer et al. 1990). The apoCIII gene is completely methylated in sperm,

kidney and leukocytes, none of which express apoCIII. In the liver (where the apoCIII gene is predominantly expressed), by contrast, this gene is highly demethylated (Breslow 1988). The tissue specificity of the methylation pattern of the apoCIII or apoAIV genes in adult tissues is not observed in embryonic tissues, which is a good indication that these genes undergo tissue-specific de-novo methylation later in development, following tissue-specific expression in the embryo (Shemer et al. 1991). In all three genes, the rule is that the gene undergoes complete demethylation when it is highly expressed, and partial demethylation in tissues where levels of expression are relatively low. It appears that the three genes in the cluster have no effect on each other's expression (Shemer et al. 1991).

Section 1.5.5 ApoAI-CIII-AIV gene cluster and rare dyslipidaemias

Defects in the apoAI-CIII-AIV gene cluster have been implicated or identified in several rare disorders of lipid metabolism (hereditary analphalipoproteinaemias). Complete deficiency for apoAI & apoCIII or apoAI, apoCIII and apoAIV is associated with virtual absence of HDL and severe premature atherosclerosis (Reichl & Miller 1989). Deficiency of apoAI, apoCIII and HDL has been associated with a DNA insertion in the apoAI gene (Karathanasis et al. 1983c) or a DNA inversion involving the apoAI and apoCIII genes (Karathanasis et al. 1987) in patients with premature atherosclerosis. A complete deletion of the apoAI-CIII-AIV gene cluster underlies a reported case of familial apoAI, CIII and AIV deficiency and premature atherosclerosis associated with marked HDL deficiency (Ordovas et al. 1989). Recently, a nonsense mutation in the signal peptide region of apoAI, transmitted as an autosomal co-dominant trait, was identified as the underlying cause of isolated complete apoAI deficiency with significantly increased risk of atherosclerosis in a kindred (Ng et al. 1994). A Japanese family with a nonsense mutation in codon 84 of apoAI and a presumed HDL assembly defect also appeared to be suspectible to atherosclerosis (Matsunaga et al. 1991). It appears that inability to synthesize apoAI or assemble HDL particles may be the common denominator among the kindreds with hereditary analphalipoproteinaemias that are predisposed to develop premature atherosclerosis (Ng et al. 1994).

However other genetic dyslipidaemias involving apoAI and/or HDL do not result in a greatly increased suspectibility to atherosclerosis, due to presence of circulating apoAI

variants. Tangier disease is characterized by very low levels of HDL and apoAI with elevated VLDL and abnormally TG-rich HDL with unique abnormal deposition of lipids and hypercatabolism of apoAI (Herbert et al. 1983), but the apoAI gene is normal. The prevalence of CHD in Tangier disease patients is only moderately increased despite HDL and apoAI concentrations that are below 5% of normal (Reichl & Miller 1989). Studies have suggested that normal HDL is rapidly removed from the circulation of patients with Tangier disease (Brewer et al. 1985). Cellular studies have implicated a defect in the way macrophages metabolize HDL from these patients (Schmitz et al. 1987). Normally HDL associates with macrophages, accepts cholesterol from these cells and subsequently dissociates. In Tangier disease, macrophages bind HDL as normal, but then internalize and degrade the HDL particle, resulting in low levels of circulating HDL. Fish-eye disease patients have severe HDL deficiency as a result of specific structural mutations of the LCAT gene which may cause specific loss of HDL-LCAT interaction (Funke et al. 1991a, Klein et al. 1992) but are spared from premature CHD. There is another genetic trait which does not predispose to premature CHD, despite low apoAI and HDL levels in plasma; this is $apoAI_{Milano}$ (Franceschini et al. 1980), due to an R173C susbstitution in apoAI (Weisgraber et al. 1983), leading to the formation of disulphide-linked $apoAI_{Milano}$ homodimers and heterodimers between $apoAI_{Milano}$ and apoAII. $ApoAI_{Milano}$ is discussed in Section 1.8.4 with regard to postprandial lipaemia. One frameshift and two missense mutations in the apoAI gene are not associated with increased risk of CHD (Nichols et al. 1990, Deeb et al. 1991, Funke et al. 1991b).

Section 1.5.6 ApoAI-CIII-AIV gene cluster polymorphisms and associations with lipids, lipoproteins, dyslipidaemias and atherosclerosis

Over ten common polymorphisms have been detected within this gene cluster, with strong linkage disequilibrium reported between pairwise combinations of these polymorphisms (eg. Stocks et al. 1987, Thompson et al. 1988, Benlian et al. 1991, Haviland et al. 1991, Ordovas et al. 1991, Paul-Hayase et al. 1992, Marasco et al. 1993). Table 1.5.6.1 shows six of these polymorphisms which will be discussed further here. Many studies have found associations between genotypes of some of these polymorphisms and variations in levels of lipids and lipoproteins (reviewed in Humphries et al 1988, 1992, Mehrabian et al 1992). Most of these polymorphisms are caused by sequence changes

Table 1.5.6.1

Selected polymorphisms at the human apoAI-CIII-AIV gene cluster

Restriction endonuclease	Position	Reference
XmnI	AI, 5' flanking, 4Kb from transcription start site	Kessling et al. 1985, Coleman et al. 1986
MspI (G_{-75} to A)	AI, 5' flanking 75bp from transcription start site	Jeenah et al. 1990
PstI	AI, 3' flanking region	Kessling et al. 1985
SstI	CIII, non-coding region of 4th exon	Karathanasis 1985, Coleman et al. 1986
PvuII-CIII	CIII, 1st intron	Colemann et al. 1986
PvuII-AIV	CIII-AIV, intergenic region	Kessling et al. 1988b

outside the coding regions of these genes. Altered allele frequencies of some of these polymorphisms in patients with hyperlipidaemia compared to healthy controls have been reported by some investigators. For example the frequency of the rare allele (S+) of the SstI polymorphism in the 3' untranslated region of exon 4 in the apo CIII gene and the first polymorphism identified in the apoAI-CIII-AIV gene cluster (Rees et al. 1983) has consistently been higher in patients with hyperlipidaemia or with CAD (Tybaerg-Hansen et al. 1993 and reviewed in Ordovas et al. 1991, with relative risk of hypertriglyceridaemia (HTG) in presence of the S+ allele being 3.9 in all Caucasians) and associated with HTG. Recently haplotypes of the SstI and apoCIII promoter polymorphisms were found to confer either protection against or suspectibility to severe HTG (Dammerman et al. 1993), with the haplotype distribution being significantly different between NTG and HTG individuals. Haplotypes containing the S+ allele were associated with a relative risk of HTG of 3.85. The S+ allele has been associated with higher TG levels in healthy individuals (Ordovas et al. 1991, Tybaerg-Hansen et al. 1993). The frequency of the A_{-75} S+ haplotype was significantly more frequent in individuals with serum cholesterol levels above the 90th percentile than in those with cholesterol levels below the 90th percentile (Marasco et al. 1993).

Other studies failed to demonstrate an association between the SstI polymorphism and HTG (Morris & Price 1985, Kessling et al. 1985). These divergent results may be due to the considerable variation in the rare allele frequency in normolipidaemic populations of different racial groups, in which frequencies ranging from 0 to 0.12 in Caucasian subjects (reviewed in Ordovas et al. 1991) to 0.35 in Japanese subjects (Paul et al. 1987). The P-allele of the PstI polymorphism 3' of apoAI occured at a higher frequency in patients with CAD compared to controls (Ordovas et al. 1986, Monsalve et al. 1989, Wile et al. 1989), but this was not confirmed in other studies (Deeb et al. 1986, Paulweber et al. 1988). The most likey reason for the differences observed between different laboratories is the different ethnic origin of the populations studied (reviewed in Humphries 1988, Mehrabian et al. 1992).

The frequency of the rare allele (X+) of the XmnI RFLP located 4Kb up-stream of the apoAI gene (Kessling et al. 1985) was higher in patients with HTG (Kessling et al. 1985,

1986), in FCHL patients (Kessling et al. 1985, Shoulders et al. 1986, Hayden et al 1987), and in individuals with combined hyperlipidaemia (Tybaerg-Hansen et al. 1993) compared with controls. Wile et al (1989) reported the association of the X+ allele with higher HDL-cholesterol and apoAI levels. This might be explained by linkage disequilibrium between the X+ and A_{-75} alleles; the A_{-75} allele is associated with higher apoAI and HDL-cholesterol levels (discussed later in this section). Wojciechowski et al. (1991) found strong linkage disequilibrium between the X+ allele and FCHL, and strong linkage in 7 out of 16 FCHL families to the apoAI-CIII-AIV gene cluster (LOD = 6.86 at θ = 0), confirming that these FCHL pedigrees have a defect in or close to the apoAI-CIII-AIV locus which results in hyperlipidaemia. However two recent studies (Wijsman et al. 1992, Xu et al. 1994) have failed to confirm the co-segregration of alleles of the apoAI-CIII-AIV gene cluster with FCHL, and thus whether mutations in the apoAI-CIII-AIV gene cluster can cause FCHL is still in debate.

It is biochemically feasible that either apoCIII or apoAIV could be involved in FCHL. In-vitro studies have shown that apoCIII inhibits the action of LPL and HL, and the hepatic uptake of VLDL and chylomicron remnants, thus mutations in apoCIII might affect TG and cholesterol levels (Windler & Havel 1985, Ginsberg et al. 1986 and discussed in Section 1.5.2). An increase in VLDL apoCIII occurs in some HTG patients (Chivot et al. 1990). ApoAIV might also modulate LPL activity by enhancing the transfer of apoCII to chylomicrons (Goldberg et al. 1990). Because apoCII is an essential co-factor for LPL (LaRosa et al. 1970), decreased levels of apoAIV or defects in apoAIV may cause HTG and thus could have a role in causing FCHL. ApoAI is unlikely to be implicated in the pathogenesis of FCHL as the main reported effect of mutations in this gene is a lowering of HDL levels (Breslow 1989 and see sections 1.5.1 and 1.5.4).

The XmnI X+ allele occured at a lower frequency in MI survivors (Hegele & Breslow 1987) and in patients with CAD (Frossard et al. 1987). Discrepant results obtained with the XmnI polymorphism may, in part, be due to the different X+ allele frequencies amongst different racial groups (Cole et al. 1989). It is disappointing that 10 years after the first reports of these associations, the mechanism of their effects is not understood, but it is assumed to be due to alterations of the levels or function of one or more of the

apolipoproteins in the cluster, with apo CIII being the most likely due to its known role in lipoprotein metabolism (eg see Aalto-Setala et al 1992). Recent studies have reported associations with the SstI and PvuII-CIII polymorphisms and levels of apoCIII, with the S+ allele having an elevating effect and the V- allele having a lowering effect (Shoulders et al 1991, Paul-Hayase et al 1992), but the mechanism of these associations is unclear. It is likely that variation in the apoCIII-AIV intergenic region may be involved; this is supported by strong linkage disequilibrium between the SstI polymorphism and polymorphisms in the apoCIII promoter (Dammerman et al. 1993). This intergenic region has been implicated as containing important elements that are necessary for correct expression of the apo CIII gene in transgenic mice (Walsh et al 1993 and discussed in Section 1.5.4).

Associations between variation in the gene cluster and levels of HDL or apoAI have been reported, with the PstI polymorphism [approximately 300bp 3' of the apoAI gene (Kessling et al. 1985)], the PvuII polymorphism in the 1st intron of apoCIII (Coleman et al. 1986) and the G_{-75} to A polymorphism in the apoAI promoter. The frequency of the P- allele was 8-fold higher in patients with HDL-cholesterol levels below the 5th percentile (Ordovas et al. 1986). Patients with the P+P- genotype had significantly lower levels of apoAI and HDL-cholesterol than patients homozygous for P+ (Wile et al. 1989). A large increase in the P- allele frequency was found in patients with hypoalphalipidaemia (Sidoli et al. 1985, Ordovas et al. 1986). In constrast, healthy men with the P+P- genotype had higher mean apoAI levels than men homozygous for P+ (Kessling et al. 1988a, Wile et al. 1989). The effect of haplotypes of the XmnI, PstI and SstI polymorphisms on apoAI levels was also studied (Kessling et al. 1988a). There were five common haplotypes deduced from these three polymorphisms. Haplotypes X-P-S- and X-P+S- are associated with large and small, respectively, increases of apoAI, while the other three haplotypes are associated with a reduction in apoAI. This suggests that there are three functionally distinct haplotypes that are affecting apoAI levels (Kessling et al. 1988a).

There may be secondary factors, either genetic or enviromental, in patients with CAD, that interact with genetic variation in linkage disequilibrium with the P- allele, and that are required for an individual to develop low levels of HDL. The rare allele of the PvuII-CIII polymorphism (V-) was more common in CHD patients (Frossard et al. 1987) and more

recently has been associated with lower apoAI levels (Table 1.5.6.2). The rare allele (V+) of the PvuII polymorphism in the apoCIII-AIV intergenetic region (Oettgen et al. 1986) was non-significantly associated with lower apoAI and HDL-cholesterol levels (Kessling et al. 1986b); this has been confirmed in most studies (Table 1.5.6.2). Both PvuII polymorphisms show allele frequency differences in different racial groups; in Caucasians, frequency of the PvuII-CIII V- allele is 0.3 (Coleman et al. 1986) and frequency of the PvuII-AIV V+ allele is 0.05 (Kessling et al. 1988b). However both frequencies are zero in blacks (Oettgen et al. 1986) and Dogrin Indians (Cole et al. 1989).

However, the strongest and most consistent associations have been with the G_{-75} to A polymorphism in the apoAI promoter (Jeenah et al. 1990). In seven independent studies (Table 1.5.6.2), the A_{-75} allele has been associated with higher levels of both HDL-cholesterol and apoAI (Jeenah et al 1990, Pagani et al 1990, Paul-Hayase et al 1992, Sigurdsson et al 1992, Xu et al 1993, Talmud et al 1994b, Saha et al 1994), and because of its position in the promoter region of the apo AI gene, it is likely that the G_{-75} to A sequence change itself may have a direct effect on the transcription of apo AI gene, and thereby the secretion of apo AI either from the liver or the intestine. The DNA segment between nucleotides -256 and -41 of the apoAI promoter is necessary and sufficient for maximal expression levels in HepG2 cells (Sastry et al. 1988). The G_{-75} to A polymorphism lies within a 51bp fragment containing two 14/15bp inverted repeats; when G is present at -75 the homology and self complementarity of the inverted repeats is disrupted (Pagani et al. 1990). In the mouse β-globin gene, an A_{-75} to G substitution within the sequence -81 AGGGCCA -75, identical to the -81 to -75 region in the human apoAI promoter (Pagani et al. 1990), dramatically reduces transcription rate (Myers et al. 1986) Using DNase I footprint analysis on the proximal part of the apo AI promoter, four protected areas (Panagiota et al 1991) have been identified, with one of the protein binding regions being from -128bp to -77bp. This is in close proximity to the G_{-75} to A sequence change and raises the possibility that this substitution alters the affinity for a trans-acting nuclear protein which affects the transcription rate of the apo AI gene and hence alters the rate of synthesis of apoAI.

Recently two independent studies attempted to elu ate mechanisms underlying the

TABLE 1.5.6.2
Comparison of published association studies between apolipoprotein levels and Apo AI-CIII-AIV RFLPs in healthy individuals

Polymorphism (rare allele)	Traits	Rare allele Assoc'n	Sample (Age in years) (1)	Reference
MspI	ApoAI	↑p<0.006	136F (45-59)	Pagani et al 1990
G/A	ApoAI	↑NS	108M (27-51)	Pagani et al 1990
polymorphism	ApoAI	↑p<0.025	96M (45-59)	Jeenah et al 1990
	ApoAI	↑p<0.025	162M (7-23)	Paul-Hayase et al 1992
(A_{-75})	ApoAI	None	54MF	Smith et al 1992
	ApoAI	↑p<0.004	104M (15-78)	Sigurdsson et al 1992
	ApoAI	↑NS	315MF (15-78)	Sigurdsson et al 1992
	ApoAI	↑p<0.05	204MF (8-11)	Xu et al 1993
	ApoAI	↑p<0.02	1657MF	Talmud et al 1994b
	ApoAI	↑p<0.01	148M	Saha et al 1994
	ApoAI	↑pNS	139F	Saha et al 1994
PstI	ApoAI	↑p<0.050	110M (45-59)	Kessling et al 1988a
	ApoAI	None	118M (> 55)	Paulweber et al 1988
(P-)	ApoAI	↑NS	103M (> 60)	Wile et al 1989
	ApoAI	↓NS	145MF(53-40)	Ordovas et al 1991
	ApoAI	↓p<0.056	162M (7-23)	Paul-Hayase et al 1992
SstI	ApoAI	↓NS	145MF(53-40)	Ordovas et al 1988
	ApoAI	None	154MF(> 60)	Shoulders et al 1988
(S+)	ApoAI	↑NS	58F (> 20)	Anderson et al 1989
	ApoAI	↓NS	162M (7-23)	Paul-Hayase et al 1992
	ApoCIII	↑p<0.040	154MF(> 60)	Shoulders et al 1991
	ApoCIII	↑NS	162M (7-23)	Paul-Hayase et al 1992
	ApoAIV	↑NS	79MF(> 60)	Shoulders et al 1991
	ApoAIV	↓NS	162M (7-23)	Paul-Hayase et al 1992
PvuII-CIII	ApoAI	↓p<0.025	209MF(8-10)	Xu et al 1990b
(V-)	ApoAI	↓NS	162M (7-23)	Paul-Hayase et al 1992
	ApoCIII	↓p<0.023	162M (7-23)	Paul-Hayase et al 1992
PvuII-AIV	Apo AI	↓NS	92M (45-59)	Kessling et al 1988b
(V+)	Apo AI	↑NS	145MF(53-40)	Ordovas et al 1991
	Apo AI	↓p<0.010	206MF(8-11)	Xu et al 1990b
	Apo AI	↓p<0.035	162M (7-23)	Paul-Hayase et al 1992
	ApoAI	↓p<0.01	204MF (8-11)	Xu et al 1993
	Apo CIII	↓p<0.085	162M (7-23)	Paul-Hayase et al 1992
XbaI-AIV	Apo AI	None	145MF (53-40)	Ordovas et al 1991
	Apo AI	None	162M (7-23)	Paul-Hayase et al 1992
	Apo AIV	None	162M (7-23)	Paul-Hayase et al 1992

(1) M = male, F = female.
(2) PvuII-CIII and PvuII-AIV show strong linkage disequilibrium (eg. Kessling et al. 1988b).
NS Not Significant
↑ Associated with high levels of the trait
↓ Associated with low levels of the trait

association of the G_{-75} to A polymorphism with apoAI levels. Tuteja et al. (1992) demonstrated that, in-vitro, the promoter strength of the G_{-75} allele in a -300 to +1 construct was double that of the A_{-75} allele, but that in a -1469 to +397 construct, the promoter strength of the A_{-75} allele was slightly higher. Thus the effect of the G to A polymorphism in-vitro appears paradoxical. Smith et al. (1992) showed that GA heterozygotes had a lower in-vivo apoAI production rate than did individuals homozygous for the G allele, which might be accounted for by the promoter activity in-vitro of the A allele being only 68% that of the G allele. The G_{-75} to A polymorphism might lead to the formation of DNA secondary structures which might interfere with transcription (Smith et al. 1992). The precise mechanism underlying the association between the A_{-75} allele and higher apoAI and HDL levels in epidemiological studies, still awaits eludication.

Section 1.6 LIPOPROTEIN LIPASE (LPL)

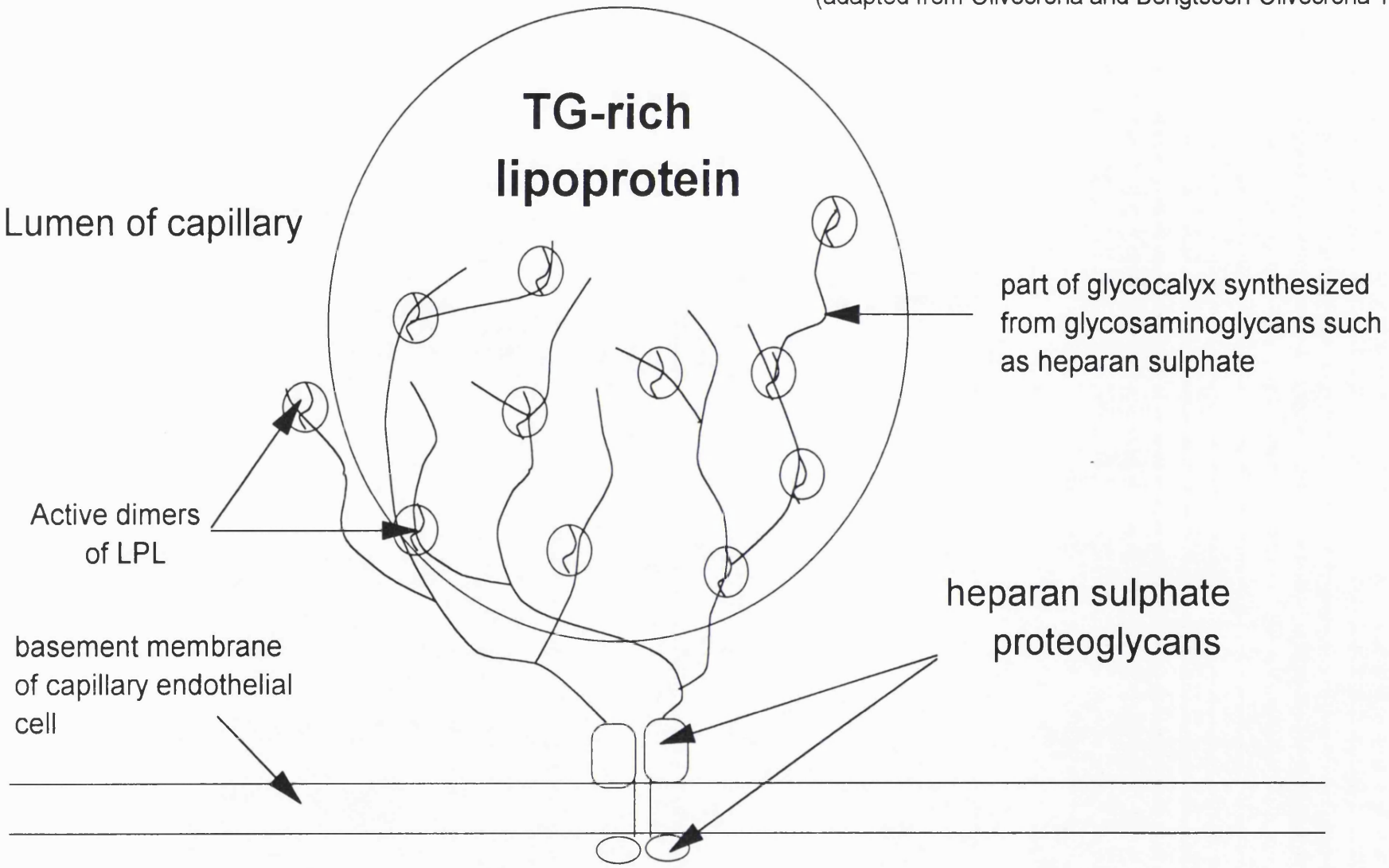
Section 1.6.1 LPL: its role, structure and functional domains

LPL (EC 3.1.1.34) catalyses the hydrolysis of core triglycerides of circulating VLDL and chylomicrons generating fatty acids for storage in adipose tissue or oxidation in muscle, thus producing smaller TG-poor remnant lipoproteins. LPL is therefore critical in plasma lipoprotein metabolism and energy metabolism in general (Olivecrona & Bengtsson 1984, Eckel 1987). The major physiological function of LPL is twofold; to direct TGs (as free fatty acids) into tissues where they either undergo β-oxidation, yielding metabolic energy, or reesterification, to be stored as TGs. LPL is a glycoprotein synthesized and secreted by a variety of parenchymal cells, including adipocytes, skeletal and cardiac muscle cells and macrophages (Nilsson-Ehle 1980, Chait et al. 1982, Mahoney et al. 1982). After secretion, LPL is located on the luminal surface of capillary endothelial cells, via interaction with surface glycosaminoglycan polymers such as heparan sulphate, and upon intravenous administration of heparin, LPL is released into the circulation (Jackson 1983, Brunzell 1989).

Circulating lipoprotein particles attach briefly to LPL at the luminal surface (Figure 1.6.1.1); this allows hydrolysis of the TGs to di- and monoglycerides and free fatty acids (Nilsson-Ehle 1980). LPL preferentially hydrolyzes the 1- and 3- ester bonds in TGs, generating 2-monoglyceride, which is converted to 1-monoglyceride by isomerization for further hydrolysis (Brunzell 1989). The end products of lipolysis are fatty acids and

Figure 1.6.1.1 Schematic model of LPL action at the capillary endothelium

(adapted from Olivecrona and Bengtsson-Olivecrona 1987)



glycerol. LPL is only active as a homodimer (Olivercrona et al. 1985) and requires apoCII activation for optimal activity (LaRosa et al. 1970); apoCII increases basal LPL activity 5-fold (Faustinella et al. 1992). Recently, Peterson et al. (1993) demonstrated, using monoclonal antibodies directed against purified LPL in an ELISA, that loss of activity is closely linked to dimer dissociation into monomer and loss of native conformation, confirming earlier observations that the active enzyme species is a non-covalent homodimer (Olivecrona et al. 1985). Osborne et al. (1985) proposed that active LPL dimer is in equilibrium with LPL monomer of native conformation, but that the monomer form is prone to undergo denaturation and this was confirmed by Peterson et al. (1992).

The mature LPL protein has 448 amino acids and is predicted, from the cDNA sequence, to have a molecular weight of approximately 50.4kDa (Wion et al. 1987). Since bovine LPL is about 8% carbohydrate (Iverius & Ostlund-Linqvist 1976), the molecular weight of the glycosylated monomeric subunit would be predicted to be approximately 54.4kDa (Brunzell 1989). There are 3 potential asparagine-linked glycosylation sites (Asn-X-Ser, Asn-X-Thr) in the LPL sequence (Wion et al. 1987), at Asn43, Asn257 and Asn359 (Kirchgessner et al. 1987). Studies in cultured cells using tunicamycin, an antibiotic that blocks N-linked glycosylation (Elbein 1987), demonstrated decreased LPL activity in adipocytes (Olivecrona et al. 1985) and in macrophages (Stray et al. 1990). This loss of LPL activity was accompanied by the presence of lower molecular weight (unglycosylated) forms of LPL (Olivecrona et al. 1985), indicating that N-linked glycosylation of LPL is required for catalytic activity. Semenkovich et al. (1990) investigated the importance of Asn43 and Asn359, using site-directed mutagenesis; substitution of the N-linked glycosylation site at Asn43, but not at Asn359 resulted in the intracellular accumulation of inactive enzyme. These studies indicate that glycosylation of Asn43 is important for the correct synthesis, secretion and biological activity of LPL.

All the lipases investigated so far share at least four common structural features (reviewed in Lawson et al. 1992). They are 1 or 2 domain α/β proteins, with the catalytic domain containing a central β-pleated sheet of 8-11 strands, with a common core of 5 parallel β strands that can be identified in all structures. The catalytic Serine is contained within a conserved Gly-X1-Ser-X2-Gly motif where X1 is either His or Tyr and X2 is Leu in human

lipases (Derewenda et al. 1991, Smith et al. 1992). This motif is typical of all serine hydroylases (Yang et al. 1989) and seen in virtually all lipases sequenced to date. The high degree of homology in the primary sequence (Hide et al. 1992) and the conservation of most disulphide bonds, of which there are five in human LPL (Kirchgessner et al. 1987) suggest a similar tertiary stucture for LPL, HL (hepatic lipase) and PL (pancreatic lipase). This has allowed the identification of potentially functionally important residues in LPL (and HL) by extrapolation from the 3-dimensional molecular model of PL (Winkler et al. 1990), shown in Figure 1.6.1.2. LPL and HL are folded similarly to PL, with a larger N-terminal and a smaller C-terminal domains; in PL, the 335 amino acid-N-terminal domain is joined by a small spanning region, to the 111 amino acid C-terminal domain (Winkler et al. 1990). LPL has several distinct functional domains; the surface lid mediating interfacial activation (see section 1.6.2), the catalytic triad and sites for apoCII-binding, heparin-binding, glycosylation, dimerization and lipid binding.

The residues, in human LPL, that comprise the catalytic triad are Ser132, Asp156 and His241, determined by sequence alignment with PL [in PL, Ser153 is hydrogen-bonded to His264, which, in turn makes a hydrogen bond with Asp177 (Winkler et al. 1990)]. Site-directed mutagenesis of any one of these LPL catalytic triad residues completely abolished enzyme activity, by comparsion to wild-type enzyme or other LPL mutants which had amino acid substitutions elsewhere (Faustinella et al. 1991a, 1991b, 1992, Emmerich et al. 1992). The site of interaction with apoCII has been localized to the N-terminal domain of LPL; chimeras consisting of the N-terminal domain of LPL and C-terminal domain of HL required apoCII for activation (Dichek et al. 1993), in contrast to the non-requirement of apoCII by HL (Jackson 1983). The importance of apoCII for LPL activity is illustrated by the observation that mutations in the apoCII gene can cause Type I hyperlipoproteinaemia (presence of chylomicrons in fasting plasma, which will be discussed more fully in Section 1.6.5). To date, ten different mutations in the apoCII gene, resulting either in absence or decreased synthesis of abnormal apoCII variants have been reported (reviewed in Santamarina-Fojo 1992). ApoCII deficiency is a rare genetic disorder inherited as an autosomal recessive trait and was first reported by Breckenridge et al. 1978. In constrast to heterozygosity for LPL deficiency predisposing to familial combined hyperlipidaemia, heterozygosity for apoCII deficiency appears, in most cases, to be associated with a normal

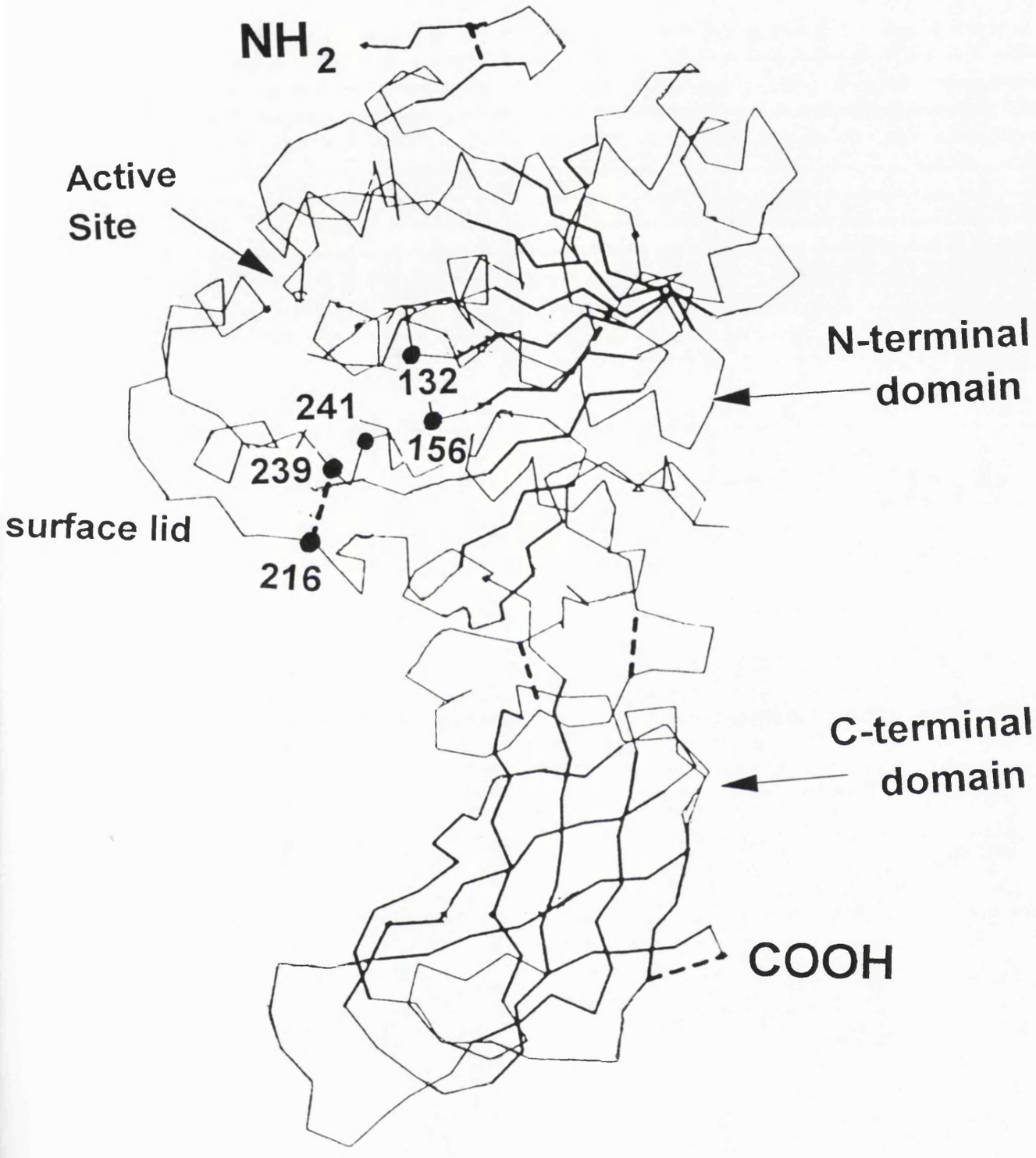


Figure 1.6.1.2 adapted from Santamarino-Fojo (1992)

Representation of 3-D structure of LPL, based on that of PL.

Location of catalytic triad, surface lid and the 5 disulphide bridges in LPL, superimposed on the 3-D structure of PL (taken from Winkler et al. 1990). Numbers denote the two cysteines which form the disulphide bridge holding the surface lid together (216 and 239) and the three catalytic triad residues (Ser at 132, Asp at 156 and His at 241). The β-sheet segments are represented by heavier lines and the α-helices and random structure are represented by lighter lines. The five disulphide bridges are represented by heavy dashed lines.

lipid profile, supporting the contention that half-normal levels of apoCII in plasma are not rate limiting for lipolysis. However truncated mutant apoCII peptides may act as an competitive inhibitor against intact apoCII in the activation of LPL (Wang et al. 1992), since the N-terminal peptide of apoCII can interact with LPL (Cheng et al. 1990) but lacks activation properties (Musliner et al. 1979). ApoCII is discussed further with regard to regulation of LPL activity (Section 1.6.3).

There is still some confusion about the exact localization of the heparin-binding domain(s). Earlier studies, involving proteolytic digestion of LPL, indicated that the region between residues 260 and 330 (which contains 16 basic residues in LPL, but the corresponding regions of HL and PL contain only 10 and 6 basic residues, respectively) was important in heparin-binding (Bengtsson-Olivecrona et al. 1986). However, two naturally occuring mutations which reduced LPL-heparin binding were reported outside this region in the N-terminal domain; A176T (Beg et al. 1990) and G188E (Emi et al. 1990). More recently, experments with chimeras of human LPL and rat HL indicated that heparin-binding avidity was primarily associated with the C-terminal domain (Davis et al. 1992). When C-terminal domains (residues 313 to 448 in LPL) were exchanged between LPL and HL, relative heparin affinities were also exchanged; both HL and the LPL/HL chimera were eluted from heparin-Sepharose by 0.75M NaCl, whereas 1.1M NaCl was required to elute LPL and the HL/LPL chimera, indicating that heparin-binding occurs in the C-terminal. These data strongly suggested that the C-terminal 45 amino acid residues are pivotal in heparin-binding; alignment of the published sequences of LPL, HL and PL shows that 29%, 26% and 10% of the 45 C-terminal residues were Lys or Arg respectively. Affinity of LPL for heparin is stronger than that of HL, and PL does not bind to heparin (Borgström & Erlanson-Albertsson 1984). In another series of experiments on chimeras of human LPL and human HL (N-terminal 314 residues of LPL and C-terminal 146 residues of HL), Dichek et al (1993) found that the affinity of this chimera for heparin was intermediate between LPL and HL, the difference between the results obtained by Davis et al. (1992) and Dichek et al. (1993) is probably attributable to the sequence divergence between rat and human HL at the C-terminus. Dichek and co-workers suggest that three separate sites, spanning residues 260 to 267, 292 to 306 and 441 to 446, in the LPL molecule are required to maintain heparin affinity; all these three sites are rich in basic residues. Another recent study showed, using

site directed mutagenesis of specific residues in the complete LPL molecule, that five basic residues (R279, K280, R282, K296 and R297) in two distinct segments (279-282 and 292-304) of the primary structure of human LPL are critical determinants of the high affinity for heparin exhibited by active LPL (Hata et al. 1993). Replacement of any one of these five basic residues by alanine led to significant reduction in the affinity of LPL for heparin, but this affinity could not be reduced below a threshold, suggesting that other heparin-binding determinants exist elsewhere in the molecule. Overall, these studies suggest that maximal binding of LPL to heparin is achieved by several sites, in both the amino- and carboxyl-terminal domains of the LPL molecule, acting in concert.

Mammalian LPL exists both as a momomer and dimer but is only active as a dimer, and Winkler et al. (1990) described crystals of PL, with two molecules per asymmetric unit. However, fungal lipases which lack the C-terminus domain, are active as monomers (Brady et al. 1990, Schrag et al. 1991). There are two possible models for dimer formation, a head-to-head dimer, where N-terminal and C-terminal domains of both molecules interact and a head-to-tail dimer, where the N-terminal domain of the first molecule interacts with the C-terminal domain of the other molecule and vice versa (Wong et al. 1991). The second model (head-to-tail dimer) is the most attractive since it allows for the interaction of the C-terminal domain of one LPL molecule with the desired substrate, which will bring the substrate close to the catalytic N-terminal domain of the other LPL molecule. Studies with chimeras of LPL and HL strongly indicated that the C-terminal domain modulates substrate specificity. Relative phospholipase activities of the LPL/HL chimera was higher than that of the HL/LPL chimeria [HL has 10-fold higher phospholipase activity than LPL (Rojas et al. 1991)] (Davis et al. 1992). Incubation with an antibody directed against the C-terminus abolished the ability of the enzyme to hydrolyze long-chain TGs but not short-chain TGs (Dichek et al. 1993). These authors proposed that the C-terminal domain is required for the initial interaction with lipid substrate, this interaction would induce the conformational change that opens up the lipase 'lid' (this is discussed in more detail in Section 1.6.2). Other studies with chimerias of LPL and HL and with chymotrypsin-digested LPL, which removed the 58 C-terminal residues reached similar conclusions (Wong et al. 1991, Davis et al. 1992, Lookene & Bengtsson-Olivecrona 1993). The importance of the C-terminal domain in mediating the activity of LPL is reinforced by recent observations, in which a naturally

occuring nonsense mutation generating LPL-381 underlied a case of familial LPL deficiency (Gotoda et al. 1991), confirmed by defective in-vitro secretion and activity of LPL-381 produced by site-directed mutagenesis (Kozaki et al. 1993). Kozaki et al. also observed a stepwise reduction in specific lipolytic activity of in-vitro generated trucated LPL variants, from LPL-437 (68%) to LPL-434 (3%) in culture medium. Whether these particular truncated species of LPL exist in-vivo is not yet known.

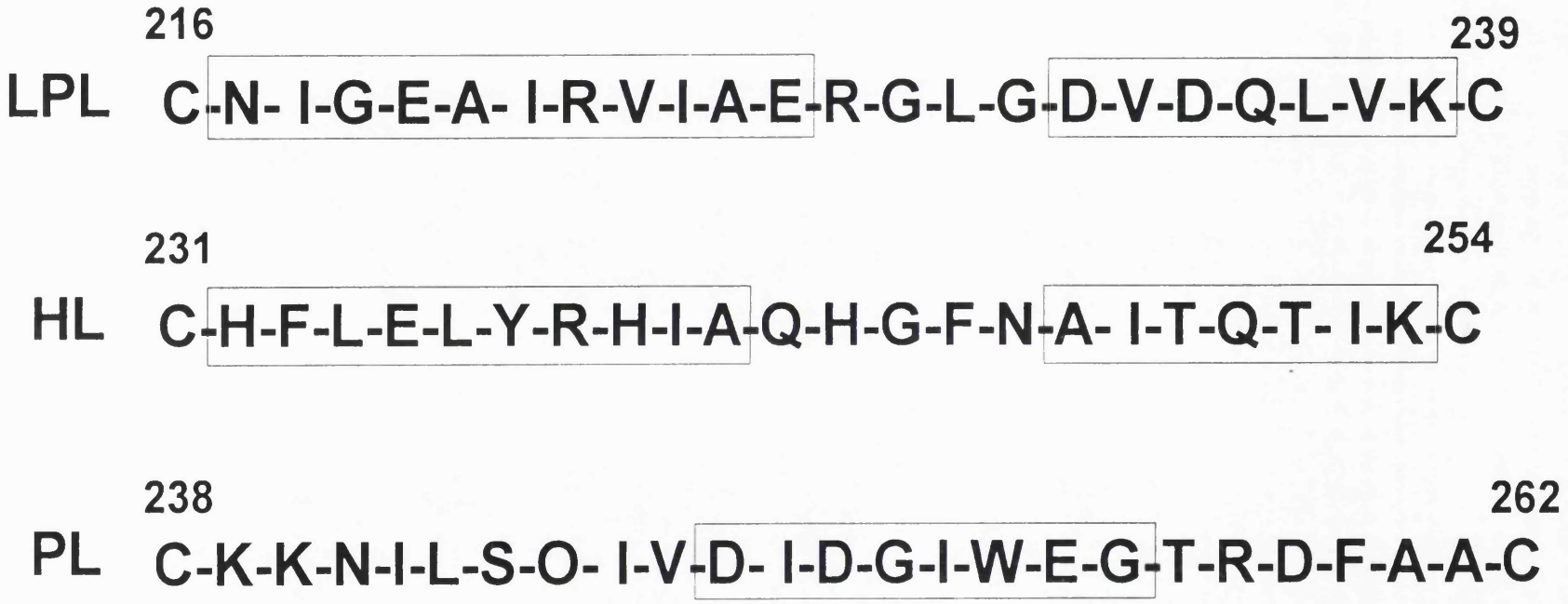
Section 1.6.2 The LPL surface lid and mechanism of Interfacial activation

Sarda & Desnuelle (1958) proposed the involvement of a lipid binding site in LPL, distinct from the catalytic domain, in the interaction of the enzyme with the lipid substrate. Studies by Chapus et al. (1976) demonstrated enhanced hydrolytic activity of LPL in the presence of lipid and established a model of an 'interfacial binding site' for LPL. It was proposed that amino acids 125 to 142 inclusive would form the lipid recognition site of LPL (Wion et al. 1987). However when the 3-dimensional structure of PL was eludicated, this area was found to be buried within the catalytic pocket of the lipase molecule (Winkler et al. 1990), therefore it was concluded that a role of residues 125 to 142 in lipid recognition is unlikely. The 3-dimensional structure of PL revealed a 23-amino acid disulphide-bridged lid covering the catalytic domain, which prevents access of the substrate to the catalytic pocket (Winkler et al. 1990). This amphipathic lid, overlaying the channel leading to the residues of the catalytic triad, is one of the structural motifs that is conserved between fungal and mammalian lipases (Brzozowski et al. 1991, Winkler et al. 1990, Derewenda et al. 1992). The amino acid sequence within the lid region is poorly conserved between LPL, PL and HL, but the adjacent regions are highly conserved (Hide et al. 1992). The lid of human LPL comprises 22 amino acids (217 to 238 inclusive) and is formed by disulphide bridging between Cys216 and Cys239. Lid sequences of the loop region in human lipases are shown in Figure 1.6.2.

Secondary structural analysis of the 22-amino acid lid of LPL identified the presence of two α-helical structures within residues 217 to 238 (Dugi et al. 1992). Helix A contains a stretch of 11 amino acids (217 to 227 inclusive) followed by a four-amino acid β-turn or coil formation. Helix B encompasses amino acids 232 to 238. The two α-helic es are highly amphiphilic, displaying opposite hydrophobic and polar faces, a structural characteristic of

Figure 1.6.2 Lid sequences in human lipases

(adapted from Henderson et al. 1993)



Numbering of residues is from Wion et al. (1987) for LPL, Datta et al. (1988) for HL and Lowe et al. (1989) for PL. The alpha-helicies that are present in the lid when in the closed conformation are boxed.

proteins known to be involved in lipid binding (Segrest et al. 1990). McLean et al. (1991) demonstrated that short amphipathic helices like helix B in the LPL lid are capable of mediating lipid binding. By disrupting the conserved secondary structure of the lid by site-directed mutagenesis, Dugi et al. (1992) established the essential role of the lid in LPL and HL for hydrolysis of long chain fatty acid triglycerides (e.g. triolein) and the requirement of the amphipathic helicies within the loop for lipid interaction. The inactive LPL mutants with part of the lid deleted are predicted to have significant changes in secondary structure, which would prevent appropriate movement and repositioning of the lid (Faustinella et al. 1992).

To date, only one naturally occuring mutation in the lid has been reported for human LPL; this is an I225T substitution, which significantly impairs catalytic activity towards long-chain triacylglycerol substrates (Henderson et al. 1993) due to the replacement of a hydrophobic residue with a hydrophilic one. This substitution lies within a triplet of hydrophobic residues, and presumably abolishes a critical hydrophobic interaction of the lid residues with either the lipid substrate or main part of the N-terminal domain of the LPL molecule. A number of in-vitro generated lid mutants have been reported that have varying effects on LPL activity and mass, depending on the amino acid residue(s) deleted (Dugi et al. 1992, Faustinella et al. 1992, Henderson et al. 1993). These in-vitro mutants are summarized in Table 1.6.2.1.

The retention of significant specific activity in LPL, when the LPL lid is replaced by the HL loop, underlies the high degree of functional similarity between these two lipases (Faustinella et al. 1992). The physiological substrates for both LPL and HL are lipoproteins, the one difference being that LPL almost exclusively acts on large TG-rich lipoproteins while HL acts on both TGs and phospholipids in a wider range of lipoproteins. The diameters of these macromolecular complexes range from 9-12nm for HDL_2 to 1200nm for chylomicrons. In constrast, PL acts on bile salt micelles with a diameter of 7nm (Jackson 1983). Therefore, from the perspective of the lipid-water interface of the respective substrates, the repositioning of the lid region of these lipases and their possible role in substrate binding would be similar for LPL and HL, but quite different for PL (Faustinella et al. 1992). This is underlined by the totally inactive chimeric enzyme produced when the LPL lid is replaced by the PL lid

Table 1.6.2.1 In-vitro generated mutants in the LPL lid

Mutant	Remarks	Reference	Specific secreted activity of LPL towards triolein or tributyrin substrate (as %age of control)	
			Triolein	Tributyrin
Replacement of residues 217-224 with corresponding part of hepatic lipase lid	Secondary structure is unaffected	Faustinella et al. 1992	100	N/D
Complete replacement with hepatic lipase lid	Secondary structure is unaffected	Faustinella et al. 1992 Dugi et al. 1992	62.9 41.4	N/D 64.5
Replacement of residues 217-224 and 231-238 with corresponding parts of pancreatic lipase lid	Replacement with less amphiphilic lid	Faustinella et al. 1992 Dugi et al. 1992	0 0	N/D N/D
Complete replacement with pancreatic lipase lid	Replaces loop with less amphiphilic lid	Faustinella et al. 1992 Dugi et al. 1992	0 0	N/D 75.5
Deletion of 5 amino acids	Shortens length of helicies	Faustinella et al. 1992	0	N/D
Deletion of 11 amino acids	Shortens length of helicies	Faustinella et al. 1992	0	N/D
Only 1st helix of lid changed	Disruption of Helix A	Dugi et al. 1992	1.1	100
Only 2nd helix of lid changed	Disruption of Helix B	Dugi et al. 1992	1.0	87.3
Both helicies changed	Disruption of both helicies	Dugi et al. 1992	0	18.5
(+) charged amino acids at 223 and 238 replaced by (-) charged amino acids	Makes helicies unstable	Dugi et al. 1992	0	100
Deletion of 18 amino acids (219-236)	Removes almost entire lid while preserving the disulphide link	Henderson et al. 1993	0	N/D
Deletion of 8 amino acids (224-231)	Deletes central region of lid while maintaining symmetry and net charge of lid	Henderson et al. 1993	112.5	N/D
Deletion of 2 amino acids (227 and 228)	As above	Henderson et al. 1993	87.1	N/D
I225T	Naturally occuring mutation	Henderson et al. 1993	14.5	N/D
4 different substitutions of Gly229	Gly229 is the only amino acid to be conserved across all three lipases	Henderson et al. 1993	G → K 54.2 G → Q 71.4 G → T 101.7 G → P 105.3	N/D N/D N/D N/D
Replacement of loop by 4 amino acid lid of protein kinase C	Deletes the lipsae lid	Dugi et al. 1992	0	237.7

N/D = not determined. Tributyrin is water-soluble (short-chain triglycerides)

(Faustinella et al. 1992, Henderson et al. 1993) and the divergence of the secondary structure of the PL lid [which has an extended conformation comprising a short apical helix with flanking sections of random coil (Henderson et al. 1993)] from those of the LPL and HL lids, which both contain two highly amphipathic helicies (Dugi et al. 1992).

The phenomenon of 'interfacial activation' (the great increase of catalytic activity in lipases when they come into contact with a lipid/water interface) has been investigated by several groups; in Rhizomucor lipase (Brozowski et al. 1991, Derewenda et al. 1992, reviewed in Smith et al. 1992) and in the human PL-colipase complex (van Tilbeurgh et al. 1993). Earlier studies involving mild proteolytic digestion of LPL with trypsin (Bengtsson & Olivecrona 1981) suggested that an intact lid is essential for normal lipid binding. More recently, digestion of LPL with trypsin, which nicks between residues 230 and 231 within the loop resulted in a selective loss of hydrolyzing activity using triolein but not tributyrin substrates (Persson et al. 1991). Movement of the lid appears to be necessary to allow hydrolysis of lipid substrates; x-ray crystallographic studies recently demonstrated movement of a similar loop in the lipase from the fungus, Rhizomucor meihei (Brzozowski et al. 1991, Derewenda et al. 1992); this lipase was isolated as a crystallized transition state complex with N-hexyl chloro-phosphate ethyl ester, which inhibited the enzyme fully and irreversibly (Brzozowski et al. 1991). An electron density map of the lipase-inhibitor complex, at 3Å resolution revealed that the α-helical lid (residues 85-92) had moved, accompanied by a movement of Trp88 so that it no longer blocked the entrance to the active site. A direct covalent bond was formed between the oxy-anion of Ser144 and the phosphorous atom of the ester. The Ser82 side chain assumes a favourable conformation for oxy-anion interaction only after the lid has moved away from the active site. The helical part of the lid rotates through 167°, and the hinge regions on each side of the lid (residues 82-85 and 92-95) allow this movement. During this rotation, the previously exposed hydrophilic side of the lid becomes partly buried in a polar cavity previously filled by water molecules. Simultaneously, the hydrophobic side of the lid becomes completely exposed, greatly expanding the nonpolar surface around the active site. This marked increase in non-polarity of the surrounding surface accompanies the exposure of the catalytic residues. Interfacial activiation is therefore explained by the stabilization of this non-polar surface by the lipid environment which would effectively create a catalytically competent enzyme, able to attack

the triglyceride molecules within the lipid phase.

PL requires a protein co-factor, colipase to overcome the inhibitory effect of the amphipathic bile salts on binding of PL to the lipid-water interface (Chapus et al 1988). Induction of a conformational change upon binding of pancreatic lipase to the lipid-water interface was anticipated long before the crystal structure of PL was known (Desnuelle et al. 1960). The blocking α-helix (residues 248 to 255) is connected to the body of the PL molecule by two enlongated polypeptide stretches which join through a disulphide bridge (Cys237-Cys261). The opening of the active site is caused by a structurally complicated reorganization of the complete lid between Cys237 and Cys261 (van Tilbeurgh et al. 1993). Movement of the helical lid changes the hydrophobic-hydrophilic balance of the exterior surface of the lipase (Faustinella et al. 1992). The α-helicies that are present in the lid of the closed (in absence of mixed micelles) and open (in presence of mixed micelles) conformations of PL, are formed by different residues. The helix of the closed conformation (residues 248 to 255) partially unwinds and two new helicies are formed (residues 241 to 246 and 251 to 259). In this open conformation, residues 247 to 250 form a tight turn.

The three amino acids that form the catalytic triad in lipases are identical to those in serine proteases. It is known that the presence of an oxy-anion hole assists the functioning of the catalytic triad of serine proteases (Krau 1977). The oxy-anion hole is an electrophilic region that stablizes the negative charge generated during the nucleophilic attack of the carbonyl carbon of the substrate ester bond by the serine oxy-anion (Krau 1977). The active site serine is involved in a charge relay hydrogen-bonded network of Ser...His...Asp (or Ser...His...Glu in G.candidum lipase sequenced by Schrag et al. 1991). The chemistry of ester and amide hydrolysis is very similar (March 1985), the structure of the inferred tetrahedral intermediate complex is reminiscent of that found in serine proteases, with one important difference. In serine proteases, the oxy-anion hole is preformed whereas in the lipase this hole is generated by the interfacial activation process. The stereochemistry of the active atoms in this triad is closely conserved and the residues are always ordered Ser...Asp...His with respect to sequence. The active Serine adopts an unusual conformation, usually only seen in Glycine residues, due to being located on a tight turn between a β strand and a α helix. The mechanism of TG hydrolysis involves a nucleophilic attack by the

activated serine on the carbonyl oxygen of the scissile ester bond to give a tetrahedral enzyme-substrate complex. This tetrahedral complex gives rise to an acyl-enzyme intermediate and releases a diglyceride molecule. The second half of the reaction involves replacement of the diglyceride molecule in the active site by a water molecule and release of a fatty acid molecule.

Section 1.6.3 Regulation of LPL activity by extrinsic factors.

Regulation of LPL is complex and shows tissue specificity, eg. heart and adipose tissue respond differentially to the same physiologic and hormonal signals (Cryer 1981, Garfinkel and Schotz 1987). There are a number of extrinsic factors that regulate LPL activity, eg. obesity, alcohol intake and hormones (reviewed in Taskinen 1987). Nutritional status was the first physiological regulator of LPL activity to be studied in detail, and seems to influence both LPL expression and synthesis. The distribution of enzyme between adipose tissue and muscle appears to be dependent on nutritional status (Lithell et al. 1978). Postprandially, LPL activity is elevated in adipose tissue compared with heart and muscle tissue, resulting in the channelling of circulating fatty acids produced as a result of TG hydrolysis into lipid deposits in adipose tissue. In the fasting state, the reverse situation occurs, with high LPL activity in heart and muscle tissues re-directing fatty acids towards these tissues, away from TG deposits in adipose tissue (Cryer 1981, Nilsson-Ehle 1982). Maintainence of TG homeostasis requires the co-ordinated regulation of LPL activity in tissues that store fatty acids and tissues that oxidize fatty acids during feeding/fasting. The increased TG deposition in obesity may be due to overexpression of LPL activity in adipose tissue (Eckel 1987).

The decrease in LPL activity observed in fat pads of fasting guinea pigs exceeded the decrease in LPL synthesis and LPL mRNA levels (Semb & Olivecrona 1986, 1989), while glucose refeeding intiated a rapid increase in LPL activity with a smaller increase in LPL mRNA (Semb & Olivecrona 1986). Similar results were obtained in human adipose tissue after a carbohydrate-rich meal (Ong & Kern 1989). This suggests that changes in LPL activity upon fasting/feeding are regulated at both transcriptional and post-translational levels (Doolittle et al. 1990). Doolittle and coworkers observed that the reduction in LPL activity in adipocytes of fasting rats occured without a concurrent change in total LPL mass. They

suggested a 'sliding' mass model in which LPL is redistributed from an active endothelial cell pool to an inactive pool in adipocytes, and in which redistribution in the fasting state proceeds by diverting LPL from a secretory pathway to an intracellular degradative pathway. Increased synthesis of inactive LPL precursor would maintain total LPL mass at a constant level. This nutrition-state regulated balance between inactive (high mannose forms) LPL and active LPL does not occur in the cld/cld mouse, which exhibits lipase deficiency not due to mutations in the genes for LPL or HL. Brown adipocytes in these mice synthesize substantial amounts of inactive LPL-protein, which is mostly in the high-mannose form and not secreted (Mashes et al. 1990, Davis et al. 1990). The cld mutation is thought to interfere with LPL and HL oligosaccharide processing, or with the transport of enzyme from the rough ER to the Golgi-system, confirming the importance of glycosylation on LPL generation and activity.

A number of studies have consistently shown significant increases in LPL activity with exercise in human subjects (reviewed in Huttunen 1982). Significant increases in both muscle, adipose and post-heparin LPL activity resulted from endurance training in human subjects (reviewed in Nikkila 1987), with post-training LPL activities up to 10 times greater than basal LPL activities. Many studies however did not control for weight loss during exercise; weight loss independently causes an increase in adipose LPL activity (Kern et al. 1990). Recently, Simsolo et al. (1993) demonstrated a significantly increased ratio of adipose tissue/muscle LPL activity (which may be an important indicator of the tendency for storage of circulating lipids in adipose tissue) following detraining of athletes (short-term changes in exercise). This was due to a decrease in muscle LPL and an increase in adipose tissue LPL, both of which occured through post-translational changes, yielding a condition favouring storage of fatty acids in adipose tissue. The regulation of LPL in adipose tissue and muscle has important implications for the disposal of TGs from lipoproteins. LPL is able to divert TGs to storage in fat or to oxidation in muscle, therefore conditions that tend to increase adipose LPL activity and/or decrease muscle LPL activity would result in a shunting of circulating lipid towards storage in adipose and hence favour the development of obesity (Simsolo et al. 1993). Exercise is believed to be essential for weight-maintenance after weight loss and this could be mediated via a decrease in the adipose/muscle LPL ratio, which would divert more TGs towards muscle for oxidation and less towards adipose for

storage (Simsolo et al. 1993).

Insulin is a major regulator of LPL activity (Pollare et al. 1991). Insulin deficiency is associated with low LPL activity which is increased by treatment with insulin, however the exact mechanism of insulin action in-vivo still awaits eludication. Hypertriglyceridaemia is a common lipid abnormality in patients with non-insulin dependent diabetes (Garg & Grundy 1990). Numerous studies have described decreases in LPL activity in diabetic individuals who are under poor glycaemic control, subsequent treatment elevates LPL activity with a fall in plasma TGs (reviewed in Simsolo et al. 1992). This increase in LPL activity was accompanied by an increase in LPL mass and synthesis but there was no change in LPL mRNA levels, suggesting that glycemic control of LPL occurs at a translational level or through a possible change in degradation (Simsolo et al. 1992). The effects of insulin on LPL in adipose tissue in-vitro are well documented; insulin produces an increase in LPL-antigen on the surface of adipocytes (Ailhaud 1990) and enhances the rate of LPL-release into the medium (Chan et al. 1988, Pradines Figueres et al. 1988). Insulin may act by increasing the half-life of LPL mRNA, since it increases cellular LPL activity, rates of LPL synthesis and LPL mRNA levels in adipocytes without affecting the transcriptional rate (Speake et al. 1985, Raynolds et al. 1990). However posttranslational as well as posttranscriptional mechanisms are implicated by another study which showed that when cultured adipocytes were treated with insulin, LPL activity increased and LPL mass decreased, without a change in LPL mRNA levels (Semenkovich et al. 1989).

LPL displays basal hydrolysing activity, but the presence of apoCII, a physiological activator of LPL (La Rosa et al. 1970) significantly stimulates this activity; purified apoCII was found to stimulate LPL activity 5-fold (Faustinella et al. 1992). The requirement of apoCII for maximal LPL activity probably prevents the expression of lipase activity at its site of intracellular synthesis (Wang et al. 1992). There is apoCII enrichment of TG-rich lipoproteins due to transfer from HDL to the surface of chylomicrons and VLDLs soon after secretion of lipoproteins into the plasma compartment (Havel et al. 1973). It has been suggested that the lipoprotein particle rich in both apoCII and TGs attaches to the endothelium via its interaction with LPL. Lipolysis may be terminated by the disappearance of apoCII from the TG-rich lipoprotein; this seems to coincide with the appearance of

lysophosphatidylcholine in the lipoprotein particle and consequently with reduced affinity of apoCII for the lipoprotein surface (Windler et al. 1986). ApoCII deficiency is clinically indistinguishable from LPL deficiency (Breckenridge et al. 1978). However heterozygotes for apoCII deficiency do not exhibit HTG (Cox et al. 1978). The HTG induced by apoCII deficiency can be relived by supplementation of about 10% of the normal plasma level of apoCII (Breckenridge et al. 1978). These observations suggest that apoCII is not rate limiting for LPL-mediated lipolysis in the normal situation. The C-terminal tetrapeptide, Lys-Gly-Glu-Glu, of apoCII has been implicated in mediating the initial ionic interaction between apoCII and LPL (Cheng et al. 1990). The N-terminus of LPL has been suggested to be the location of the apoCII binding domain (Davis et al. 1992). Recently the Lys147-Lys148 domain in LPL was implicated to be the ionic apoCII binding site; site-directed mutagenesis of these two Lys residues to Ala produced a mutant LPL that showed a 35% reduction of apoCII-activation compared to either wild type LPL or two other mutants in which two adjacent basic residues (at residues 279 + 280 and 445 + 446) were replaced by alanine (Bruin et al. 1994a).

In individuals with hyperlipidaemia, apoCIII levels were found to be significantly postively correlated with the plasma LPL-inhibition activity and apoCIII was identified as one of the most important plasma factors involved in the regulation of LPL activity (Wang et al. 1985). More recently, studies showed that overexpression of apoCIII was a direct cause of the hypertriglyceridaemia observed in transgenic mice (Ito et al. 1990), further substantiating the suggestion of a direct relationship between apoCIII levels and HTG and a modulating effect of apoCIII on LPL activity. The concentration of apoCIII required for 50% inhibition of LPL activity in-vitro decreased when apoCII was also present, suggesting that apoCII actually increased the affinity between LPL and apoCIII, without involving apoCIII competing for the activator site of apoCII (McConathy et al. 1992), consistent with previous observations that apoCIII is a non-competitive inhibitior of LPL. ApoE has also been found to exhibit inhibitory activity (Wang et al. 1981, McConathy & Wang 1989), but its correlation with plasma LPL inhibitory activity may be masked by the stronger correlation between apoCIII and LPL activity (Wang et al. 1985). The requirement for high concentrations of apoCIII and apoE to inhibit LPL (Wang et al. 1985) is likely to ensure the unhindered lipolysis of TG-rich lipoproteins in the vascular compartment of normolipidaemic

individuals and apoCIII and apoE will exert their effect only when plasma TG concentration is high (Wang et al. 1992). Since the increase of TG is associated with increased levels of apoCIII and apoE, it is likely that these apolipoproteins are the signals and modulators responsible for the in-vivo inhibition of LPL activity (Wang et al. 1992). A study on transgenic mice provided direct evidence that overexpression of apoCIII can be a primary cause of hypertriglyceridaemia.

The binding of LPL to the endothelium is thought to be weakened by local fatty acid accumulation resulting from lipolysis of plasma triglycerides. This has been indicated in a study of healthy men, in whom the lipolytic system has been overloaded by infusion of triglyceride emulsions, resulting in a substantial increase in levels of free fatty acids in the plasma of some of the individuals, which correlated closely to a rise in plasma LPL activity (Olivecrona & Bengtsson-Olivecrona 1990, Peterson et al 1990). Peterson et al. (1990) suggested that in situations when free fatty acids are generated more rapidly by LPL than they are used by the local tissue, these fatty acids cause dissociation of the enzyme from its binding to endothelial heparan sulphate, releasing the LPL enzyme-lipoprotein complex into the circulation. Excess of free fatty acids released from TG-rich lipoproteins have therefore been proposed to exert feed-back control of lipolysis, due to dissociation of LPL from its endothelial binding sites. These suggestions were supported by experiments on cultured endothelial cells (Saxena et al. 1989). Significant positive correlations have been found between postprandial LPL response and free fatty acids derived directly from lipolysis of postprandial TG-rich lipoproteins, indicating that the magnitude of response was similar for LPL and postprandially derived fatty acids (Karpe et al. 1992). This is evidence for a simultaneous and possibly coordinated metabolism and supports the hypothesis of free fatty acid control of endothelial LPL under physiological conditions in humans (rate of lipolysis of TG-rich lipoproteins can be partly regulated by accumulating free fatty acids that dissociate LPL from its endothelial binding sites). The synergistic effects of apoCII and albumin allow the optimium activiation of LPL for the hydrolysis of long-chain triacylglycerols (Wang et al. 1993), due to the combination of apoCII-mediated activiation of LPL (La Rosa et al. 1970) and the transfer of fatty acids to albumin in plasma. The requirement for serum albumin in the LPL-catalyzed reaction is well documented (Wang et al. 1990) and the rate of transfer of fatty acid to albumin has been shown to be much faster

than the chemical cleavage step of catalysis (Foster & Berman 1981, Wang et al. 1990).

Section 1.6.4 The LPL gene and its regulation

The human LPL gene is localized in the p22 region of chromosome 8 (Sparkes et al. 1987). The structure and organization of the LPL gene in human (Deeb & Peng 1989, Kirchgessner et al. 1989, Oka et al. 1990) and in other mammalian species; bovine (Senda et al. 1987), guinea pig (Enerback et al. 1989) and rat (Brault et al. 1992) has been elucidated. The human LPL cDNA sequence was elucidated (Wion et al. 1987, Kirchgessner et al. 1987), and cloning and characterization of the LPL gene followed shortly afterwards (Deeb & Peng 1989, Kirchgesser et al. 1989). The human LPL gene spans about 30Kb and comprises 10 exons coding for a 475-amino acid protein (Deeb & Peng 1989, Kirchgessner et al. 1989). Exons 1 to 9 are of average size (105-276bp) whereas exon 10, which specifies the entire 3' untranslated sequence is 1948bp in length. Exon 1 encodes 188 nucleotides of the 5' untranslated region, the 27-residue signal peptide and the first 2 amino acids of the mature protein. Table 1.6.4 is a summary of the characteristics of the LPL gene (Deeb & Peng 1989, Kirchgessner et al. 1989), and a schematic representation of the human LPL gene is shown in Figure 1.6.4.

There are two alternative polyadenylation signals (sequence AATAAA) where the poly(A) tail is attached to the mRNA, starting at nucleotides 3126 and 3522, respectively, and separated by 395 nucleotides, in exon 10; this accounts for the two distinct species of LPL mRNA (about 3350 and 3750 nucleotides long) produced in approximately equal amounts in different tissues (Wion et al. 1987). The human cDNA sequence predicts a translated molecular weight of 50.4kD for the mature LPL monomer (Wion et al. 1987).

The sequence of the 5' flanking region of the LPL gene up to 730 nucleotides upstream from the CAP site was determined (Kirchgessner et al. 1989). The most frequently used inititation site is 188bp 5' of the translation inititation codon (CAP site). Within this reported 5' flanking sequence, there are several potential binding sites for known transcriptional factors, which may modulate LPL gene expression, have been identified. All nucleotide numbers given are the 5'-most nucleotide relative to the CAP site. The TATA box is at -27, and two CCAAT motifs, which are potential binding sites for the transcription

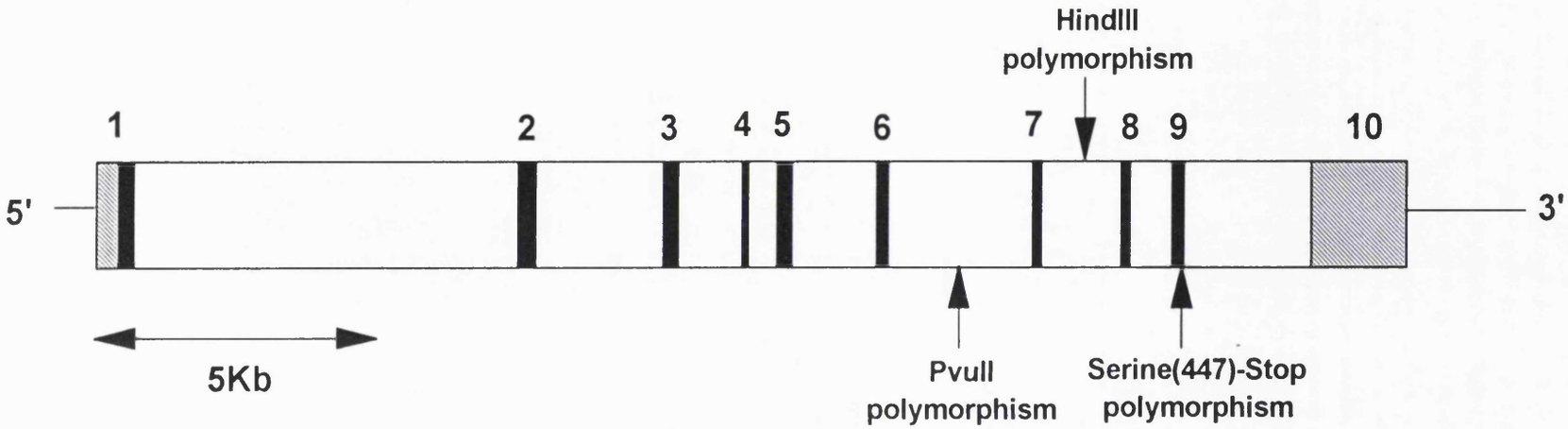
Table 1.6.4 Characteristics of the LPL gene

Exon	Exon size (bp)	Intron phase	codons interrupted	3' Amino acid encoded by exon *	No. of amino acids encoded	Approximate size of intron (in Kb)
1	276	I	C↓AA	Gln3	29	9.2
2	161	0	ACG↓GTA	Thr56	54	4.3
3	180	0	GAG↓GAG	Glu116	60	0.9
4	112	I	ACT↓GGC	Thr153	37	0.8
5	234	I	G↓AT	Asp232	78	1.9
6	243	I	C↓TC	Val313	81	2.8
7	121	II	CT↓G	Leu353	41	1.6
8	183	II	AA↓G	Lys414	61	0.9
9	105	II	TG↓A	Gly448	34	3.1
10	1948	-		-	-	

*numbering of amino acid residues as in Wion et al. 1987

Intron phase; introns in phases I, II and 0 interrupt coding sequence after the first, second and third nucleotides, respectively, in a codon.

Figure 1.6.4 Schematic Representation of the human LPL gene



The transcribed region of the gene is represented by the rectangle; with filled and shaded boxes being translated and untranslated regions respectively. 5' and 3' untranscribed regions of the gene are shown by lines. Exons are numbered 1 to 10 and scale of diagram is shown. The approximate positions of the three polymorphisms that are discussed further in Section 1.6.6.

factor NF-1, are at -506 and -65 (Dorn et al. 1987). Two octonucleotide sequences, ATTTGCAT, at -580 and -46, have been identified (Kirchgessner et al. 1989, Hua et al. 1991, Previato et al. 1991). This octanucleotide sequence is also found in immunoglobulin gene promoters (Parslow et al. 1984) and is a potential binding site for known octamer transcription factors (Singh et al. 1986). Previato et al. (1991) showed that the ubiquitous nuclear factor Oct-1 (likely to be important in modulating LPL expression in adipocytes) binds specifically to the octamer sequence at -46. Sequences that share partial homology with the consesus binding site for C/EBP are located at -509 and -68 (Previato et al. 1991). C/EBP is a well known transcription factor that modulates the expression of liver- and adipocyte-specific proteins during differentiation (Metzger et al. 1990, Umek et al. 1991). However C/EBP may not play a major role in the modulating LPL expression in adipocytes (Previato et al. 1991).

There is a Ca^{2+}-responsive motif (TGAGGTTT) at -54, which may allow transcriptional activation in response to intracellular Ca^{2+} mobilization (Sheng et al. 1988) and a GAGAGGA motif at -355, also found in the 5' upstream regions of murine genes coding for adipsin, adipocyte P2 protein and glycerophosphate dehydrogenase, that are transcriptionally induced as cells differentiate into adipocytes in the mouse (Distel et al. 1987). This fits in with the strong expression of the LPL gene in adipocytes (Wion et al. 1987). Nucleotide sequences with partial identity with the hexanucleotide portion of the glucocorticoid-responsive element (Scheidereit & Beato 1984) and the cAMP-responsive element (Rupp et al. 1990) are present at -644 and -306, respectively. Enerback et al. (1992) identified two cis-regulatory elements in the human LPL promoter; LPα spanning from nucleotides -702 to -666 and LPβ spanning from nucleotides -468 to -430; both LPα and LPβ are important for the gradual activation of the LPL gene during adipocyte differentiation.

Previato et al. (1991) studied the region up to 730bp upstream from the transcription initiation site and they established that LPL expression in adipocytes is modulated by multiple positive and negative regulatory elements in the 5' flanking region of the LPL gene. The presence of tissue-specific regulatory elements in the -724 to -368 region was also demonstrated, and deletion of this region caused an increase in LPL promoter activity. They also demonstrated the presence of proximal elements that modulate LPL expression in the

-232 to -35 region; deletions in this region caused a 65% decrease in transcriptional activity in both adipocytes and hepatocytes in culture. Glucocorticoid receptor elements (at -644), the fat-specific element 2 (identified in promoters of genes expressed during adipocyte differentiation (Phillips et al. 1986) and thyroid response element are found in the LPL promoter of all species (Enerback & Gimble 1993). It has been suggested that the 5' untranslated region of the LPL gene is involved in post-translational control of LPL by agents such as insulin and thyroid hormone.

The lipase gene family includes three "vertebrate" specific genes (coding for LPL, HL and PL) and three "invertebrate" genes coding for the Drosophila Yolk proteins (YP 1,2,3), all thought to be dervived from a common ancestral gene. The six proteins share significant amino acid sequence homology to one another, though this homology is limited between the lipases and YPs (Hide et al. 1992). The amino acid (aa) sequences of all the published lipase sequences can be readily aligned. The relatedness among the lipases was deduced and a phylogenetic reconstruction proposed; this model suggests that LPL and HL share a more recent ancestor and PL has branched off earlier that LPL and HL (Hide et al. 1992). HL from different mammalian species is much less conservative that LPL (Datta et al. 1988, Kirchgessner et al. 1989). This is demonstrated by the aa sequence divergence between human and rat HL of 23.6%, but only 2.2% for human and mouse LPL (Hide et al. 1992) i.e. LPL has evolved much more slowly than HL in the higher vertebrates, illustrated by a conservation index of 1.0 along most of the LPL sequence from human and mouse. Human and canine PL have a sequence divergence of 26.8%.

Kirchgessner et al. (1989) compared the sequence of the genes encoding human LPL, human HL, canine PL and Drosophila YP-1, and revealed a close similarity in the structures of the LPL and HL genes, which are more similar to one another than to PL. Both LPL and HL genes have 10 exons interrupted by 9 introns in identical locations and phases (introns 2, 4, 6, 7, 9 and 11-14 of the primordial gene) but the PL gene has 13 exons and 12 introns. These authors proposed a model for the evolution of the lipase gene family, in which the primordial gene had 14 introns. After early duplication, there was a loss of introns 1,3,5,8 and 10 followed by a second duplication giving rise to the mammalian LPL and HL genes. Introns 4 and 14 were lost from the primordial gene to give rise to the mammalian PL gene

and according to their model, the PL gene most resembles the primordial gene. Introns 1, 3, 5, 8 and 10 of the PL gene have no counterpart in the LPL or HL genes. These unpaired introns include some that result from intron-loss or intron-gain events and one (intron 2) that may be due to a process called 'intron-sliding' - this is when new splice donor or acceptor sites are created within exons or introns during evolution, replacing pre-exisitng sites. This change results in a shift of intron position between related genes with an insertion or deletion of coding sequence (Kirchgessner et al. 1989). In the third path taken after early duplication of the primordial gene, there was an addition of an exon encoding a tyrosine sulphation site, the loss of introns 1,3-11 and those mediating the exon-shuffling event plus further gene duplications giving rise to YP-1, YP-2 and YP-3.

Section 1.6.5 Instrinsic factors affecting LPL activity

Familial LPL deficiency leading to Type I hyperlipoproteinaemia (HLP) is a rare disease with an autosomal recessive mode of inheritance and a frequency of about 1 in 1 million and usually manifests itself in childhood (reviewed in Brunzell 1989). Most of the reported missense and nonsense mutations result in severe Type I HLP (the chylomicronemia syndrome), due to absent post-heparin LPL activity, which is characterized by a massive accumulation of chylomicrons in post-absorptive plasma and a corresponding increase of plasma TG concentrations (2000mg/dl or higher) accompanied by one or more other symptoms such as eruptive xanthomata, lipaemia retinalis and pancreatitis; often these three symptoms occur together. Type I hyperproteinaemia is not associated with atherosclerosis, but is often accompanied by recurrent pancreatitis which may be life-threatening.

Auwerx et al. (1989) put LPL defects causing Type I HLP that are due to changes in amino acid sequences, into three classes; class I defects are those where neither LPL activity nor mass are released into plasma upon heparin adminstration, and class II and III defects are those resulting in catalytically defective LPL that is heparin-releasable and non heparin-releasable, respectively. LPL activity can be affected by changes within the primary structure of LPL, which are due to missense or nonsense mutations in the gene for LPL. Most instances are missense mutatations leading to an amino acid substitution (reviewed in Lalouel et al. 1992, Henderson et al. 1993); these show clustering in exons 3, 4, 5 and 6, which correspond to the most highly conserved domains of LPL. Only two mutations

directly involving the catalytic triad residues have been reported, both involve Asp156, replaced by Gly or by Asn (Faustinella et al. 1991a, Ma et al. 1992). Recently a naturally occuring mutation within the LPL lid region (I225T, Henderson et al. 1993) was reported; see section 1.6.2. Several nonsense mutations occur in exon 3 at Tyr61 (Gotoda et al. 1991), Tyr73 (Wilson et al. 1993) and Gln106 (Emi et al. 1990). A frameshift mutation, due to a 5bp insertion was reported at residue 102 in exon 3 (Henderson et al. 1990), which causes LPL deficiency. Nonsense mutations are reviewed in Lalouel et al. 1992. Two mutations leading to abnormal LPL mRNA splicing, due to G to A substitutions in the conserved splice site have been identified, one is a splice donor mutant in intron 2 (Gotoda et al. 1990) and the other is a splice acceptor mutant in intron 2 (Hata et al. 1990). Three major rearrangements of the LPL gene, a 6Kb deletion, involving exons 3 to 5, a 2Kb insertion (due to duplication of exon 6) (Langlois et al. 1989, Devlin et al. 1990) and a 3Kb deletion involving exon 9 (Benlian et al. 1991) resulting in no detectable post-heparin plasma LPL activity have been reported.

Almost all amino acid substitutions identified so far in the LPL gene are in buried or partly buried residues, therefore it is likely that the inactivation of mutant LPL occurs through unfolding of the protein rather than inactivation of the catalytic centre, indicating that the LPL molecule may be labile and sensitive to changes in the packing patterns of amino acid side chains (Derewenda & Cambillau 1991). Most of the missense mutations leading to inactive LPL are distributed over the amino-terminal domain (Lalouel et al. 1992).

Several 'less severe' LPL defects are known, requiring a 'second hit', either genetic or environmental, for the manifestation of chylomicronaemia and pancreatitis. The chance to identify less severe mutations in the LPL gene is much lower compared to more severe mutations, because individuals only have moderately elevated TG levels in the absence of a 'second hit'. At least some of these milder variants may encode amino acids on the surface of the LPL enzyme rather than in buried residues, therefore the extent of misfolding of the LPL protein would be smaller. The first example was homozygosity for S172C interacting with an increased level of estrogen during pregnancy to markedly increase TG levels (Ma et al. 1993b). Normal pregnancy is associated with alterations in lipid levels, this physiological hypertriglyceridaemia is believed to be induced by an increased level of estrogen during

pregnancy (Knopp et al. 1982). The female proband had mildly elevated TG levels (3.4mmol/l), due to a defective LPL with 10-20% residual catalytic activity, so was not diagnosed as having LPL deficiency until she developed pancreatitis during pregnancy, when TG levels increased to 22.5mmol/l (Ma et al. 1993b). Another example was nutritional stress or alcohol abuse interacting with compound heterozygosity for V69L and G188E to bring about hyperchylomicroaemia and pancreatitis (Bruin et al. 1994b). Other environmental factors, like a low fat and a healthy life style, are suggested to mitigate the expression of Type I HLP in childhood (Bruin et al. 1994b).

Most of the missense mutations in the LPL gene occur uniquely in unrelated families and distinct population groups. However some, like the G188E and P207L substitutions account for a high percentage of LPL defects in certain populations e.g. the French Canadians in Quebec, suggesting the presence of a founder gene (Monsalve et al. 1990, Ma et al. 1991, Normand et al. 1992). The P207L substitution accounts for 73% of mutant LPL alleles in Quebec and the estimated heterozygote carrier rate is 1 in approximately 210 (Normand et al. 1992). Over 30 different defects in the LPL gene are known, all give clues to the importance of certain domains and sequences in LPL. Without doubt, more will be discovered in the near future, with site-directed mutagenesis on known and undiscovered defects being used in the eludication of functional roles of affected domains and sequences.

Section 1.6.6 Common DNA polymorphisms in the LPL gene

Several polymorphisms in the LPL gene have been reported. The common ones are the PvuII polymorphism in intron 6 (Fisher et al. 1987), the HindIII polymorphism in intron 8 (Heizmann et al. 1987), the BamHI polymorphism 3' to exon 10 (Fisher et al. 1987, Heizmann et al. 1991), the C to G tranversion in nucleotide 1595 of the cDNA sequence (the Serine447 to Stop polymorphism) in exon 9 causing production of a protein truncated by 2 amino acids (Hata et al. 1990) and the tetranucleotide repeat $(TTTA)_n$ polymorphism in intron 6 (Oka et al. 1989). Less well-known polymorphisms, some of which the localization has not yet been determined, are the BstNI (EcoRI) in intron 8, which shows almost complete linkage disequilibrium with the HindIII polymorphism (Funke et al. 1988, Groenemeyer et al. 1993), BstI (Li et al. 1988), Bg1II (Hegele et al. 1989), XbaI in intron 9 (Heizmann et al. 1991), PstI and TaqI (Hegele et al. 1989) polymorphisms, dinucleotide

$(CA)_n$ repeat polymorphisms at two distinct loci within the LPL gene (Narcisi et al. 1993) and an A to C transversion in intron 3 (Gotoda et al. 1992). All these, with the exception of the XbaI polymorphism, occur in Caucasians. The XbaI polymorphism has only been reported in US blacks in which the rare allele occurs at a very low frequency (Heizmann et al. 1991). Of these, the PvuII, HindIII and Serine/Stop polymorphisms have been studied most extensively and these will be the only three discussed in the next two sections.

Table 1.6.6 shows the frequencies of the rare alleles of the PvuII, HindIII and Serine to Stop polymorphisms in healthy individuals; the frequency of the V- allele is about 0.45 to 0.55 in most Caucasians, but noticeably higher in Blacks (Heizmann et al. 1991) and lower in Japanese individuals (Chamberlain et al. 1989, Gotoda et al. 1992). The frequency of the H- allele is more consistent across different ethnic origins. Strong linkage disequilibrium has been reported between alleles of the PvuII and HindIII polymorphisms (Chamberlain et al. 1989, Ahn et al. 1993a) but not in US black or Mediterranean populations (Heizmann et al. 1991) and between the HindIII and Serine to Stop polymorphisms (Stocks et al. 1992), with the Stop allele being only found on the same chromosome as the H- allele. This suggests that little or no recombination has occured within the LPL gene in evolutionary history. The presence of strong linkage disequilibrium in Caucasian or Hispanic population samples means that combining any two polymorphisms does not significantly increase their informativeness for association studies.

Section 1.6.7 Associations of common LPL polymorphisms with fasting levels of lipids, lipoproteins and insulin and with atherosclerosis.

The PvuII, HindIII and Serine447 to Stop polymorphisms have been used in several association studies (references given in Tables 1.6.6 and 1.6.7), and as expected from the physiological function of LPL, the primary association of LPL genotypes is with TG and HDL-cholesterol levels. In healthy individuals, the largest and most consistent effect seen was between genotypes of the HindIII polymorphism and plasma TG levels, usually with smaller effects seen on levels of HDL-cholesterol (Table 1.6.7). This is in concordance with the effect of LPL on HDL levels being indirect via transfer, to the HDL fraction, of surface components from TG-rich lipoproteins that have been acted upon by LPL (Patsch 1987).

Table 1.6.6

Frequencies of rare alleles of PvuII and HindIII polymorphisms in the LPL gene in healthy individuals reported over the past five years

Ethnic origin (nos. of individuals in brackets)		Rare allele of polymorphism in LPL gene		
	Reference	V- (PvuII)	H- (HindIII)	$Stop_{447}$
Caucasian (172)	Chamberlain et al. 1989	0.46	0.41	-
Japanese (82)	"	0.28	0.34	-
Utah (86)	Hata et al. 1990	-	-	0.17
Caucasian (122)	Thorn et al. 1990	0.45	0.42	-
Caucasian (189) *	Heizmann et al. 1991	0.46	0.27	-
Mediterranean (132)	"	0.42	0.24	-
Afro-Carribeans (78)	"	0.78	0.32	-
Japanese (70)	Gotoda et al. 1992	0.24	0.27	-
Caucasian (151)	Stocks et al. 1992	-	-	0.11
Caucasian (539)	Ahn et al. 1993a	0.55	0.26	-
Hispanic (384)	"	0.54	0.22	-
Russian (90)	Stepanov & Lemza 1993	0.52	-	-
Caucasian (1074)	Humphries et al. (in preparation)	-	0.30	-

Ethnic group	V- (PvuII) Weighed mean (range) No. of individuals	H- (HindIII) Weighed mean (range) No. of individuals
All Caucasians	0.498 (0.42 - 0.55) n = 1244	0.299 (0.24 to 0.41) n = 2228
All Hispanic	0.54; n = 384	0.22; n = 384
Japanese	0.262; n = 152	0.308; n = 152
Afro-Carribeans	0.78; n = 78	0.32; n = 78

* combined sample of healthy individuals and individuals undergoing elective coronary arteriography

Table 1.6.7 shows the reported associations observed with the PvuII and HindIII polymorphisms in healthy individuals. These findings suggest that the H+ allele is associated with a variant in or near the LPL gene that predisposes to hypertriglyceridaemia, but the underlying mechanism is not yet known. That the haplotype V+H+ may be associated with a particularly atherogenic lipoprotein profile, in healthy individuals compared to other haplotypes, is suggested by the associations between the V- or H- alleles and lower TGs or higher HDL-cholesterol (Table 1.6.7). However none of the population-based studies have studied associations between LPL haplotypes and lipids; small sample sizes, high linkage disequilibrium and high degree of heterozygosity, particularly for studies of the PvuII polymorphism have, so far, precluded attempts to detect such associations. Associations have also been found between genotypes of the HindIII polymorphism and total cholesterol levels but these have been less consistent, with homozygosity for the H- allele being associated both with higher levels ($p<0.03$, Heizmann et al. 1991) and (non-significantly) lower levels (Cole et al. 1993).

Significant associations have been reported between genotypes of the PvuII (Ahn et al. 1993a) and HindIII (Cole et al. 1993) polymorphisms and fasting insulin levels in normoglycemic Hispanic males, with the V- and H- alleles being associated with higher levels, and a clear gene dosage effect was observed; this effect was not seen in the females. These authors suggest that the regulation of LPL by insulin may be different in Hispanics with different PvuII genotypes. The frequency of the H+ allele (0.86) was significantly higher in individuals with insulin resistance syndrome (IRS), which is the co-occurrence of sustained hyperglyceridaemia, dyslipidaemia (↑TG and ↓HDL-cholesterol) and hyperinsulinaemia (DeFronzo et al. 1991, Cole et al. 1993b). Ahn et al. (1993b) speculated that the H+ allele may be associated with decreased sensitivity of LPL to insulin regulation or leads to the expression of LPL protein with reduced activity. This would explain the higher TG levels observed in IRS. The reduced HDL-cholesterol levels would arise from reduced remodelling of circulating TG-rich particles by LPL enzyme encoded by the H+ allele. Alternatively the H+ allele encodes LPL enzyme with elevated activity, leading to increased storage of TG in adipocytes, a decrease in adipocyte insulin sensitivity and a resultant increase in insulin secretion. Ahn et al. (1993b) suggest that expression of IRS is affected by genetic variation at the LPL locus, i.e. that LPL is an important candidate gene

Table 1.6.7 - Associations observed with genotypes of the PvuII and HindIII polymorphisms in the lipoprotein lipase gene in healthy individuals.

Effects of the rare alleles (no cutting site) V- (PvuII) and H- (HindIII) are given

Reference	Sample (age in years; ranges or mean ± SD given)	HDL-cholesterol		Plasma triglycerides	
		V-	H-	V-	H-
Chamberlain et al. 1989	93M (48 ± 12)	↑ NS	↑ NS	↓ $p<0.02$	↓ NS
Thorn et al. 1990	122M (48 ± 12)	not given	not given	not given	not given
Heizmann et al. 1991	189MF (33-70)[1]	none	↓ $p=0.015$	↓ NS	↓ NS
Stocks et al. 1992	149M (40-65)	not given	not given	not given	not given
Ahn et al. 1993a	923MF (20-74)	none	↑ $p=0.005$	↓ NS	↓ $p<0.001$
Cole et al. 1993	101M	None	None	↑ NS	↓ NS
Humphries et al. (in prep.)	571MF (18-26)[2]	not given	↑ NS	not given	↓ $p<0.01$
Humphries et al. (in prep.)	1074MF (18-26)[3]	not given	↓ NS	not given	↓ NS

M = males; F = females

[1] combined sample of healthy individuals and individuals undergoing elective coronary arteriography
[2] offspring of myocardial infarction survivors (father had vertified heart attack before 55 years of age)
[3] offspring of controls who did not have a vertified heart attack.

for the pathogenesis of IRS, but this requires confirmation by other epidemiological studies.

The frequency of the H+ allele of the HindIII polymorphism was significantly increased in HTG individuals compared to controls of both Japanese and Caucasian origin (Chamberlain et al. 1989). These authors also found that healthy men who were homozygous for V+ had higher plasma TGs than did those lacking the V+ allele. Two studies found that the Stop447 allele was significantly less frequent in HTG individuals compared to controls (0.04 in Hata et al. 1990 and 0.05 in Stocks et al. 1992). These authors proposed that this allele would be protective against the development of HTG. Studies have reported different allele frequencies between patients with CAD and healthy individuals. The H+ allele was significantly more common in Causcasian individuals with severe atherosclerosis compared to healthy controls (0.77 vs. 0.58, $p<0.001$) (Thorn et al. 1990). In-vitro expression assays with the LPL protein (LPL-446) truncated by 2 amino acids, coded for by the Stop allele, have produced conflicting results; LPL-446 had a full catalytic activity (Faustinella et al. 1991), but had 45% of normal lipolytic activity in another study with long-chain triacylglycerols (Kobayashi et al. 1992), leading these authors to suggest that LPL-446 may have altered lipid binding properties (altered lipid interface recognition ability) and thus substrate specificity. However, more recently, LPL-446 was found to have almost 2-fold higher specific activity in-vitro than wild-type LPL, reproducible in nine experiments (Kozaki et al. 1993). These discrepant results are most likely to be due to variations in the assay substrates and conditions used in the different studies. Further studies are required to eludicate more precisely the specific activity of LPL-446 towards different substrates.

Section 1.6.8 LPL and atherosclerosis

Zilversmit (1979) proposed, in the 'atherogenic remnant' hypothesis, that LPL may promote atherosclerosis via the production of cholesteryl-ester (CE) rich remnant lipoproteins which are deposited in the artery wall. Evidence has accumlated that LPL may be important in the atherogenesis process via several different mechanisms. Corey & Zilversmit (1977) showed, in rabbits, that LPL activity is low in normal arteries, which have few macrophages, and increases during progression of the atherosclerotic plaque, which contains large numbers of macrophage-like cells. LPL seems to cause CE and TG accumulation in macrophages via facilitation of uptake of TG-rich lipoproteins and remnants (Lindqvist et al. 1983). LPL is

synthesized and secreted in-vitro by macrophages (Mahoney et al. 1982) and has been identified as a constitutive secretory product of macrophages (Khoo et al. 1981). Recently it was reported that macrophages express both LPL mRNA and protein in atherosclerotic lesions in-vivo (Yla-Herttuala et al. 1991). LPL is also produced by smooth muscle cells (SMCs) (Vance et al. 1982).

Macrophages are thought to be the precursors of foam cells in atherosclerotic lesions (Fowler et al. 1979, Schaffner et al. 1980). Recent in-situ hybridization studies strongly suggested that macrophages, and in particular macrophage-dervived foam cells, rather than SMCs are the primary cellular source of LPL (O'Brien et al. 1992). Macrophage-dervived LPL could play a role in CE accumulation in cells during atherogenesis, due to LPL-mediated binding of remnant lipoproteins to heparan-sulphate, followed by endocytosis of the entire particle or translocation of CE into cells without endocytosis. Since LPL, by hydrolyzing TGs, generates remnant lipoproteins locally, that are taken up avidly by macrophages (Goldstein et al. 1980), it may facilitate the macrophage conversion into foam cells and consequently promote the atherogenic process. LPL modifies LDL to a form that is more readily taken up by cultured macrophages (Aviram et al. 1988). In-vitro, mouse peritoneal macrophages take up acetylated LDLs and VLDLs by receptor-mediated endocytosis, hydrolyze the CEs and then reesterify the cholesterol in the cytoplasm. There is evidence that this CE cycle also occurs in foam cells of atherosclerotic lesions in-vivo (St Clair 1976, Brown et al. 1980). LPL-mediated hydrolysis of VLDL was associated with enhanced transport of LDL across endothelial cell monolayers, and enhanced LDL retention in the subendothelial matrix in-vitro (Saxena et al. 1992). These authors postulate that LPL-mediated LDL retention potentiates conversion of these lipoproteins to more atherogenic forms. Thus, LPL is suggested to have a stimulating effect on conversion of macrophages to foam cells, via LPL-mediated hydrolysis of TG-rich lipoproteins and LPL-mediated LDL retention within the arterial wall.

The likely mechanism of this effect is that LPL acts as a 'biochemical bridge', anchoring LDL particles by binding to heparin sulphate glycosaminoglycans on one hand and lipoproteins on the other hand, bringing about the appropriate conditions for formation of LDL-LPL complexes that also bind to matrix proteoglycans such as those on the arterial wall

(Saxena et al. 1992); dissociation of LPL-LDL complexes decreases LDL binding to heparan sulphate glycosaminoglycans. This mechanism is supported by recent in-vitro observations indicating that LPL may enhance the interaction of LDL with artery-dervived extracellular matrix proteoglycans and thus facilitate LDL retention in the artery wall (Edwards et al. 1993). This would be possible because LPL has separate lipid/lipoprotein and heparin-binding sites (Kirchgessner et al. 1987). Both LPL mRNA and LPL protein were associated with macrophage-dervived foam cells, endothelial cells and, to a lesser extent, with SMCs in well-developed plaques from coronary artery segments removed from patients (O'Brien et al. 1992). Another mechanism for LPL-mediated foam cell formation was proposed by Beisigel et al. (1991), who showed that LPL enhances binding of chylomicrons and β-VLDLs to cells via the LDL-receptor-related protein.

A recent study on strains of mice resistant and susceptible to atherosclerosis demonstrated that macrophage mRNA expression and LPL secretion in susceptible strains (which have an inherited tendency to develop atherosclerosis) were double those in resistant strains (Renier et al. 1993). LPL gene expression, secretion and activity were significantly (about 3-fold) higher in recombinant in-bred strains typed as susceptible compared to those typed as resistant (Renier et al. 1993). These authors suggested a contributive role for LPL in development of atherosclerosis due to the concordance of the phenotypes of lesion formation and LPL activities. They suggest that macrophage LPL secretion contributes towards the state of susceptibility or resistance to atherosclerosis and may be one of the inherited components of atherosclerosis, underlying variability in levels of LPL in the atherosclerotic plaque. High LPL secretion could facilitate macrophage uptake of lipoproteins by accelerating the degradation of VLDLs or remnants, thus promoting the atherogenic process. In-vitro incubation of macrophages with LPL resulted in induction of macrophage tumour necrosis factor α (TNFα) gene expression at both transcriptional and post-transcriptional levels (Renier et al. 1994), underlying the importance of LPL in macrophage biology. TNFα is an important secretory product of macrophages, which is directly involved in inflammatory reactions after bacterial infection (Beutler & Cerami 1989) and may play a role in the development of atherosclerosis (Barath et al. 1990). Thus, Renier and coworkers demonstrated a new role for LPL, of modulating macrophage TNFα gene expression and this effect may be one mechanism by which LPL may favour the development

of atherosclerosis.

A second role for LPL in promoting atherogenesis is through its involvement in FCHL. Heterozygosity for LPL deficiency is associated with an increased risk of atherosclerosis, being characterized by variable expression of hyperlipidaemia and reduced HDL-cholesterol levels (Babirak et al. 1989). Obligate heterozygotes had post-heparin activity and mass for LPL that were below the 95% confidence limits for normal controls. This reduced LPL activity may not be sufficient to keep the plasma TG level within normal limits when stress is placed on the plasma lipid transport system. Some of the heterozygotes had elevated plasma apoB levels, making them clinically similar to individuals with FCHL (FCHL is discussed in more detail earlier in Sections 1.3.7 and 1.5.6). Babirak et al. (1989) suggested that the heterozygote state for LPL deficiency forms one subset of individuals with FCHL, confirmed by reduced LPL activities (in the same range as individuals who are obligate heterozygotes for LPL deficiency) in 20 of 56 FCHL patients (Babirak et al. 1992). Partial LPL deficiency has been suggested to underlie about 30% of FCHL cases (Babirak et al. 1989, 1992).

Williams et al. (1990) showed that the net secretory output of apoB from cultured HepG2 cells can be regulated by rapid re-uptake of nascent lipoproteins shortly after their export across the membrane. LPL reduced net output of apoB from cultured hepatoma G2 (HepG2) cells to 25-50% of control by tripling the cellular re-uptake of nascent lipoproteins, thus LPL deficiency would reduce local reuptake of apoB; a mechanism suggested to underlie the hepatic apoB overproduction characteristic of FCHL (Williams et al. 1991). LPL is able to sustantially remodel nascent particles before they have diffused away from the vicinity of the hepatocytes (Williams et al. 1991) due to the rapid enzymatic action of LPL on mature TG-rich lipoproteins (Olivecrona & Bengtsson-Olivecrona 1987) and the presence of LPL in and near the space of Disse in adult mammalian liver (Vilaró et al. 1988). Hepatic uptake of LPL from the circulation has been demonstrated (Wallinder et al. 1979, Peterson et al. 1985). This LPL remains enzymatically active intra-hepatically (Wallinder et al. 1984). LPL enhances receptor-binding and cellular uptake of a wide range of apoB-containing lipoproteins e.g. LPL (Aviram et al. 1988), VLDL (Catapano et al. 1979, Ishibashi et al. 1990), β-VLDLs (Hui et al. 1984) and chylomicrons (Ostlund-Linqvist et al. 1983,

Borensztajn et al. 1988).

Several epidemiological studies have demonstrated lower LPL activities in patients with premature CAD (Brier et al. 1985) or young MI survivors (Johansson et al. 1991a), relative to healthy controls, but this was not confirmed in other studies (Barth et al. 1983, Mendoza et al. 1983, Babirak et al. 1992), though LPL activities tended to be lower in normotriglyceridaemic CAD patients. A subset of patients with both premature CAD and reduced LPL activity was defined as having FCHL by Babirak et al. (1992), however future studies will be needed to delineate the significance of reduced LPL activities in patients with premature CAD.

Finally, there are two other mechanisms in which LPL may be atherogenic. In-vitro studies (Tall et al. 1984) demonstrated that when CETP (cholesteryl ester transfer protein) and LPL were both present, the transfer of CEs from HDL into VLDL remnants was enhanced 2-8 fold compared to the effects of CETP alone, i.e. CETP and LPL act synergistically on CE transfer and LPL enhances CETP-mediated transfer; this property of LPL is related to lipolysis (Tall et al. 1984). Therefore LPL affects the balance between HDL and non-HDL cholesterol, and may adversely affect the process of reverse cholesterol transport. Secondly Factor VII coagulant activity, which is a predictor of CHD (Meade et al. 1986), is dependent on LPL activity, due to liberation of free fatty acids (FFAs) during lipolysis on the surface of large TG-rich lipoprotein particles, which generates a potent contact surface, promoting Factor XII activation (Mitropolous & Esnouf 1991, Mitropolous et al. 1992). This activated Factor XII, in turn, cleaves Factor VII with a resultant increase in Factor VII activity (Mitropolous et al. 1990).

Section 1.7 HIGH DENSITY LIPOPROTEINS (HDLs) AND SUBCLASSES

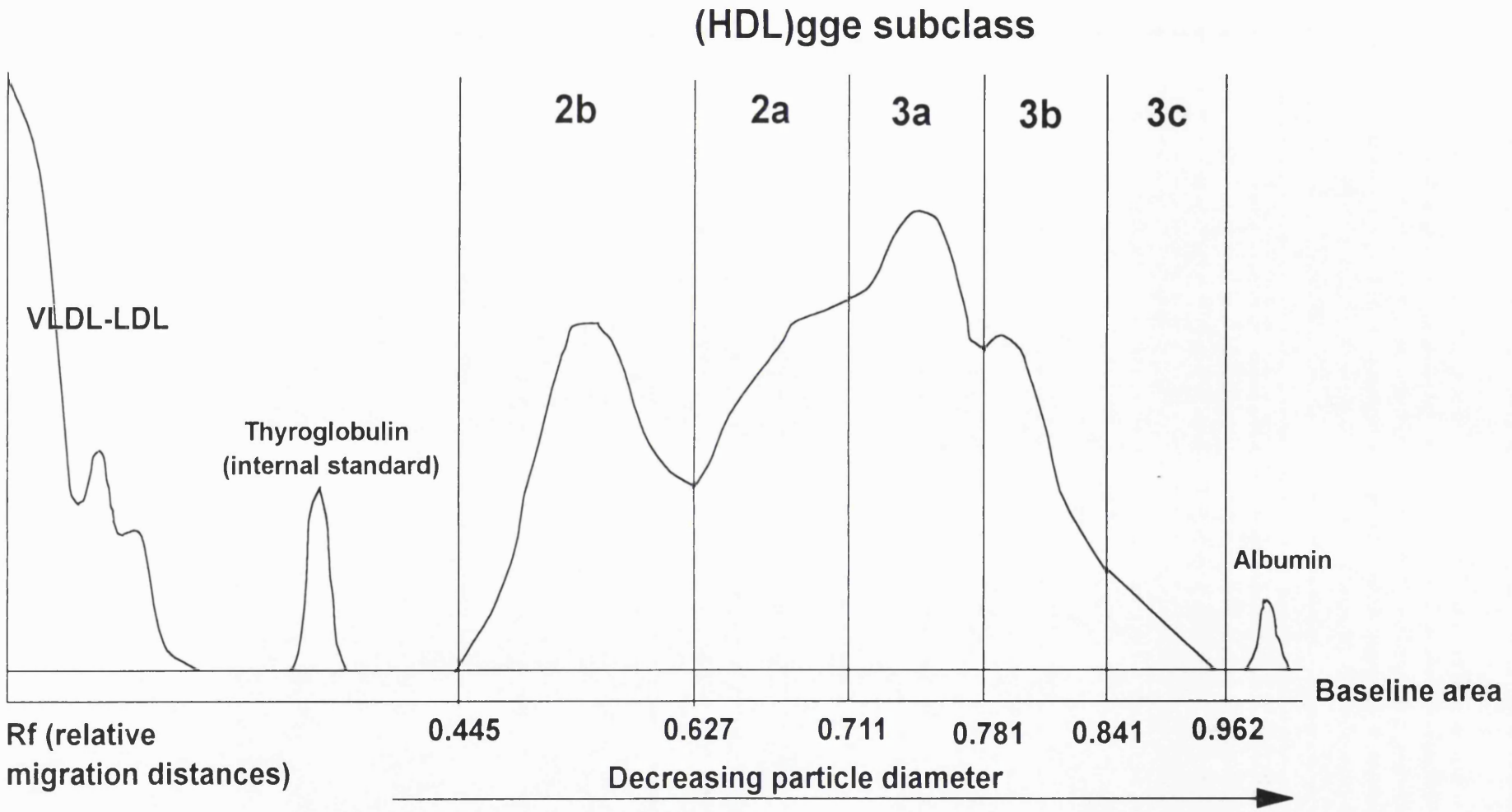
Section 1.7.1 Classification of HDL subclasses

The high density lipoprotein (HDL) class, in the density range 1.063-1.21g/ml, is a heterogenous mixture of lipoprotein particles with varying sizes (70 to 100Å), molecular weights (200 to 400KDa) and apolipoprotein composition (Eisenberg 1984, Tall 1990). HDLs are small dense spherical lipid-protein complexes, containing 50-80% lipid and 20-50% protein by weight. The major lipids are phospholipids (on the surface), cholesterol,

cholesteryl esters and triglycerides (in the core). The two major apolipoproteins of HDL are apoAI (70%) and apoAII (20%), but there are also minor apolipoproteins; apoE and apoC's (CI, CII and CIII) (reviewed in Tall 1990). Early studies (De Lalla et al. 1954) demonstrated heterogeneity in the density and size distributions of the HDL class, and defined two major subclasses HDL_2 (density interval 1.063-1.125) and HDL_3 (density interval 1.125-1.210), by their flotation characteristics in the analytic ultracentrifuge. Later, two subpopulations in the HDL_2 density range were defined by particle size, using gradient gel electrophoresis (Anderson et al. 1977) and two apparent subpopulations within the HDL_3 subclass were described (Laggner et al. 1977, Patsch et al. 1980). But it was not until the use of gradient gel electrophoresis in conjugation with automated densitometry that the two HDL_2 and three HDL_3 subclasses were definitively classified (Blanche et al. 1981) according to hydrated density. Five distinct subclasses are now known, termed HDL2b, HDL2a, HDL3a, HDL3b and HDL3c, in order of decreasing particle diameter and increasing hydrated density. The different HDL subclasses have different mobilities on non-denaturing polyacrylamide gels reflecting the impeded migration of larger HDL. Five apparent frequency distributions with characteristic maxima are observed for peak Rf values in the HDL patterns seen in healthy individuals (Blanche et al. 1981), Figure 1.7.1.1 shows an example concentration distribution of HDL subclasses and Table 1.7.1.1 shows characteristics of the HDL (gge) subclasses (gge = gradient gel electrophoresis).

The second method of classifying HDLs is based on apolipoprotein composition. This method of classifying lipoproteins was first advocated by Alaupovic (1972) and Osborune & Brewer (1977). Cheung & Albers (1984), ultilizing sequential immunoaffinity chromatography, demonstrated the presence of two major HDL populations, HDL with apoAI but not apoAII (LpAI) and HDL with both apoAI and apoAII (LpAI:AII), LpAI:AII being most abundant. In normal individuals about 1/3 of plasma apoAI is in LpAI and 2/3 is in LpAI:AII (Cheung & Albers 1984, Koren et al. 1987, Parra et al. 1990). This classification system is gaining popularity and appears to be the most effective codification system for the plasma lipoproteins. The justification of this classification system is that the plasma catabolism of apoAII is slower than that of apoAI in normal human subjects (Schaefer et al. 1982, Zech et al. 1983, Brinton et al. 1989, 1990, Rader et al. 1991), reflecting the divergent in-vivo metabolism of LpAI and LpAI:AII. Based on this system, the major HDL

Figure 1.7.1.1 Densitometric scan of HDL subclass distribution (adapted from Blanche et al. 1981)



Gradient gel is stained with Coomassie G-250 (stains protein) and scanned with a densitomer at a wavelength of 596nm. The resulting densitometric scan describes the concentration distribution of HDL protein versus migration distance, which is inversely correlated with particle size. The reference proteins (and radius) used for calibrating HDL particle sizes are thyroglobulin (8.5nm), apoferritin (6.1nm), lactate dehydrogenase (4,1nm) and bovine serum albumin (3.6nm). Radii of reference proteins were plotted against Rf to give a calibration curve, and particle size of HDL subclasses were dervived from this calibration curve.

Magnitude of scan area within each HDL subclass depends both on the concentration of protein and proportion of total HDL protein within the subclass. Scan areas bounded by Rf intervals of 0.445 to 0.711 and 0.711 to 0.962 respectively, approximate to mass of protein in HDL_2 and HDL_3 subclasses. However each interval does not include all of the area for each HDL subclass because of the spread of peak area beyond the characteristic Rf intervals.

Table 1.7.1.1 Characteristics of the HCL (gge) subclasses

Subclass	Particle diameter range (nm)	Mean particle size (nm) *	Hydrated density range (g/ml)	Mean hydrated density * (g/ml)	Peak Rf ●	Rf range
HDL2b	9.7-12.0	10.57	1.063-1.100	1.085	0.578	0.445-0.627
HDL2a	8.8-9.7	9.16	1.100-1.125	1.115	0.678	0.627-0.711
HDL3a	8.2-8.8	8.44	1.125-1.200	1.136	0.747	0.711-0.781
HDL3b	7.8-8.2	7.97		1.154	0.807	0.781-0.841
HDL3c	7.2-7.8	7.62		1.171	0.863	0.841-0.962

Footnote; * = corresponding to those HDLs with relative migration distances (Rf) of the frequency maxima.

● Relative migration distance of frequency maxima

Table 1.7.1.2 Classification of small pre-beta LpAI particles.

		Composition (%age of weight)		
Group	Size (nm)	Protein	Surface lipids	Core lipids
I (spherical)	7.3-7.6	56.3-64.3	20.5-33.9	9.7-21.8
II (spherical)	7.8	57.9-63.6	31.5-39.8	2.2-4.9
III (discoidal)				
pre-beta$_1$ (71KDa)	8.4 x 4.1	45.8-47.5	52.2-54.2	-
pre-beta$_2$ (325KDa)	13.5 x 4.4	29	71	-

Group I pre-HDLs have elevated core lipids, while group II pre-HDLs have little core lipid and accumulate in LCAT deficiency (Chen et al. 1984). Group III have essentially no core lipids.

particles are LpAI, LpAI:AII, LpAI:AIV and LpAI:AIV:AII. All HDLs contain at least one apoAI molecule and most also contain apoAII. A subclass of the large discoidal particles are rich in apoE (Utermann et al. 1975, Marcel et al. 1980, Mitchell et al. 1980) and probably contain relatively little apoAI or apoAII. This apoE-rich HDL (HDL_1) which has been suggested to provide a vehicle for transport of cholesterol from perpherial tissues to liver migrates largely as HDL2b (Griffin et al. 1988). LpAI largely corresponds to the HDL2b and HDL3a subclasses (Puchois et al. 1987).

The third classification system for HDLs is based on electrophoretic mobility and is intricately linked with the apolipoprotein composition. ApoAI-containing lipoproteins are divided into 2 major subfractions, those with alpha mobility and those with pre-beta mobility with the majority of HDL, containing both apoAI and apoAII, migrating with alpha-mobility in agarose electrophoresis (Castro et al. 1988). The average concentration of pre-beta HDL is about 5% (range 0-10%) of the apoAI in the plasma of normolipidaemic individuals (Ishida et al. 1987, Neary et al. 1987). HDLs of pre-beta mobility were first described by Levy & Fredrickson (1965). Pre-beta HDLs are small and protein-rich, being composed predominantly of apoAI, and are structurally distinct from alpha-HDLs (Kunitake et al. 1985, 1987) Pre-beta and alpha HDLs have an intricate metabolic inter-relationship, as evidenced in a number of reports in the literature. There are several lines of evidence that discoidal HDLs with pre-beta mobility and apoAI but not apoAII are the precursors of alpha-migrating HDL particles containing apoAII in addition to apoAI.

Discoidal HDLs are the preferred substrate for the enzyme lecithin:cholesterol acyl-transferase (LCAT) which converts free cholesterol to cholesteryl esters (Fielding 1989). When LCAT is inhibited, these discoidal pre-beta HDLs accumulate (Hamilton et al. 1976), this also occurs in the plasma of LCAT deficient individuals (Chen et al. 1984) and in HepG2 conditioned medium (McCall et al. 1988). Discoidal HDL incubated in-vitro with purified LCAT forms spherical HDL (Fielding & Fielding 1971). LpAI particles are more efficient at promoting cholesterol efflux from adipocytes (Barbabras et al. 1987) and from fibroblasts (Oram et al. 1987) compared to LpAI:AII particles. Small LpAI, generally containing 1 or 2 apoAIs, are considered to be the primary acceptors of cellular cholesterol and therefore are crucial in the first stage of reverse cholesterol transport (Reichl et al.

1989). These particles in the HDL3c density range have a diameter of <7.2 to 7.8nm (Nichols et al. 1983). These small LpAI are divided into three groups (Table 1.7.1.2) according to core lipid content (reviewed in Nichols & Gong 1990).

Section 1.7.2 Effect of lipoprotein and hepatic lipases on HDL subclass abundance and distribution

Lipoprotein lipase (LPL) and hepatic lipase (HL) have different impacts on HDL turnover (Kuusi et al. 1989) and regulate the plasma HDL_2 concentration in a reciprocal manner. Both LPL and HL are important determinants of HDL cholesterol in populations (Applebaum-Bowden et al. 1985, Kuusi et al. 1989). Experiments using antisera to LPL injected into fasted roosters, to selectively inhibit LPL, demonstrated that the existence of HDL_2 requires the action of LPL, and that the accumulation of triglyceride-rich lipoproteins, due to blocked lipolysis, resulted in the conversion of HDL_2 to HDL_3 and a fall in HDL-cholesterol (Behr et al. 1981). LPL and HL act on different lipoproteins, despite having the same general 2-step mode of lipase action (Tikkanen 1985). These two steps are adsorption of enzyme to lipoprotein particle via reversible binding to lipid/water interface followed by alignment and hydrolysis of single substrate molecules in the active site. LPL acts on chylomicrons and large VLDLs and LPL deficiency causes accumulation of these lipoproteins (Manzato 1984), while HL acts on HDL and on remnants of chylomicrons and VLDLs formed by LPL action. Thus, in HL deficiency VLDL remnants and HDLs accumulate (Demnant et al. 1988), particularly HDL2b, with a virtual absence of the smaller HDL particles (Carlson et al. 1986). When LPL activity is low, as in young post-infarction patients, HDL2b becomes more triglyceride-enriched due to increased lipid-transfer activity and is acted upon more avidly by HL, resulting in a shift towards small HDL particles and a low total HDL-cholesterol concentration (Johansson et al. 1991b). In hypertriglyceridaemia, LPL activity is one major determinant of HDL2b concentrations (Johansson et al. 1991b). Reduced LPL activity might have contributed to the evolution of coronary atherosclerosis in young post-infarction patients (Hamsten & de Faire 1987) by promoting VLDL-triglyceride elevation and lowering of HDL2b levels. This is in agreement with relationships between low postheparin LPL activity and severity of coronary lesions in an angiographic study (Breier et al. 1985).

The generation of HDL_2 is a consequence of the catabolism of triglyceride-rich lipoproteins by LPL and transfer of apolipoproteins and surface phospholipids released from these TG-rich lipoproteins, which fuse with pre-existing HDL_3 (Patsch et al. 1978, Nikkila et al. 1980, Taskinen and Kuusi 1987). Formation of HDL_2 depends on LPL activity and is promoted under conditions of high VLDL turnover (Nikkila et al. 1984). HDL_2 levels and post-heparin LPL activity are postively associated (Nikkila et al. 1984, Taskinen & Kuusi 1987). HDL_2 is subsequently acted upon by HL to produce smaller and denser HDL particles (Groot et al. 1983, Patsch et al. 1984, Hopkins & Barter 1986, Deckelbaum et al. 1988). HL acts on surface phospholipids and triglycerides of HDL particles (Shirai et al. 1981, Groot et al. 1981, Hopkins et al. 1986). Usually, HL has a small effect on HDL particle size and distribution, producing particles with a Stoke's radius of 4.3nm. However, the particle size and distribution of triglyceride-enriched human HDL is dramatically transformed by HL (Hopkins & Barter 1986), producing core-lipid depleted particles of Stoke's radius 3.7nm. HL does not appear to be rate-limiting in the catabolism of VLDL or VLDL remnant particles (Auwerx et al. 1989). Composition of HDL_2 rather than HL activity determines whether or not a HDL_2 particle is converted to HDL_3 (Patsch et al. 1984). Reduced LPL activities in hypertriglyceridaemic (HTG) patients and reduced HL activities plus an inverse association between HDL2b concentrations and severity and rate of progression of atherosclerosis in normotriglyceridaemic (NTG) patients who survived a myocardial infarction, were found, compared to healthy age-matched controls (Johansson et al. 1991a, 1991b). These data unequivocally points to the interpretation that the HDL2b concentration is not primarily governed by cholesterol uptake and LCAT-mediated cholesterol esterification, but rather by the sequential actions of LPL, CETP and HL (Johansson et al. 1991a).

The rabbit can be considered as an animal model of HL deficiency, as activity of HL in this animal is naturally very low (Clay et al. 1989), with the consequence that the HDL fraction in rabbit plasma is relatively depleted of cholesteryl esters and markedly enriched with triglycerides, and thus has a density comparable to human HDL_2 (Clay et al. 1989). In-vitro incubation of rabbit plasma with HL extensively hydrolyzes HDL triglycerides accompanied by conversion of HDLs into particles of size and composition resembling human HDL_3 (Clay et al. 1989).

Section 1.7.3 Associations of HDL subclasses with presence and severity of atherosclerosis.

The hypothesis that high levels of plasma HDL are protective against CAD was first proposed by Barr et al. (1951); later several large epidemiological studies confirmed this, showing an inverse relationship between HDL levels and incidence of CAD. These included prospective (Gordon et al. 1977, Miller et al. 1977, Enger et al. 1979, Goldbourt et al. 1979) and case-control studies (Puchois et al. 1987, Stampfer et al. 1991, Parra et al. 1992). Observations made in 13 major epidemiological studies in 8 countries (Miller 1989) show that on average, CHD risk increased by about 3% with each 0.026mM decrease in HDL-cholesterol concentration. HDL as a whole (Miller & Miller 1975, Gordon et al. 1977) and the HDL_2 subfraction (Miller et al. 1987) have been suggested to be protective against development of coronary atherosclerosis. Case-control studies indicate that the well-known inverse relation between HDL and CHD incidence is due mainly to HDL_2 (Ballantyne et al. 1982, Hamsten et al. 1986, Griffin et al. 1988, Johansson et al. 1991b). HDL_2 levels are selectively decreased in patients with coronary and peripherial atherosclerosis (Fellin et al. 1985, Franceschini et al. 1987, Wallentin et al. 1987, Roche et al. 1991).

Recently Johansson and coworkers (1991b) studied a group of young post-infarction patients and suggested that this inverse relation is accounted for by the largest HDL particles, the HDL2b subclass. Levels of HDL2b were strongly inversely related to baseline severity and progression of atherosclerotic lesions in those patients who were normotriglyceridaemic (NTG). CHD risk may be increased when HDL2b is reduced relative to HDL3b and HDL3c (Wilson et al. 1990, Cheung et al. 1991, Johansson et al. 1991b). Individuals with low coronary risk indices had a pronounced HDL2b peak, absent in indiviudals with high coronary risk indices (Griffin et al. 1988).

Plasma levels of HDL-C are strongly correlated with HDL_2 but not with HDL_3 levels (Anderson et al. 1978, Patsch et al. 1983, Patsch 1987), this was confirmed recently by Johansson et al. (1992). Studies have also demonstrated a strong inverse correlation between HDL_2 and TGs (Calabresi et al. 1990) or VLDL-TGs (Johansson et al. 1992). Levels of the HDL_2 subfraction (Anderson et al. 1978, Krauss et al. 1982) and LpAI (Cheung & Albers 1984, Cheung et al. 1991) are more variable in human populations. HDL_2 is the main

determinant of total HDL-C, while HDL_3 varies only within narrow limits (Patsch et al. 1983, Eisenberg 1984).

LpAI largely corresponds to the HDL2b and HDL3a subclasses (Puchois et al. 1987); the relative proportion of LpAI is higher in HDL_2 than in HDL_3 (Koren et al. 1987). In a normolipidaemic sample, average LpAI concentrations were 10% higher in women than in men (Parra et al. 1990). The decrease in apoAI in patients with significant CAD relative to healthy individuals was due to a decrease in LpAI (Puchois et al. 1987). Evidence that LpAI but not LpAI:AII may be the specific anti-atherogenic particle within HDL is reinforced by two recent studies. Schultz et al. (1993) recently demonstrated, in transgenic mice fed on an atherogenic diet, that the area of atherogenic lesions was 15x greater in mice expressing both human apoAI and apoAII compared to mice expressing apoAI but not apoAII, thus demonstrating that the protein composition of HDL significantly affects its role in atherogenesis and that LpAI is more anti-atherogenic than LpAI:AII. The genetic syndromes of complete (classic) or partial (fish eye disease) LCAT deficiency both result in low plasma HDL levels but not in an increased risk of atherosclerosis (Norum et al. 1989). Recently Rader et al. (1994) carried out in-vivo investigations into apoAI and AII metabolism of patients with LCAT deficiency, revealing that the characteristically low levels of HDL are due to preferential hypercatabolism of LpAI:AII but not LpAI. Rader et al. (1994) suggested that this selective effect is a potential explanation of why patients with LCAT deficiency are not at increased risk for atherosclerosis.

Section 1.7.4 Possible Mechanisms underlying the protective effect of HDLs

HDL has been thought to be directly protective via the mechanism of reverse cholesterol transport (Tall 1990). Glomset (1968) suggested the involvement of HDL in reverse cholesterol transport and coined this term. For convenience, reverse cholesterol transport, which is the transport of cholesterol from peripherial tissues back to the liver, is described in terms of three sequential stages, extravascular, intravascular and intrahepatic (Reichl & Miller 1986, 1989). The extravascular stage consists of the transfer of unesterified cholesterol from cells to cholesterol acceptors in interstitial fluid (reviewed in Reichl & Miller 1989). These cholesterol acceptors are small apoAI-containing particles called pre-beta LpAI (Castro 1988) which are nascent HDLs, postulated to serve a key role in reverse

cholesterol transport (Fielding & Fielding 1980, Castro & Fielding 1988, Francone et al. 1989). Of all tissues, the arterial wall may depend most on reverse transport mediated by these small pre-beta LpAI (Reichl & Miller 1989), which are present at relatively high levels in human aortic intima and atherosclerotic lesions (Heideman & Hoff 1982, Castro et al. 1988).

The intravascular stage occurs in the plasma. The nascent HDLs [small spherical particles or apolipoprotein/phospholipid bilayer discs with pre-beta mobility (Tall 1990)] from the extravascular stage are rapidly modified by LCAT with apoAI as activator, into mature spherical HDL particles, rich in cholesteryl esters along the $HDL_4 \rightarrow HDL_3 \rightarrow HDL_2 \rightarrow HDL_1$ cascade (Tall 1990, 1992). HDL enlargement occurs due to lipid influx and LCAT action (Deckelbaum et al. 1982, Tall 1990). The phospholipids and cholesterol in HDL act as substrates for LCAT, generating cholesteryl esters and lysolecithin which is bound to albumin or taken up by tissues (Tall 1992). The terminal intrahepatic phase consists of delivery of CE to heptacytes. The CE produced by the LCAT-catalysed reaction can undergo three fates (Tall 1992). Firstly CE can be transferred to TG-rich apoB-containing lipoproteins in a process involving cholesteryl ester transfer protein (CETP) and eventually taken up by heptacytes via receptor mediated catabolism of CE-enriched apoB-containing remnant lipoproteins eg. IDL and LDL; this is the major pathway of HDL CE catabolism in man (Tall 1992). Secondly, CE can be transferred directly into cells by selective non-particulate CE uptake, however its role in man remains speculative (Goldberg et al. 1991). Thirdly, CE can be catabolized with HDL particles; large HDL particles rich in apoE are taken up by receptor-dependent pathways (Mahley & Innerarity 1983).

There is a widespread, although still unsubstantiated, belief that these processes are antiatherogenic by virtue of their potential to promote the efflux of cholesterol from the artery wall (Barter 1993). However, it is not clear whether "reverse cholesterol transport" mediates the protective effect of HDL noted in epidemiological studies (Tall 1990). An inverse relationship between plasma HDL-C and tissue C stores, as measured by isotopic turnover studies, in humans was not found in a large group of subjects (Blum et al. 1985) and the HDL deficiency states, Tangier disease, associated with accumulation of CEs in tissue macrophages and hepatosplenomegaly (Breslow 1989) and LCAT deficiency do not

result in markedly increased atherosclerosis (Tall 1990).

The alternative hypothesis is that low HDL is a marker for increased development of atherosclerosis via postprandial accumulation of chylomicron or VLDL remnants (Patsch 1987, Tall 1990). Low levels of HDL result from both inefficient lipolytic transfer of lipids into HDL and accelerated CE-TG interchange due to the accumulation of TG-rich lipoproteins (Tall 1990). Postprandial lipaemia is strongly inversely correlated with HDL_2 levels (Patsch et al. 1983) and postprandial enrichment of HDL_2 with TGs is strongly postively associated with postprandial lipaemia (Patsch et al. 1984). It has long been known that patients with coronary heart disease exhibit an exaggerated postprandial triglyceride response after fat ingestion (eg. Moreton 1947, Angervall 1964), and recent case-control studies confirm this (Simpson et al. 1990, Groot et al. 1991, Patsch et al. 1992).

High levels of postprandial TG-rich lipoproteins lead to enchanced CETP-mediated exchange with a shift of cholesterol from HDL to atherogenic apoB-containing lipoproteins (Tall 1986). CETP, a plasma protein, mediates the heteroexchange of CE in HDL for TGs in chylomicrons and VLDLs (Tall 1986). Accumulation of CEs on VLDLs and LDLs decreases when levels of TG-rich lipoproteins are reduced with gemfibrozil in both fasting and postprandial states (Bhatnagar et al. 1992). Hayek et al. (1993) proved using transgenic mice, that CETP is a major mediator of the well-known inverse relationship between TG and HDL-C (eg. Schaefer et al. 1978, Deckelbaum et al. 1984, Gordon et al. 1977) but that, in addition, this inverse relationship, on the background of human apoAI, was still evident in the absence of CETP, most apparent in animals with marked HTG, where HTG increased the fractional catabolic rate and decreased the transport rate of HDL-CE. Recently Bhatnagar et al. (1993) demonstrated in-vitro an increased rate of accumulation of CEs on VLDLs and LDLs in plasma from patients with CAD compared to healthy controls with similar lipid levels, which could be due to increased free cholesterol content of patients' VLDLs - this has been demonstrated to accelerate the transfer of CEs from HDL to LDL and VLDL in hypercholesterolaemic plasma (Begdade et al. 1991) and patients with CAD have VLDLs enriched in free cholesterol (Mancini et al. 1988).

HDL composition (Sparks et al. 1989), VLDL mass (Tall et al. 1987) or VLDL

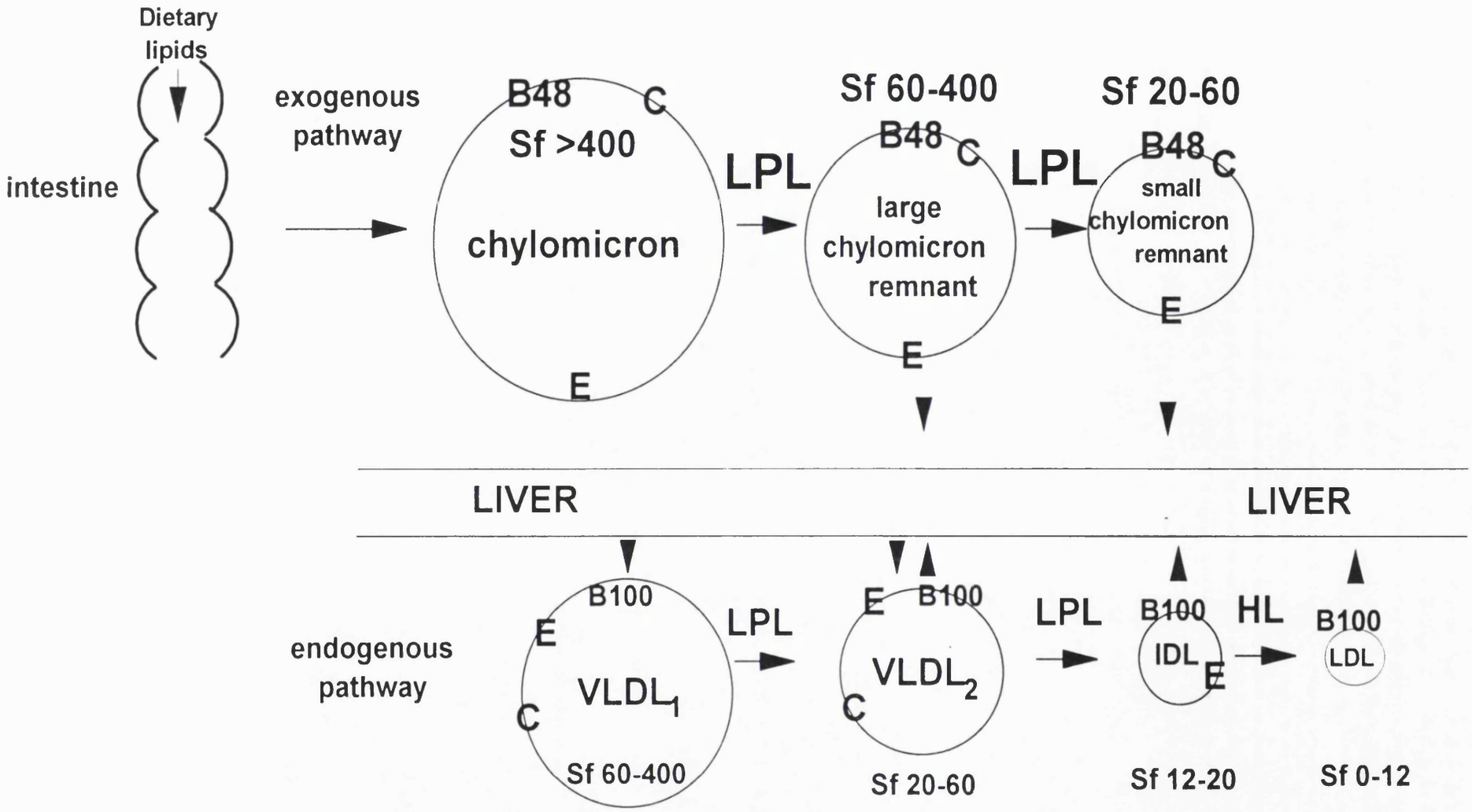
composition (Mann et al. 1991) may be more important than CETP activity in the transfer process. In man, the CETP pathway is of major importance in HDL catabolism, influencing size, composition and quantity of HDL (Tall 1992), as illustrated by the altered lipoprotein profile in individuals with genetic CETP deficiency (Brown et al. 1989, Inazu et al. 1990, Koizumi et al. 1991). The lipoprotein profile in the absence of CETP (ie. high HDL, high apoAI and low LDL) has low atherogenic potential, resembling that in animals (rats, pigs, dogs) lacking plasma CETP activity (Tall 1986) caused partly by greatly reduced catabolism of both apoAI and apoAII (Ikewaki et al. 1993). On the other hand, transgenic mice expressing CETP had much worse atherosclerosis than did non-expressing controls, largely due to CETP-induced alterations in the lipoprotein profile (Marotti et al. 1993).

Section 1.8 Postprandial lipid metabolism

Section 1.8.1 Overview of normal postprandial metabolism in the circulation

The major part of life is spent in the postprandial state i.e. the time period between food ingestion and 6 to 8 hours later. The postprandial state is characterized by the presence of chylomicrons of intestinal origin. When the plasma lipid transport system of normolipidaemic individuals is subjected to a physiological challenge such as the intake of a fatty meal, the magnitude of the ensuing postprandial lipaemia varies greatly among individuals (Patsch et al. 1983). This is due to highly variable capacities to metabolize dietary fat. In normolipidaemic individuals, the magnitude of postprandial lipaemia shows, by far, the greatest interindividual varability among all parameters of lipid transport, matched only by levels of HDL_2 in the fasted state (Patsch et al. 1983, Patsch 1987, Brown & Roberts 1991). The input of newly synthesized chylomicrons into the circulation causes significant perturbations in other plasma lipoproteins (reviewed in Patsch 1987). The increase in plasma TGs after a meal is dervived from both exogenous (dietary) and endogenous (hepatic) sources, as indicated by increased levels of both apoB-48 and apoB-100 in TG-rich lipoproteins (Genest et al. 1986, Cohn et al. 1988, 1989, 1993, Karpe et al. 1993a). A schematic representation of metabolism of TG-rich lipoproteins is shown in Figure 1.8.1.

The predominant TG-rich particle after ingestion of an 'ordinary' meal with 38% of total calories from fat, as opposed to a fatty emulsion (e.g. where 60%, Cohn et al. 1988, or 70%, Gylling & Miettinen 1993, of the total caloric intake is from fat), is VLDLs rather



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Figure 1.8.1 Schematic representation of chylomicron and VLDL metabolism

Chylomicrons are secreted from the intestine after ingestion of dietary fat. In the plasma, newly synthesized chylomicrons acquire apoE and apoC and rapid LPL-mediated lipolysis occurs, accompanied by transfer of redundant surface material to HDL and heteroexchange of core lipids with HDL. The latter is mediated by cholesteryl ester transfer protein (CETP). These two processes modulate the final composition of the lipoprotein particle and receptor-mediated uptake of remnants occurs.
VLDL is secreted from the liver and largely shares the same metabolic pathway, involving lipases and CETP, as chylomicrons. The one difference is the uptake of LDL particles via apoB-100 via the LDL-receptor; these particles cannot be recognised by the LDL-receptor-related-protein.
For simplicity, only one apoE and one apoC are shown per lipoprotein particle, though multiple apoE and apoC particles are known to be present on lipoprotein particles.

than chylomicrons (Schneeman et al. 1993). The generation of chylomicrons may also be influenced by the fatty acid composition of a fat meal. An oral fat containing medium chain triglycerides did not result in an acute elevation of plasma TGs (Barr et al. 1985), due to direct transfer of these fatty acids from the intestinal enterocytes to the liver via the portal vein. Magnitude of chylomicronaemia observed was significantly smaller in individuals who had an oral fat load containing polysaturated fats, than in those who ingested saturated fats (Weintraub et al. 1988). This was partly explained by faster in-vitro lipolysis of chylomicrons containing polysaturated fat.

'Ordinary' meals contain at least 100 times more TG than cholesterol, by mass (Havel et al. 1973). More than 95% of TGs are absorbed in the intestine, while absorption of cholesterol is only 20-50%, and dietary fat serves both as a source of energy and a carrier of lipid-soluble vitamins (A, D and E). Pancreatic lipase acts on dietary TGs in the duodenum, cleaving the fatty acid ester bonds at postions 1 and 3 in the triacylglycerol molecule. Bile salts and phospholipids emulsify intestinal lipids, and water-soluble micelles containing neutral lipids are subsequently formed. The end products of lipid hydrolysis that are presented for absorption include fatty acids, glycerols, monoglycerides, some di- and triglycerides, cholesterol and phospholipids. Absorption into enterocytes (cells lining the surface of the intestine) is greatly enhanced by solubilization of the end products of lipolysis by bile acid micelles. The lipids enter the enterocytes by passive diffusion and are rapidly esterified inside the cells, maintaining a favourable concentration gradient from the lumen into the enterocyte.

Within the intestinal epithelial cells, absorbed TGs and cholesterol are reesterified. The fate of fatty acids depends on their chain length; short fatty acids with a chain length of less than 10-12 carbons, together with glycerol, are transported directly to the liver via the portal vein. In constrast, long-chain fatty acids are re-esterified into TGs (dietary cholesterol also undergoes reesterification) within the intestinal epithelial cells; this absorbed dietary fat is packaged into chylomicrons, which are secreted into the circulation. Chylomicrons contain a core of TGs and CEs which is wrapped with a surface film of phospholipids, free cholesterol and apolipoproteins. Chylomicrons are the largest lipoprotein particles with a diameter ranging from 80 to 500nm, density of <0.96g/ml, and Svedberg floation rate (Sf)

of >400. Lipid absorption and chylomicron production take place mainly in the jejenum (proximal intestine). The size and composition of chylomicrons depend on the fat content of the meal ingested, with ingestion of small quantities of fat resulting in production of small chylomicrons of size and density similar to lipoproteins produced in the fasted state (diameter of 30-80nm and Sf of 20-400) (reviewed in Carey et al. 1983, Patsch 1987).

The major structural apolipoprotein of chylomicrons is apoB-48. ApoB-48 is synthesized in the microsomes of the intestinal enterocyte, and this comprises the amino-terminal 48% of the full length apoB polypeptide (Kane et al. 1980). There is always a certain concentration of apoB-48 maintained in the enterocyte, and apoB-48 synthesis in the enterocyte shows bile salt dependance (i.e. it is stimulated by the presence of biliary lipids in the lumen) and is not regulated by acute TG flux across the epithelium (Davidson et al. 1986). ApoB-48 synthesis is also not affected by increasing the TG content of an isocaloric diet from 0 to 30% by weight for 6 weeks (Davidson et al. 1986). Other apolipoproteins are also synthesized in enterocytes; AI, AII, AIV, CI, CII, CIII and E (reviewed in Patsch 1987). However, with the exception of apoB, little is known about the secretory regulation of apolipoproteins in response to diet. The chylomicron lipid-synthesizing enzymes in the enterocyte seem to be influenced by the composition of dietary fat (Chautan et al. 1991).

Once secreted into plasma, chylomicrons (which contain apoB-48, apoAI and apoAIV) undergo rapid modification, with some components leaving the particle and others joining the particle. Only apoB-48 remains with the parent chylomicron particle throughout its catabolism and subsequent removal by the liver. All the other apolipoproteins are partitioned between different lipoprotein fractions as their relative affinities for different lipoproteins alter. This is due to changes in surface pressure of the lipoproteins as they lose or gain surface components. ApoAI, which is secreted with the chylomicron diffuses to the HDL fraction and apoAIV diffuses to the lipoprotein-free fraction of the plasma (Tall et al. 1979). Conversely, apolipoproteins (the C and E proteins) which are already circulating in plasma, mainly bound to HDL are rapidly transferred to the chylomicron (Havel et al. 1973), such as apoCII, the essential activator of LPL (LaRosa et al. 1970). The subsequent catabolism of chylomicrons is a two-step process. The first step involves LPL-mediated hydrolysis of TGs, producing TG-poor particles called remnant lipoproteins (this process is described in

more detail in Section 1.6). These remnants have only about 5% of the mass of the original chylomicron particle (Patsch 1987). Under normal conditions (in normolipidaemic individuals) chylomicrons (Sf>400) disappear rapidly, with a half life averaging a few minutes (Cortner et al. 1987, Berr et al. 1992). During the successive delipidation of the chylomicron core, redundant surface materials, such as phospholipids and apolipoproteins are transferred back to HDL (Redgrave et al. 1979, Tall et al. 1979). These components are an important source of HDL mass and influence HDL structure; phospholipids from lipolysed chylomicrons are transferred to both HDL_2 and HDL_3 with a resultant decrease in HDL density (Tall et al. 1982, Patsch et al. 1984) and subsequent action of hepatic lipase on HDL surface phospholipids, and, in TG-enriched HDL, core TGs as well (discussed in more detail in Section 1.7.3).

The delipidated chylomicron remnant is subsequently taken up by hepatic receptors which recognize apoE (Hoeg et al. 1985). ApoE appears to be the principal ligand for binding of remnants of both chylomicrons and VLDLs; evidence for this comes from the observation that the removal of apoE-depleted chylomicron remnants is only one third of the extent of native particles (Arbeeny & Rifici 1984). The LDL-related protein (chylomicron remnant receptor or LRP, a 220kDa heparan sulphate proteoglycan found on endothelial cells (Kowal et al. 1989, Saxena et al. 1991) and on cultured HepG2 cells (Hayashi et al. 1993), rather than the LDL-receptor (Brown & Goldstein 1983) is thought to be predominant in clearance of chylomicron remnants, at least in humans. In subjects (with variable degrees of expression of LDL receptor activity), TG and cholesterol levels in the density range <1.006g/ml increased to a similar extent during adminstration of a heavy intestinal fat load, indicating that the capacity for clearance of chylomicrons and their remnants is not affected by variation in LDL-receptor expression (Eriksson et al. 1991). However the removal rate of remnant lipoproteins is influenced by variable expression of the LDL-receptor, as evidenced by the accumulation of LDLs and IDLs in the plasma of individuals with heterozygous familial hypercholesterolaemia (FH) (Brown & Goldstein 1986, Eriksson et al. 1991). Of particular interest was the normal capacity to clear chylomicron remnants in these individuals (Eriksson et al. 1991), implying the existence of non LDL-receptor mediated pathways for clearance of these remnants. With less drastic fat loads, normal chylomicron clearance has been suggested to occur in a few individuals with homozygous FH (Weintraub

et al. 1987). In the Watanabe heritable hyperlipidaemic (WHHL) rabbit, which is deficient in LDL receptors (Kita et al. 1981), chylomicron remnant clearance is not impaired (Kita et al. 1982) although this has recently been questioned (Bowler et al. 1991).

Several additional ligands for LRP, apart from apoE, have been discovered recently, suggesting that LRP is mutifunctional (reviewed in Herz 1993). One of these is LPL, which is transported on lipoprotein particles (Goldberg et al. 1986), and which increased binding of β-VLDLs to LRP 40-fold and strongly enhanced binding of apoE-containing lipoproteins to LRP (Beisiegel et al. 1991, Saxena et al. 1991). However LPL cannot quantitatively compensate for a lack of apoE in-vivo; demonstrated by the inability of human patients (Schaefer et al. 1986) and mice (Plump et al. 1992, Zhang et al. 1992) deficient in apoE to efficiently clear remnant particles. Another ligand is α-macroglobulin which has been shown to compete with chylomicron remnants in binding to LRP (Hussain et al. 1991). The apoE-mediated binding of remnant lipoproteins to LRP is counteracted by the C apolipoproteins (Kowal et al. 1990) in particular apoCI and to a lesser extent apoCII (Weisgraber et al. 1990). This appears to be mediated by apoCI and apoCII displacing apoE from the surface of the lipoprotein particle, or possibly masking or altering apoE conformation so that it is no longer recognised by LRP. ApoCIII had only a small inhibitory effect on apoE binding to LRP. The implication of this is that apoCIII does not directly inhibit this binding, its effect is indirect via inhibition of lipolysis (discussed earlier in Section 1.6.3), this would slow down the generation of remnants able to bind to lipoprotein receptors.

VLDLs containing apoB-100 are secreted by hepatocytes (Demmer et al. 1986) and are the endogenous TG transporters. The question of whether synthesis of apoB-100 is increased as well as that of intestinal apoB-48 as a result of fat intake has been investigated more recently. Cohn et al. (1988) were the first to report a postprandial increase in the plasma levels of both apoB-48 and apoB-100 TG-rich lipoproteins, both of which contribute to early (0 to 6 hours) and late (6 to 12 hours) changes in TG levels (Cohn et al. 1989). VLDL synthesis was then quantified, after repeated small meals, using stable isotope incorporation into apoB-100, and newly sythesized apoB-100 TG-rich lipoproteins were found to accumulate in plasma (Cohn et al. 1990). These authors concluded that increased synthesis of TG-rich lipoproteins from the liver had taken place, but alternatively the increase

in VLDLs could be due to delayed removal of these lipoproteins from plasma as a consequence of chylomicrons and VLDLs competing for the same lipolytic pathway (Brunzell et al. 1973).

Very recently, the contribution of apoB-48 and apoB-100 TG-rich lipoproteins to postprandial lipaemia was quantified in normolipidaemic male volunteers who were given an oral fat load (Cohn et al. 1993). An average of 82% of the postprandial increase in TGs was due to apoB-48 TG-rich lipoproteins, while the contribution of apoB-100 TG rich lipoproteins to total postprandial lipaemia ranged from 3 to 27% in individual subjects. The contribution of apoB-100 containing lipoproteins to lipaemia became more significant at later time points and the association of retinyl esters [a marker of intestinally dervived chylomicrons and their remnants] (Ruotolo et al. 1992) with apoB-100 lipoproteins was observed (Cohn et al. 1993). This might be due to production of of apoB-100 containing lipoproteins, the transfer of retinyl esters to these apoB-100 TG-rich lipoproteins from other plasma lipoproteins or inhibition of plasma clearance of apoB-100 TG-rich lipoproteins (Cohn et al. 1993). This issue is complicated by the fact that evidence has been presented showing that the intestine has the capacity to synthesize apoB-100 (Hoeg et al. 1990), in addition to synthesis in hepatocytes (Demmer et al. 1986). However it is clear from several studies (Cohn et al. 1989, 1993, Schneeman et al. 1993), that the postprandial increase in TG is predominantly due to apoB-48 containing TG-rich lipoproteins. These lipoproteins accounted for 73 to 97% of postprandial lipaemia, with the other 3 to 27% coming from apoB-100 containing TG-rich lipoporoteins (Cohn et al. 1993).

Section 1.8.2 Plasma determinants of postprandial lipaemia

Fasting TG levels are a strong predictor of postprandial lipaemia, and many studies have reported significant positive correlations between fasting TGs and the total TG response during the postprandial period (e.g. Mattock et al. 1981, Patsch et al. 1983, 1992, Patsch 1987, Weintraub et al. 1987, Cohn et al. 1988, Simpson et al. 1990, Groot et al. 1991, O'Meara et al. 1992). The correlation coefficient (extent of covariance between two interdependent variables) can be as high as 0.93 (Syvanne et al. 1994). This explains the decline of interest in postprandial lipid metabolism in the 1960s; fasting TG levels were strongly related to postprandial TG levels and to the rate of disappearance of chylomicrons

(Nestel 1964, Pelkonen & Nikkila 1965). Interest was revived when the two variants of apoB, apoB-48 and apoB-100 were discovered (Kane et al. 1980). The inverse association of fasting TG levels and the rate of chylomicron metabolism (Nestel 1964) led to the speculation that impaired chylomicron TG clearance reflects a competition of chylomicrons with VLDL for removal (Grundy & Mok 1976), since both compete for the action of LPL (Brunzell et al. 1973). This is supported by the finding of an inverse correlation between magnitude of postprandial lipaemia and post-heparin activity of LPL (Patsch 1987, Weintraub et al. 1987).

In studies of normolipidaemic individuals, LPL activity and HDL-cholesterol were strongly inversely related to postprandial TG response in the chylomicron fraction (Weintraub et al. 1987) and to magnitude of postprandial lipaemia (Mowri et al. 1994), in line with current concepts on the direct relationship between rates of chylomicron metabolism and transfer of surface components to HDL (Patsch et al. 1983, Eisenberg 1984). In studies of patients, there was no relationship between LPL activity and magnitude of postprandial lipaemia e.g. in middle-aged CAD patients (Groot et al. 1991), or in NIDDM (non-insulin-dependent diabetes mellitus) individuals (Syvanne et al. 1994), implying an abnormality in lipolysis of TG-rich lipoproteins in these groups of individuals. The relation between LPL activity and postprandial lipaemia may therefore depend on several factors; the lipoprotein fraction being considered, LPL activity, age, obesity and insulin resistance (Miesenbock & Patsch et al. 1992) and presence/absence of hyperlipoproteinaemia (Weintraub et al. 1987) and NIDDM (Syvanne et al. 1994).

Strong inverse correlations between HDL (in particular HDL_2) in a number of studies (eg. Patsch et al. 1983, 1984, Patsch 1987) or apoAI levels (Patsch et al. 1983) and magnitude of postprandial lipaemia have been reported. The relationship between HDL-cholesterol & HDL_2 and postprandial TG response appears to be stronger than that between HDL (or HDL_2) and fasting TGs (Patsch et al. 1983, 1984), leading to the proposal that low HDL-cholesterol concentrations in normolipidaeic men are caused by latent defects in TG metabolism which become apparent only upon challenge with a dietary fat load (Patsch et al. 1984) but these appear not to include defective clearance of chylomicron remnants (Cohen et al. 1992). The inverse correlation between HDL and Sf 60-400 postprandial lipoproteins

was stronger than that between HDL and Sf 20-60 postprandial lipoproteins (Karpe et al. 1993b). However this inverse relationship between postprandial lipaemia and HDL-chol levels appears to be weaker in NIDDM patients (Syvanne et al. 1993, 1994), endurance-trained men (Cohen et al. 1991) and in individuals with hypoalphalipoproteinaemia and mild HTG (Ooi et al. 1992). Characteristics such as age, obesity and fasting lipid concentrations may distort the metabolic interactions, and thus weaken the inverse relationship, between TG-rich lipoproteins and HDL. Plasma levels of LpAI (HDL particles containing only apoAI; HDL_2 is comprised mainly of these), but not LpAI:AII (HDL particles containing both apoAI and apoAII) were inversely related to magnitude of postprandial lipaemia in normolipidaemic individuals (Mowri et al. 1994). This seems to be explained by the differential associations between LpAI or LpAI:AII and lipoprotein modifying enzymes; LpAI levels were postively associated with LPL activity and inversely associated with HL activity (Mowri et al. 1994).

HL activity is a moderate positive determinant of postprandial lipaemia (Patsch 1987, Syvanne et al. 1994). In normotriglyceridaemic individuals, HL activity is inversely related to levels of chylomicrons and their remnants (Weintraub et al. 1987). HL activity was negatively correlated with the postprandial TG response in the IDL fraction in NIDDM individuals (Syvanne et al. 1993) compatible with the putative role of HL in IDL catabolism in hyperlipidaemia (Taskinen & Kuusi 1987). Hyperlipidaemia (partly due to VLDL-TG overproduction) is a characteristic of NIDDM (Taskinen 1990).

Postprandial lipaemia is associated postively with fasting levels of apoB (Patsch et al. 1983, Patsch & Gotto 1987). Dyslipidaemias which are characterised by elevated fasting apoB levels, such as FCHL (Grundy et al. 1987) and LCAT deficiency (Murano et al. 1987) - see Section 1.8.3, are also associated with elevated postprandial lipaemia. More recently the TG-rich lipoprotein response to an oral fat load was positively correlated with concentration of apoB in dense LDL. Dense protein-rich LDL is a characteristic of patients with manifest CHD (Sniderman et al. 1982, Swinkels et al. 1989, Tornvall et al. 1991, Coresh et al. 1992) and a preponderance of small LDL particles is linked to increased risk of MI (Austin et al. 1988) and presence of CAD (Campos et al. 1992).

Fasting plasma insulin levels are moderately postively correlated with magnitude of

postprandial lipaemia in individuals with CAD or NIDDM or healthy controls (Syvanne et al. 1994). This is consistent with findings that elevated insulin levels (e.g. Eschwege et al. 1985) are an independent predictor of atherosclerosis and that patients with atherosclerosis have exaggerated postprandial lipaemia (see Section 1.8.3). Cross-sectional studies have shown that NIDDM patients with atherosclerosis have higher fasting plasma levels of insulin than do those without atherosclerosis (eg. Hillson et al. 1984) and hyperinsulinaemia is an indicator of CAD in both NIDDM patients and non-diabetic subjects (Ronnemaa et al. 1991). Insulin resistance (functional insulin deficiency), as in NIDDM, leads to increases in plasma TGs and free fatty acids levels (Reaven & Reaven 1974). The main abnormality in NIDDM is in the removal of TG-rich lipoproteins, which is partially due to a decrease in LPL activity in adipose tissue (Chen et al. 1979). Insulin is a known positive regulator of adipose tissue LPL activity (Tavangar et al. 1991 and see Section 1.6.3) and in insulin resistance, hepatic TG-rich lipoprotein overproduction superimposed on reduced lipolysis of chylomicrons and VLDLs will augment postprandial lipaemia (Patsch et al. 1986).

Section 1.8.3 Relationship of postprandial lipaemia with presence and severity of atherosclerosis and dyslipidaemias

The atherosclerotic remnant hypothesis states that much of the development of atherosclerosis is related to the postprandial accumulation of cholesterol-rich remnants of both chylomicrons and VLDLs such as IDLs (Zilversmit 1979). In general, triglyceride transport is considered potentially atherogenic, with the postprandial state of challenge constituting a particularly critical and revealing phase, with a low metabolic TG capacity per se constituting increased susceptibility to atherosclerosis (Miesenbock & Patsch 1992). An excessive postprandial triglyceride response to a meal high in fat can be due to the overproduction of TG-rich lipoproteins, to inadequate lipolysis, or to abnormalities in the receptor-mediated uptake of remnant lipoproteins (Slyper 1992). In the latter instance, remnant lipoproteins will accumulate in the circulation (Weintraub et al. 1987). Epidemiological studies in humans suggest that such remnant lipoproteins (Sf 12-60) are predictors of the severity (Tatami et al. 1981, Readon et al. 1985) and progression of atherosclerosis (Steiner et al. 1987). In these studies, plasma IDL or Sf 12-60 lipoprotein cholesterol concentrations were only 10-25% that of LDL-cholesterol, which probably explains the fact that LDL was a stronger independent predictor of CAD (Tatami et al. 1981, Readon et al. 1985). However,

Krauss et al. (1987) reported that changes in IDL levels predict progression of atherosclerosis better than do changes in LDL or VLDL levels. In the St Thomas' rabbit strain, an animal model with genetically elevated levels of VLDL, IDL and LDL, remnant lipoproteins whether defined as IDL (Sf 12-20) or remnant lipoproteins (Sf 12-60) were the most powerful independent predictors of the extent of atherosclerosis (Nordestgaard & Lewis 1991). Recently, levels of small postprandial chylomicron remnants (Sf 20-60) were significantly postively related to the rate of progression of CAD in young MI survivors between angiographies. This association was independent of other factors such as levels of HDL-cholesterol, concentration of apoB in dense LDL and magnitude of postprandial lipaemia, suggesting direct atherogenic action of these remnants per se (Karpe et al. 1994).

The hypertriglyceridaemic response of an individual to a fatty meal plays a key regulatory role in governing that individual's plasma HDL cholesterol concentration and subfraction distribution (Patsch et al. 1984, Patsch 1987), which are strongly associated with severity of CAD (see Section 1.7). Evidence is accumulating that many patients with CAD have postprandial abnormalities of lipid metabolism, and that these may play a role in the etiology of their disease. Chylomicron remnant metabolism was studied in two groups of NTG individuals, with CAD and with documented minimal coronary atherosclerosis and the group with mainfest CAD showed a marked delay in both clearance of retinyl esters (= intestinally-dervived lipoproteins) and normalization of plasma TG concentrations postprandially, indicating delayed clearance (Groot et al. 1991). Patients with CAD had much higher levels of TGs in the $d < 1.006$g/ml fraction at the peak of lipaemia and higher levels of retinyl palmitate concentrations in the late postprandial period, compared to healthy individuals (Simpson et al. 1990). This indicated a reduced efficiency of metabolism of TG-rich lipoproteins of intestinal origin and an excess of atherogenic chylomicron remnants, due to delayed clearance, long after ingestion of the fat load, in the patients (Simpson et al. 1990). Both the maximal TG increase and magnitude of postprandial lipaemia were higher in patients with CAD compared to controls and late single postprandial TG levels at 6 and 8 hours after a standardized test meal were highly discriminatory (Patsch et al. 1992).

The metabolic behaviour of $VLDL_1$ (Sf 60-400) and $VLDL_2$ (Sf 20-60) in the postprandial phase was studied in normal-weight and obese individuals, who consumed a

typical mixed meal (Fisher et al. 1993). The similar TG/apoB and cholesterol/apoB ratios in the $VLDL_1$ and $VLDL_2$ subfractions in the control and obese individuals demonstrated that the abnormality in the obese group was an elevated number of VLDL particles of normal composition which may predispose these individuals to CHD. This ties in with the fact that elevated levels of TG (and hence elevated levels of VLDL) are an important risk factor for CHD and that degree of obesity is postively correlated with plasma TG levels (Castelli 1986). Hence obese individuals are at risk from CHD (Hubert et al. 1983, Garrow 1991). Obesity is associated with insulin resistance which leads to significantly higher plasma insulin levels (Olefsky et al. 1982). Plasma insulin levels are, in turn, highly correlated with production of TG and apoB in VLDL (Howard et al. 1987) and hyperinsulinaemia increases the risk of CHD (eg. Pyorala et al. 1979).

Patients with NIDDM have an abnormal fasting lipoprotein profile which includes overproduction of VLDL-TG (Taskinen 1990) and these individuals are at several-fold higher risk of CAD (Bierman 1992). Levels of atherogenic postprandial lipoproteins particularly in the Sf 60-400 range (the $VLDL_1$ fraction) are increased in patients with NIDDM; impaired lipolytic processing of chylomicrons and ineffective hepatic removal of remnant particles both appear to contribute to this (Syvanne et al. 1994). However magnitude of postprandial lipaemia did not distinguish NIDDM patients with CAD from those without CAD. Thus abnormal postprandial lipid metabolism in NIDDM appears not to be related to CAD.

Damage, dysfunction or metabolic alterations of the vascular endothelium have been implicated in the patheogenesis of atherosclerosis (DiCorleto & Chisolm 1986, Scharf & Harker 1987) and high levels of circulating TG-rich lipoproteins were causally implicated in endothelial injury (Zilversmit 1976, DiCorleto & Chisolm 1986). Free fatty acid anions, which are produced from the LPL-mediated hydrolysis of TG-rich lipoproteins (see Section 1.6), have been hypothesized to cause endothelial injury by allowing increased penetration of remnants into the arterial wall (Zilversmit 1973, 1976). However, until recently the underlying mechanism(s) of injury to the vascular endothelium by TG-rich lipoproteins have not been elucidated. Exposure of cultured endothelial cells to free fatty acids resulted in increased transfer of both albumin and LDL across the endothelium (Hennig et al. 1984, 1985). In-vitro exposure of endothelial cell monolayers to lipolyzed but not oxidized

lipoproteins resulted in morphological perturbations of these cells (Hennig et al. 1992). These authors suggested that lipolytic remnants of TG-rich lipoproteins (but not native lipoproteins) may be important in the development of atherosclerosis by decreasing the barrier function of the vascular endothelium (evidenced by a significant increase in albumin transfer across endothelial monolayers); this remnant-induced injury of the arterial wall may permit the entry of cholesterol-rich lipolytic remnants as well as LDL into the arterial wall (Hennig et al. 1992). The net difference between influx into and efflux from the arterial intima, of lipoproteins may be an important atherogenic indicator. This hypothesis has been tested in both humans (Wootton et al. 1987) and in the St. Thomas' rabbit strain (Nordestgaard et al. 1992). A scheme for lipoprotein/arterial wall interactions was summarized (Nordestgaard & Tybjaerg-Hansen 1992), with a number of factors determining rate of arterial influx; these are size and concentration of lipoprotein species and arterial wall characteristics; the greater the extent of atherosclerosis, the greater the influx (Nordestgaard et al. 1992). Permeability is directly dependent on concentration and inversely dependent on particle size; large particles of diameter > 75nm seem to be excluded from entering the arterial wall (Nordestgaard & Zilversmit 1988). HDL has the highest arterial permeability into the intimia, and it is able to penetrate beyond the internal elastic lamina and thus has access to the entire arterial wall, while most of the LDL particles that penetrate the artery wall leave the intimia via the lumen of the artery (Nordestgaard et al. 1990).

Individuals with both HTG and hypoalphalipoproteinaemia (hypoalpha = low HDL-cholesterol level) but normal LDL-cholesterol levels in the fasting state, exhibited enhanced postprandial lipaemia in both the chylomicron and non-chylomicron fractions compared to NTG controls; this may account for the increased CAD risk in the HTG/low HDL syndrome (Ooi et al. 1992, Simo et al. 1993). Chylomicron remnant metabolism was normal in NTG men with low HDL-chol levels, suggesting that defects in chylomicron-TG clearance giving rise to excess postprandial lipaemia are not a common occurance in these individuals. Other factors may be more important than postprandial lipid metabolism in causing the low HDL-cholesterol levels observed in these men (Cohen et al. 1992). This is corroborated by another recent study (Miller et al. 1993) where NTG and hypoalpha subjects did not show higher peak levels of postprandial TGs or delayed postprandial response to a fat load compared to age-matched controls. These results suggest that a low HDL-cholesterol level

is not necessarily a marker of abnormal fat clearance, as previously suggested (Patsch 1987), if it is accompanied by a normal fasting TG level (Miller et al. 1993). Hypoalpha is characterized by low apoAI levels due to enhanced catabolism and reduced synthesis (Brinton et al. 1991). This reduced synthesis of apoAI may, therefore, affect intestinal TG-rich lipoprotein production because apoAI appears to be important in the formation of TG-rich lipoproteins (Schonfeld et al. 1978). Miller et al. (1993) suggest that hypoalpha is a distinct phenotype with a reduced rather than an increased number of postprandial TG-rich lipoproteins and that these lipoproteins have an abnormal composition (protein depletion and cholesterol & phospholipid enrichment), in concordance with observations in an individual with apoAI deficiency (Norum et al. 1986). An association between hypoalpha and atherosclerosis exists (Miller et al. 1990, 1992), but not all individuals with low HDL are at equal risk (Grundy et al. 1989) and the mechanism(s) underlying the association between hypoalpha and atherosclerosis is at present unknown.

Increased postprandial lipaemia or delayed clearance of remnant lipoproteins has also been reported in patients with Type IIa (increased LDL levels), III (increased β-VLDL levels) or IV (increased VLDL levels) hyperlipoproteinaemia (HLP) compared to healthy controls (Weintraub et al. 1987). These authors used retinyl palmitate as a marker of intestinally dervived lipoproteins. Type IIa patients had significantly lower levels of chylomicrons, indicating accelerated clearance of these lipoproteins, and normal levels of chylomicron remnants; the mechanism for this is unexplained. The chylomicron remnant response was much greater in Type III patients due to greatly delayed removal of these lipoproteins; this is compatible with the concept that Type III HLP is a chylomicron remnant removal disorder, due to the presence of apoE2 (discussed in Section 1.4.7). In these individuals chylomicron levels were intermediate between normal and Type IV; this may be due to a block in the conversion of chylomicrons to remnants, or inhibition of this conversion by high concentrations of remnants; this is supported by observations that the β-VLDL in type III patients is not as suspectible to lipolysis as normal VLDL (Chait et al. 1978). Type IV patients had considerably delayed clearance of both chylomicron and their remnants; this could be due either to defective TG hydrolysis or to competition between exogenous and endogenous TGs for the same hydrolysis pathway.

Delayed clearance of chylomicron remnants and smaller, more TG-rich lipoproteins in the Sf>1000 fraction have been reported in familial combined hyperlipaemia (FCHL) compared to controls (Castro Cabezas et al. 1993a). The impairment of chylomicron remnant clearance was significantly associated with elevated fasting plasma TG and apoCIII levels. Several mechanisms may be responsible for delayed chylomicron remnant clearance in FCHL. Firstly, chylomicrons and VLDLs both share a common metabolic pathway involving LPL (Brunzell et al. 1973) and there is evidence that TG-rich lipoproteins of both exogenous and endogenous origin compete for LPL (Weintraub et al. 1987, Castro Cabezas et al. 1993a). Secondly apoCIII is thought to inhibit LPL-mediated lipolysis by counteracting the action of apoCII (Brown & Baginsky 1972 and discussed earlier in Sections 1.5.2 and 1.6.3) and apoCIII also reduces apoE binding to lipoprotein receptors (van Berkel et al. 1983 and discussed in Section 1.5.2). Thirdly, Castro Cabezas et al. (1993a) suggest that in FCHL, the overproduction of VLDL-apoB (a hallmark of FCHL - Grundy et al. 1987) results in an increased number of circulating atherogenic particles, that compete for the action of LPL. This, in turn, causes reduced hepatic clearance of remnant particles, therefore promoting premature atherosclerosis, another characteristic of FCHL (Grundy et al. 1987). The characteristic hepatic overproduction of atherogenic apoB-containing lipoproteins in FCHL may be explained by elevated postprandial fatty acid concentrations observed in FCHL individuals compared to controls (Castro Cabezas et al. 1993b). This is in concordance with increased delivery of fatty acids to hepatocytes in-vitro resulting in increased synthesis and secretion of VLDL (Cianflone et al. 1990).

Postprandial HTG has been observed in individuals with lecithin-cholesterol acyltransferase (LCAT) deficiency (Frohlich et al. 1988); the major abnormality in postprandial lipid metabolism in these individuals is a retarded removal of chylomicron remnants in the density range of VLDLs and LDLs (ie. <1.006 to 1.019g/ml) (Gylling & Miettinen 1993). LCAT deficiency is characterized by an inability to esterify plasma cholesterol, presence of structurally abnormal apoB-100 lipoprotein particles in plasma and presence of HDL particles containing abundant amounts of apoE. These apoE-rich HDL particles compete with chylomicron remnants for the same receptor-mediated catabolic pathway (Norum et al. 1989), and could be one of the mechanisms underlying retarded removal of chylomicron remnants.

For all the above cases of atherosclerosis or dyslipidaemia, the underlying genetic variation, if any, causing increased postprandial lipaemia are unknown, although candidate genes are those encoding apolipoproteins and enzymes involved in lipid metabolism.

Section 1.8.4 Associations of magnitude of postprandial lipaemia with genetic variation

Large interindividual differences in postprandial responses to a standard fat meal (eg. Brown and Roberts 1991b) could be partially explained by genetic factors that modulate postprandial lipaemia. Specific genetic mutations giving rise to dysfunctional proteins, have been implicated as the cause of elevated postprandial lipaemia in two recent studies. An R173C substitution in apoAI gives rise to $apoAI_{Milano}$, associated with decreased HDL-cholesterol levels (Franceschini et al. 1980). Abnormally small HDL_3 particles comprise the major component of the HDL in carriers, while plasma HDL_2 levels are reduced (Franceschini et al. 1987). Carriers have a higher prevalence of HTG (Gualandri et al. 1985). The magnitude of postprandial lipaemia was three times greater in the $apoAI_{Milano}$ carriers, but after adjustment for different fasting TGs, it was similar to control subjects and catabolism of TG-rich lipoproteins was normal (Calabresi et al. 1993). The capacity of $apoAI_{Milano}$ HDL to accept TG from TG-rich lipoproteins was significantly reduced compared to controls, which was identified by almost identical rises in HDL-TG in $apoAI_{Milano}$ carriers and controls, despite the much greater rise of TG-rich lipoproteins in the $apoAI_{Milano}$ carriers. The two groups had similar postprandial changes in both LCAT and CETP activities, leading these authors to conclude that the reduced heteroexchange of CEs and TGs between HDLs and TG-rich lipoproteins was due to the defective HDL itself (Calabresi et al. 1993). Both <u>in-vitro</u> (Franceschini et al. 1990) and <u>in-vivo</u> (Calabresi et al. 1993) studies support the hypothesis that a primary alteration in HDL i.e. the presence of covalently-linked dimeric apoAI forms, markedly affects the interaction between HDL and TG-rich lipoproteins

The second case involves heterozygosity for a G188E missense mutation in LPL; carriers exhibited clearly reduced LPL activity, normal fasting TGs, but pronounced postprandial lipaemia (more than twice as great as controls) (Miesenbock et al. 1993), confirming a previous report (Paulweber et al. 1991). Glu188 carriers had a greatly elevated chylomicron level over the entire postpandial period, suggesting that chylomicrons were converted to remnants at reduced rates, consistent with the view that it is the initial step of

chylomicron metabolism which critically depends on LPL action (Stalenhoef et al. 1984, Sprecher et al. 1991, Miesenbock et al. (1993) proposed the term 'impaired TG tolerance' for these individuals to emphasize that a tolerance test (the oral fat load) is required to uncover heterozygosity for Glu188. Although the carriers' impaired TG intolerance became overt in the postprandial state only, it is associated with a fasting lipoprotein constellation characterized by TG-enriched HDL_2, reduced HDL_2 cholesterol, CE-enriched VLDL & IDL, increased levels of IDL and small LDL (Miesenbock et al. 1993). Low fasting HDL_2 chol levels are strongly indicative of pronounced postprandial lipaemia or impaired TG tolerance (Patsch et al. 1983, Patsch 1987).

The low frequency of these genetic conditions implicates that the underlying mutations are not responsible for a large fraction of the genetic variability in posprandial metabolism in the general population. Because of its recognized importance in remnant clearance, the apoE gene has been targeted for the study of postprandial lipid response (Mahley & Rall 1989). Consistent with the known diminished receptor-binding affinity of apoE2, several studies have shown a reduction in the clearance of postprandial lipoproteins in carriers of the apoE2 isoform (Gregg et al. 1986, Breninkmeijer et al. 1987, Weintraub et al. 1987, Brown & Roberts 1991, Superko & Haskell 1991, Boerwinkle et al. 1994). Fasting TG levels are higher in E4/3 individuals compared to E3/3 individuals (Brown & Roberts 1991, Dallongville et al. 1992), this in turn, is related to the magnitude and duration of postprandial lipaemia (Cohn et al. 1988, Brown & Roberts 1991). Homozygosity for apoE2 was associated with a strong continuing rise in the level of intestinal TG-rich lipoproteins and their remnants up to eight hours postprandially, while in individuals with one copy of apoE2 or with other apoE genotypes, levels dropped after a maximum at five hours postprandially (Brenninkmeijer et al. 1987). Differences between apoE2/2 individuals and others occured in both heparin-unbound (containing mainly nascent VLDLs) and heparin-bound (containing mainly apoE-rich and cholesterol-rich remnants of chylomicrons and VLDLs) fractions. The difference in the heparin-bound fraction was particularly striking; corresponding to poor clearance of remnants containing only the apoE2 isoform.

Recently the postprandial TG and retinyl palamite (RP) responses of approximately. 400 Caucasian individuals with apoE genotypes E2/3, E3/3 and E4/4 were investigated

(Boerwinkle et al. 1994). There were no statistical differences in average TG responses among genotypes, though it was higher in E2/3 and lower in E4/3 individuals compared to E3/3 individuals. In constrast, postprandial RP responses (measure of intestinally dervived lipoproteins, which have apoE as their sole ligand for receptors) were strikingly different amongst these apoE genotypes; average RP response was very similar for apoE3/3 and apoE4/4 but approx. 33% higher in the E2/3 group eight hours after the oral fat load. The average RP response in the apoE2/2 individuals was even higher than that of apoE2/3, potentially indicating an additive effect of the number of apoE2 alleles on postprandial response. The effect of apoE polymorphism on postprandial responses was consistent between Caucasians and Blacks (Boerwinkle et al. 1994). The proposed mechanism underlying increased RP responses in apoE2 carriers is the defective clearance of remnant lipoproteins of intestinal origin due to the well-known reduced binding affinity of apoE2 to receptors (Weisgraber et al. 1982). The subsequent up-regulation of the LDL-receptor (Weintraub et al. 1987, Boerwinkle & Utermann 1988) would 'mop up' some of the excess remnants in apoE2 heterozygotes but it would have little effect in apoE2 homozygotes. Boerwinkle et al. (1994) calculated that about 7% of the interindividual variation in postprandial RP response was attributable to the apoE polymorphism, exceeding the 4% of interindividual variation in fasting LDL-cholesterol attributable to apoE genotype. This, together with the similar average postprandial RP response, after adjustment for fasting TG levels, between apoE3/3 and apoE4/3 groups, indicate that the effects of the common apoE polymorphisms on remnant and LDL metabolism do not fully explain the observed association of the apoE4 isoform with CAD (discussed in Section 1.4.7).

Section 1.9 Intentions and aims of the studies described in this thesis

Associations between raised fasting triglycerides, atherosclerosis and common polymorphisms at the apoB, apoE, apoAI-CIII-AIV and LPL gene loci have been reported, in particular associations concerning the X+ allele of the apoB-XbaI polymorphism, the rare allele of the SstI polymorphism 3' of apoCIII and the H+ allele of the LPL-HindIII polymorphism. It was, therefore, decided to generate hypotheses of associations between a large range of common genetic variation and fasting lipids, lipoproteins and LPL activity and atherosclerosis for testing on population-based samples. This hypothesis generation, and elucidation of possible underlying mechanisms, using a group of young myocardial infarction

survivors and healthy age-matched individuals from Sweden is described in **Chapter 3.** The hypothesis that common genetic variation at the above four gene loci is associated with severity and progression of atherosclerosis was tested in an independent patient sample from London **(Chapter 4).**

A number of epidemiological studies have documented a strong positive association between magnitude of postprandial lipaemia and atherosclerosis; this was examined in **Chapter 5** on a subsample of the Swedish individuals used in **Chapter 3,** to test the hypothesis that common genetic variation, at the above four gene loci, is modulating magnitude of postprandial lipaemia and that this explains at least part of the assocation observed between common genetic variation and severity of atherosclerosis.

Chapter 6 describes the search for variation in the exons of the LPL gene, that is in linkage disequilibrium with the H+ allele of the HindIII polymorphism, in selected individuals from the Swedish sample. The subsequent screening of the Swedish population samples for the Asp291 to Ser substitution in exon 6, the search for associations with fasting and postprandial lipids and lipoproteins and eludication of the possible effect of this mutation are also described.

Descriptive data for the Swedish sample
Means ± standard errors

Trait (unit)	Healthy individuals	Patients
Age (years)	40.5 ± 0.40 (n=93)	40.4 ± 0.38 (n=87)
Body mass index (Kg/m^2)	24.6 ± 0.26 (n=92)	26.4 ± 0.35 (n=87) *
Total cholesterol (mmol/l)	6.1 ± 0.12 (n=90)	7.2 ± 0.14 (n=87) *
Total triglycerides (mmol/l)	1.5 ± 0.14 (n=90)	2.6 ± 0.19 (n=87) *
apoAI (mg/dl)	124.5 ± 2.04	111.4 ± 2.01 *
apoB (mg/dl)	106.5 ± 1.88	125.8 ± 2.23 *
Post-heparin lipoprotein lipase activity (nmol of fatty acid released/minute/ml plasma)	92.1 ± 3.64 (n=64)	74.3 ± 4.16 (n=36) *

* significantly different between the healthy individuals and patients ($p<0.01$)

Descriptive data for the St. Thomas Atherosclerosis Regression Study.
Means ± standard errors. Taken from Watts et al. (1992)

Characteristic	Usual Care only (n=24)	Diet (n=26)	Diet plus cholestyramine (n=24)
age (years)	53.9 ± 1.6	48.9 ± 1.6	49.6 ± 1.5
body mass index (Kg/m^2)	26.5 ± 0.6	26.0 ± 0.4	26.0 ± 0.5
Current smokers (%)	29.2	26.9	20.8
History of hypertension (%)	8.3	7.7	8.3
Family history of early CHD (%)	45.8	50.0	41.6
Previous MI (%)	41.7	46.1	54.2
angina at entry (%)	75.0	69.2	79.2
In-trial cholesterol (mmol/l)	6.93 ± 0.20	6.17 ± 0.09	5.56 ± 0.16
In-trial TGs (mmol/l)	2.35 ± 0.20	1.85 ± 0.20	2.21 ± 0.22
In-trial LDL-cholesterol/HDL-cholesterol	4.10 ± 0.25	3.74 ± 0.14	2.87 ± 0.19

CHAPTER TWO - SUBJECTS, MATERIALS AND METHODS

Section 2.1

Population samples screened for polymorphisms at apoB, apoAI, apoCIII, apoE and LPL gene loci

Section 2.1.1 Swedish myocardial infarction survivors and healthy individuals

A group of 87 patients and 90 age-matched healthy individuals, all male, recruited from Stockholm County, Sweden for an angiographic study of the mechanisms associated with progression of coronary atherosclerosis (Hamsten et al. 1986, 1987). They had all suffered a first MI before the age of 45 years. This group excluded those who had manifest diabetes, familial hypercholesterolaemia or other major concomitant disease such as uraemia and collagenosis. Reangiographies were done after the first angiography on 74 of the patients (mean time between angiographies = 5 years, 2 months, SD = 9 months; range 3.5 to 6.8 years). Procedures for coronary angiography, details of the scoring systems and baseline and progression scores of atherosclerosis in the patients have been described (Hamsten et al. 1986, 1987)). Characteristics of patients and healthy individuals with respect to lipids, lipoproteins and lipase activity have been previously described (see papers bound to the back of this thesis).

For the postprandial study (Chapter 6 in this thesis), a subgroup of the above population was used, described previously (Karpe et al. 1992, 1993a, 1993b, 1994). Briefly, there were thirty-three male myocardial survivors and eleven healthy individuals. More than five years had elapsed between the MI and the postprandial study in all post-infarction patients (Karpe et al. 1992).

Section 2.1.2 St Thomas' Atherosclerosis Regression Study (STARS)

A group of 74 patients who completed this study to which 90 male patients (age < 66 years, plasma cholesterol level above 6.1mM but not above 10mM and had not been treated with lipid-lowering drugs) were recruited. Details of the patients, study design, methodologies and treatment effects have been reported (Watts et al. 1992). Eligible patients had fasting plasma lipids and lipoproteins measured and coronary angiography (baseline measurements) prior to being randomly assigned to one of three treatment groups; usual care only (U), a lipid-lowering diet (D) or diet plus cholestyramine (DC) each with an

DNA preparation by the salting-out method;

Sucrose lysis buffer contains 0.32M sucrose, 10mM Tris pH 7.5, 5mM $MgCl_2$ and 1% Triton-X-100.

Nuclei lysis buffer contains 10mM Tris-HCl pH 8.2, 0.4M NaCl and 2mM Na_2 EDTA (the disodium salt of EDTA [ethylenediaminetetra-acetic acid]).

approximately equal number of individuals. Coronary angiography was carried out at entry and after approximately 39 months (Watts et al. 1992). The end-points used were change in mean absolute width of coronary segment (ΔMAWS) which is a measure of diffuse CAD and change in minimum absolute width of coronary segment (ΔMinAWS) which is a measure of focal CAD.

Sections 2.2 to 2.11 Methods

Section 2.2 DNA preparation - by the salting-out method

DNA was extracted from 5mls of thawed-out whole blood, collected after an overnight fast, using a salting-out prodecure (Miller et al. 1988). This method involved several steps (carried out on ice until the SDS was added): 1) Addition of ice-cold sucrose lysis buffer to lyse the leucocytes followed by centrifugation to collect the intact nuclei. 2) Addition of nuclei lysis buffer to lyse the nuclei followed by centrifugation to collect the DNA-protein complexes. 3) SDS and proteinase K were added and the mixture incubated overnight at 37°C. The next day, saturated NaCl was added and after the centrifugation step, the supernatant was collected. At least 3 volumes of 100% ethanol were added to the supernatant to precipitate out the DNA. Excess salt was removed by dipping the DNA briefly in 70% ethanol and the DNA was finally dissolved in TE buffer. DNA concentration was determined by taking spectrophotometric readings of the optical density (OD), using TE buffer as a blank, at 260nm and 280nm in quartz cuvettes. The 260nm reading gave the concentration of DNA present where;

concentration in ug/ml = OD_{260} x dilution factor x conversion factor (50 for genomic DNA and 20 for oligonucleotides)

The ratio OD_{260} /OD_{280} gave an estimate of the purity of the DNA preparation and is normally between 1.6 and 2.0 (ideally 1.8). Protein impurities cause the OD_{260} /$0D_{280}$ ratio to rise towards 2.

Section 2.3 DNA amplification - polymerase chain reaction (PCR)

PCR-amplification (Saiki et al. 1988) was performed in automated thermal cyclers (Cambio, Cambridge, UK and Genetic Research Instrumentation Ltd, Essex, UK). The standard PCR was in a total volume of 50μl, containing approximately 50ng genomic DNA

as template, 200ng each primer and 0.5 unit *Thermus aquaticus* (Taq) DNA polymerase (Perkin Elmer/Cetus CT. USA. and Gibco-BRL, Paisley, UK) in a buffer consisting of 50mM KCl, 10mM Tris/HCl (pH 8.3), 0.001% Gelatin, 0.1% W-1 Detergent, 0.2mM each dNTP (Pharmacia, Uppsala, Sweden) and 1.0 to 4.0mM $MgCl_2$ depending on the primers used. The PCR was overlaid with 50μl paraffin to prevent evaporation. Primer sequences and PCR conditions used for amplification of restriction fragment length polymorphisms (RFLPs) used in this thesis are shown in Table 2.3.1 Table 2.3.2 shows the primer sequences and conditions required for the signal peptide polymorphism of apoB and Figure 2.3 shows the PCR products obtained, from amplification of DNA from individuals with different genotypes, run on a 10% polyacrylamide mini-gel. The sequences of the primers and PCR conditions used to amplify exons of the LPL gene are shown in Table 2.3.3.

Section 2.4 Restriction Enzyme Digestion; detection of restriction fragment length polymorphisms (RFLPs)

PCR-amplified DNA was digested with restriction enzymes, according to the manufacturer's instructions, in the appropriate buffer either supplied by the manufacturer or from a five buffer set (Boehringer Mannheim, UK) with the addition of 100ng/μl BSA (Gibco-BRL, Paisley UK). Digestions were carried out overnight in a water bath at the recommended temperature (37°C for all the restriction endonucleases used in this thesis). For RFLP analysis, 10-15μl of the PCR reaction was digested in a total volume of 20μl with 2ul 10x buffer, 1ug 100ng/μl BSA and 10 units of thte appropriate enzyme added.

Restriction digested DNA was normally electrophoresed on 1.5-2% agarose gels (see section 2.5.1) containing 0.5μg/ml Ethidium bromide in 1xTAE buffer and subsequently visualized on a UV (260nm) transilluminator. Figures 2.4.1 to 2.4.4 (apoB RFLPs), figure 2.4.5 (the common apoE alleles by HhaI digestion of PCR-amplified DNA, Hixson & Vernier 1990), figure 2.4.6 and 2.4.7 (apoAI-CIII-AIV RFLPs) and figure 2.4.8 (LPL) show the electrophoretic patterns obtained on agarose gels, after digestion with the appropriate restriction endonuclease, and run for 1 to 2 hours at 70V.

Table 2.3.1 RFLPs used in this thesis

Polymorphism	Sequence of PCR primers (from 5' to 3') and reference	PCR program and conditions used	Size of PCR product (bp)	Size of fragments after digestion
ApoB				
5'-MspI	CAATTCAGTCCAGGAGAAGCA CAGCAACCGAGAAGGGCACTCAG Xu et al. 1990 Peacock et al. 1992a	1 x 93°C 5 mins 58°C 5 mins 30 x 92°C 1 min 58°C 5 mins Phial mode Final Mg^{2+} = 1-1.5mM	405	C_{-265} 179, 96, 76 and 54 T_{-265} 275, 76 and 54
AluI in exon 14	GGAAATCCATATTTACTTGGGGTGC CTTCTATTTTGGCTGAGGCTGGGTC Wang et al. 1988	1x 95°C 5 mins 55°C 3 mins 72°C 3 mins 35x 98°C 1 min 55°C 1 min 72°C 1.5 mins Plate mode Final Mg^{2+} = 1.5mM	223	Val_{591} 167 and 60 Alu_{591} 117,50 and 60
XbaI in exon 26	GGAGACTATTCAGAAGCTAA GAAGAGCCTGAAGACTGACT Boerwinkle et al. 1990	1 x 93°C 5 mins 58°C 5 mins 30-35 x 92°C 1 min 58°C 5 mins Phial mode Final Mg^{2+} = 4mM	709	X- 709 X+ 433 and 276
EcoRI in exon 29	CTGAGAGAAGTGTCTTCGAAG CTCGAAAGGAAGTGTAATCAC Boerwinkle et al. 1990	1 x 93°C 5 mins 58°C 5 mins 30-35 x 92°C 1 min 58°C 5 mins Phial mode Final Mg^{2+} = 1-1.5mM	480	R+ 253 and 227 R- 480
ApoE isoforms	CTGGGCGCGGACATGGAGGACGT GATGGCGCTGAGGCCGCGCTCG Myant et al. 1991	1 x 95°C 5 mins 67°C 2.5 mins 30-35 x 95°C 45 secs 67°C 1.5 mins Phial mode Final Mg^{2+} = 1.5mM	223	E2 91 and 83 E3 91, 48 and 35 E4 72, 48, 35 and 19 Constant fragments of 18, 17 and 14 b produced

Table 2.3.1 (continued)

ApoAI-CIII-AIV

Polymorphism	Sequence of PCR primers (from 5' to 3') and reference	PCR program and conditions used	size of PCR product (bp)	Size of fragments after digestion
3'ApoAI-PstI	GAGCGCTCTCGAGGAGTACAC GACTGGCTTCCACTGCTGTGC Wiseman et al. 1991	1 x 95°C 5 mins 35 x 95°C 1 min 54°C 1 min 72°C 2 mins Phial mode Final Mg^{2+} = 1.0mM	740	P+ 400 and 340 P- 730
ApoCIII-PvuII	CCAGTGTGAAAGGCTGAGATGG ACCTGCACTTGGAGCCACTTC Wiseman et al. 1991	1 x 95°C 5 mins 35 x 95°C 1 min 55°C 1 min 72°C 2 mins Phial mode Final Mg^{2+} = 1.0mM	613	V- 367 and 246 V+ 367, 129 and 117
LPL				
PvuII	ATGGCACCCATGTGTAAGGTG GTGAACTTCTGATAACAATCTC Peacock et al. 1992b	1 x 93°C 5 mins 58°C 5 mins 30 x 92°C 1 min 58°C 5 mins Phial mode Final Mg^{2+} = 1.8mM	440	V+ 330 +110 V- 440
HindIII	TTTAGGCCTGAAGTTTCCAC CCCAGAATGCTCACCAGC Peacock et al. 1992b	1 x 96°C 5 mins 55°C 1.5 mins 72°C 5 mins 35-40x 98°C 1 min 55°C 1 min 72°C 3 mins Plate mode Final Mg^{2+} = 1.5mM	c. 1250	H+ c. 625 + 625 H- c. 1250

Sequences of primers are from 5' to 3'. The upper and lower sequences are for the left and right hand primers respectively.
For apoE isoforms, the yield of PCR was greatly improved by using only 100ng per primer in some cases.

Table 2.3.2 Length polymorphisms used in this thesis

Polymorphism	Sequence of PCR primers and reference	PCR program and conditions	size of PCR products
ApoB signal peptide	CAGCTGGCGATGGACCCGCCGA ACCGGCCCTGGCGCCCGCCAGCA Xu et al. 1990	1 x 95°C 5 mins 85°C 8 mins (add Taq polymerase here) 70°C 5 mins 5 x 94°C 1 min 66°C 30 secs 72°C 30 secs 45 x 92°C 45 secs 66°C 25 secs 72°C 30 secs Phial mode Final Mg^{2+} = 1mM	'insertion' allele - 93bp 'deletion' allele - 84bp PCR products were checked on a 3% agarose gel

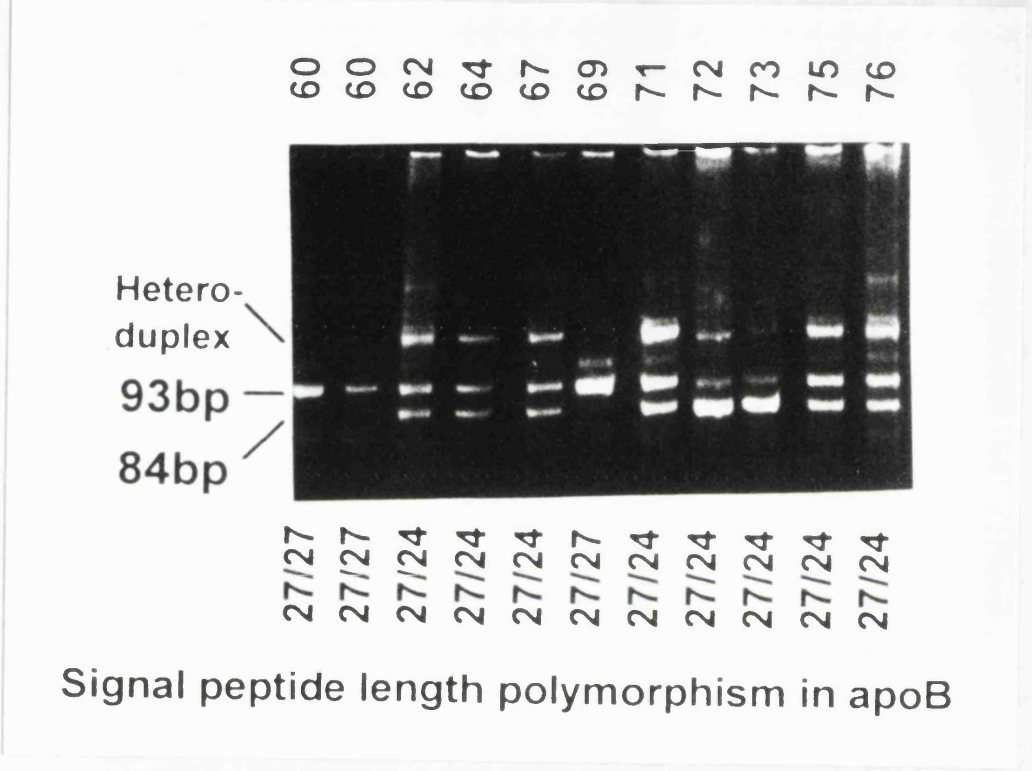


Figure 2.3

Photograph of gels showing electrophoresis of PCR-amplified products for different genotypes of the apoB signal peptide length polymorphisms. The PCR products were resolved on a 10% polyacrylamide gel run at approximately 50mA for 45 minutes. ID numbers (for STARS sample) and genotypes are shown above and below the photograph, respectively.

Gel was run on a 'Mighty Small' unit - refer to p.162

Table 2.3.3. Oligonucleotide primers used for PCR amplification of LPL exons 2 to 9.

Exon	Left hand primer	Right hand primer
2	5' CTCCAGTTAACCTCATATCC 3'	5' CACCACCCCAATCCACTC 3'
3	5' GCTTGTGTCATCATCTTCAG 3'	5' TCCCAGTCTTACCTCCATC 3'
4	5' GCAGAACTGTAAGCACCTT 3'	5' TGCTTTCTTACAGTAATTCTG 3'
5	5' TTACAAATCTGTGTTCCTGCT 3'	5' TTAAGAAAGCTTGTGTCATCA 3'
6	5' GTGGACCAGCTAGTGAAG 3'	5' GCATGATGAAATAGGACTCC 3'
7	5' AAGATTGATCAACATGTT 3'	5' ACTGCTGCCATGATGACCGC 3'
8	5' TTTAGGCCTGAAGTTTCCAC 3'	5' GGGGGTCTAAAGTGAAGG 3'
9	5' TGTTCTACATGGCATATTCAC 3'	5' GCTCACCAGCCTGACTTC 3'

Sequences of oligonucleotide primers are taken from Oka et al. 1990.



Figure 2.4.1

Photograph of gel showing electrophoresis of fragments obtained after digestion of PCR-amplified DNA with the restriction endonuclease, MspI, for different genotypes of the C_{-265} to T polymorphism in the apoB promoter. The schematic representation of the PCR products, not to scale, shows the PCR products obtained for the 'C' and 'T' alleles. The variable cutting site is starred. Sizes of fragments on photograph and schematic representation are shown as number of base pairs. ID numbers (for Swedish sample) and genotypes are shown above and below the photograph, respectively.

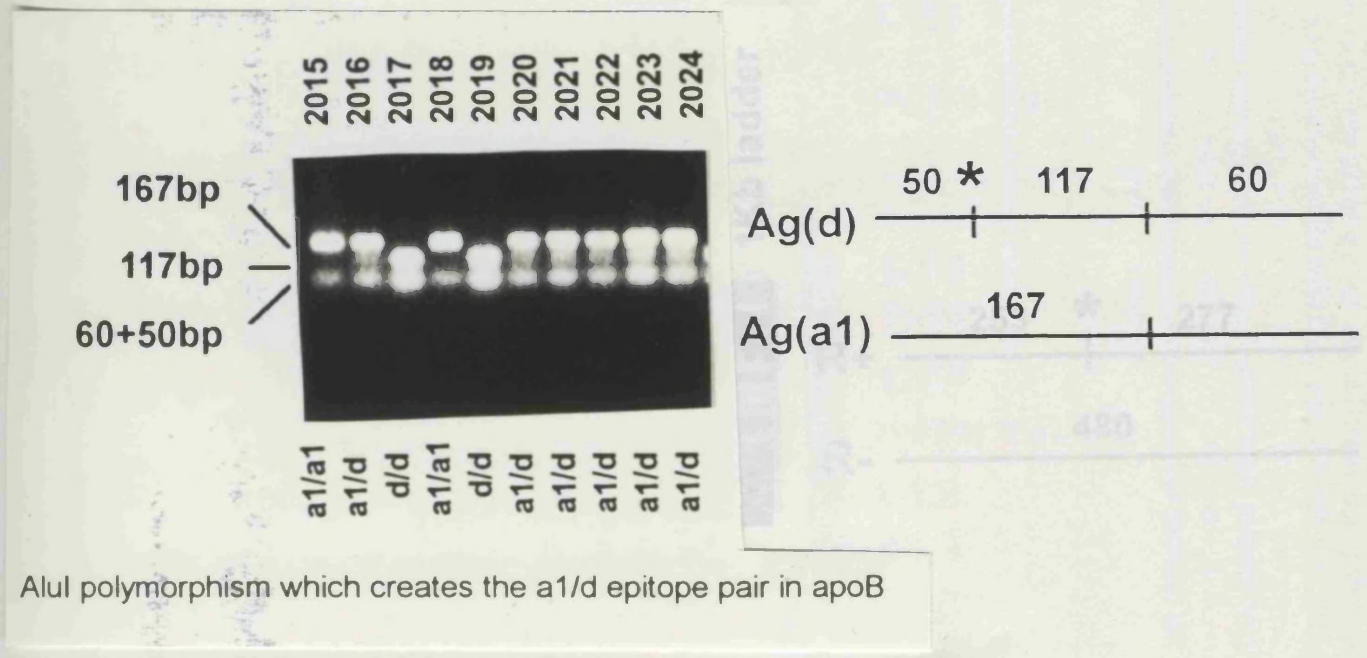


Figure 2.4.2

Photograph of gel showing electrophoresis of fragments obtained after digestion of PCR-amplified DNA with the restriction endonuclease, AluI, for different genotypes of the Ag (a1/d) epitope pair in exon 14 of the apoB gene. The schematic representation, not to scale, shows the PCR products obtained for the Ag (d) and Ag (a1) alleles. The variable cutting site is starred. Sizes of fragments on photograph and schematic representation are shown as number of base pairs. ID numbers (for Swedish sample) and genotypes are shown above and below the photograph, respectively.

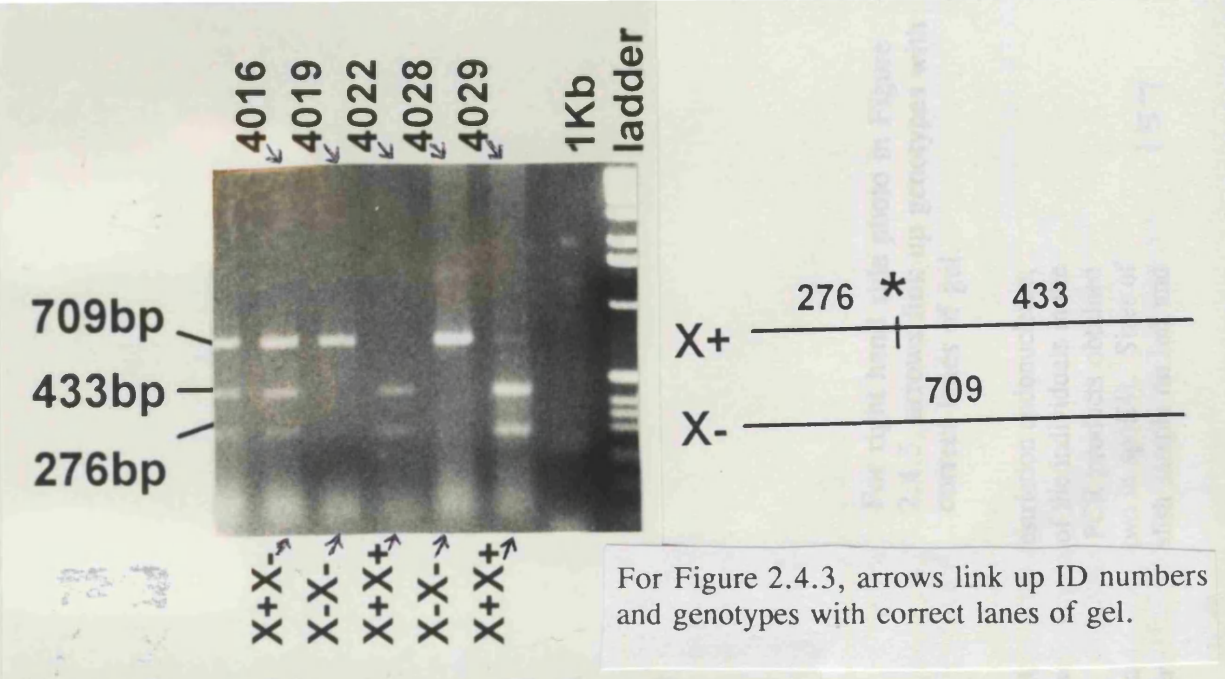


Figure 2.4.3

Photograph of gel showing electrophoresis of fragments obtained after digestion of PCR-amplified DNA with the restriction endonuclease, XbaI, for different genotypes of the XbaI polymorphism in exon 26 of the apoB gene. The schematic representation, not to scale, shows the PCR products obtained for the X+ and X- alleles. The variable cutting site is starred. Sizes of fragments on photograph and schematic representation are shown as number of base pairs. ID numbers (for Swedish sample) and genotypes are shown above and below the photograph, respectively.

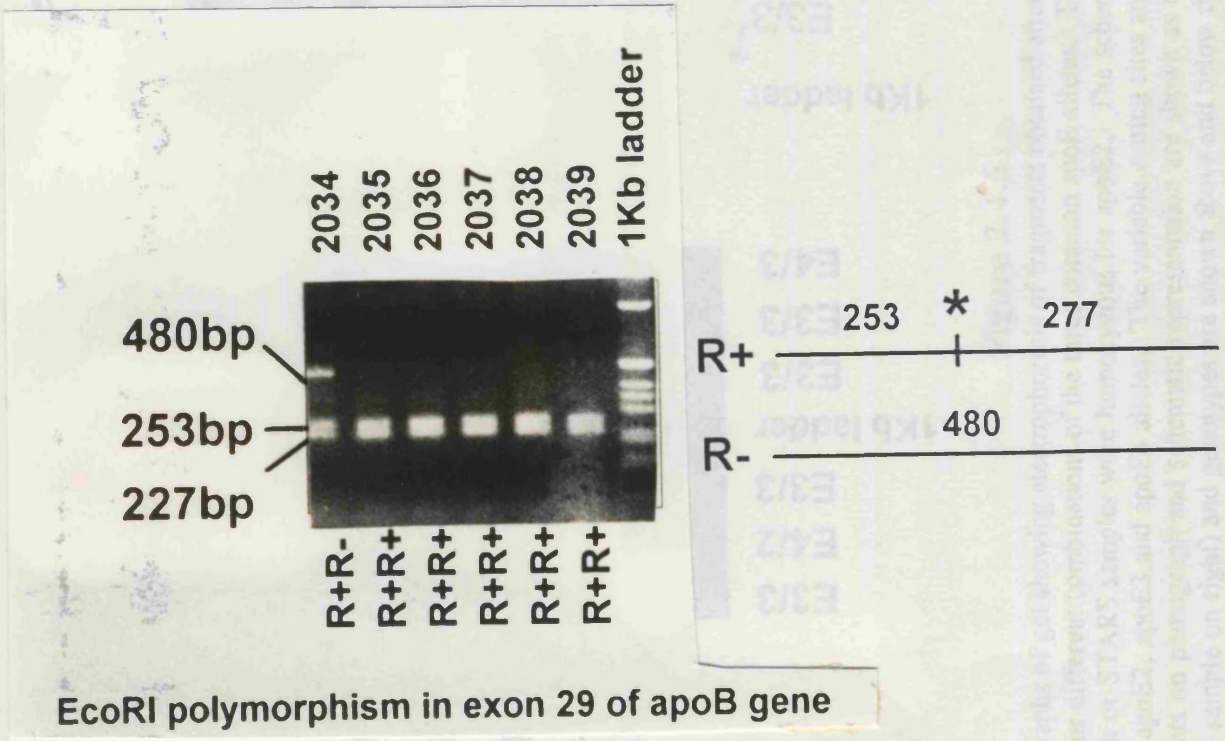
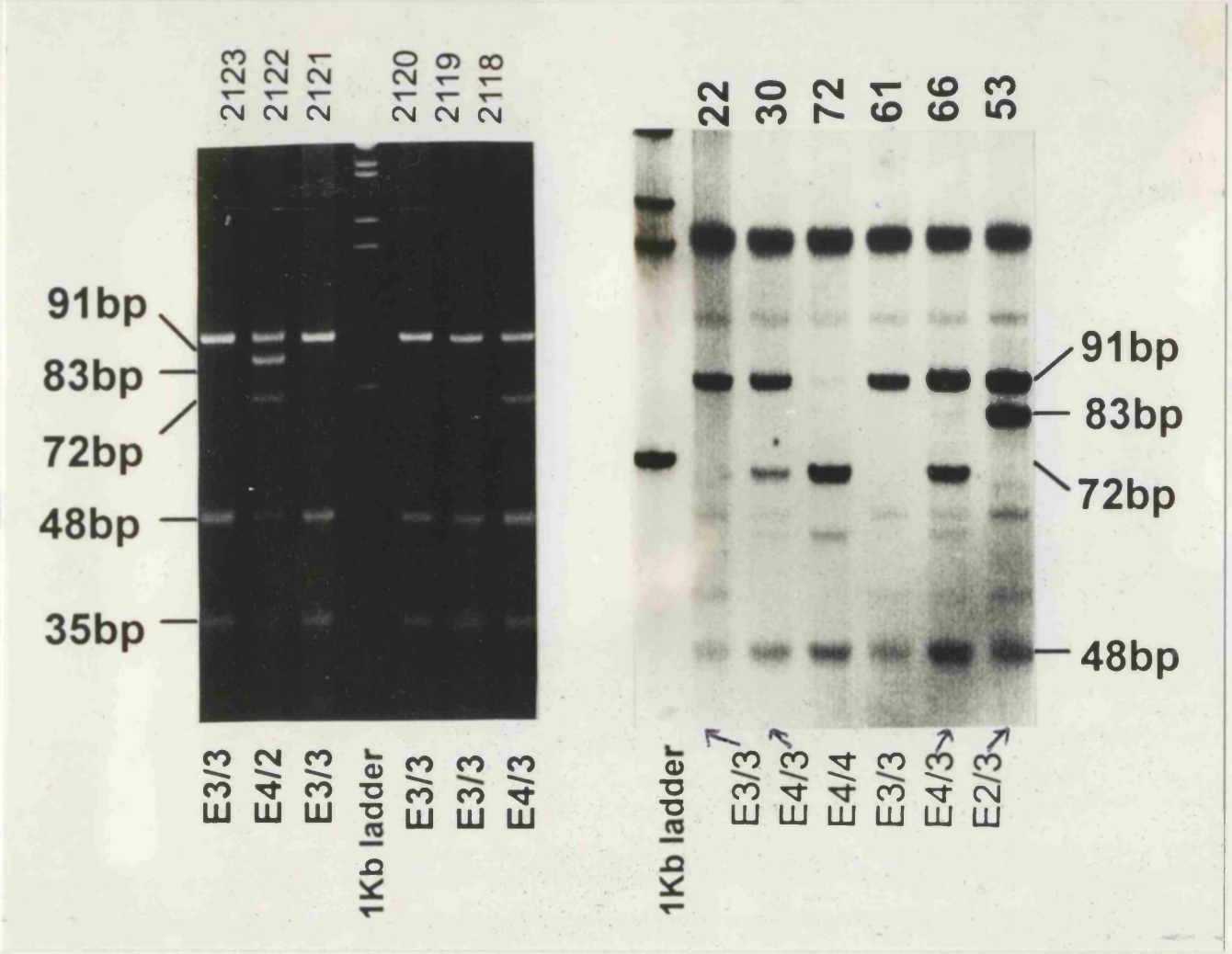
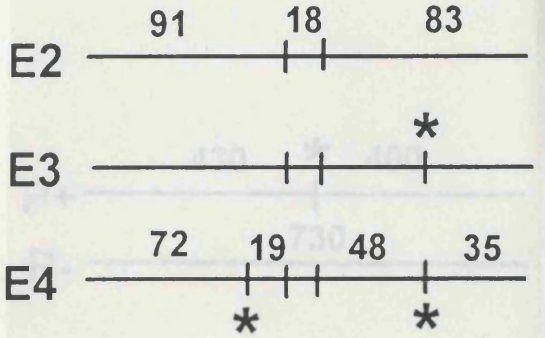


Figure 2.4.4

Photograph of gel showing electrophoresis of fragments obtained after digestion of PCR-amplified DNA with the restriction endonuclease, EcoRI, for different genotypes of the EcoRI polymorphism in exon 29 of the apoB gene. The schematic representation, not to scale, shows the PCR products obtained for the R+ and R- alleles. The variable cutting site is starred. Sizes of fragments on photograph and schematic representation are shown as number of base pairs. ID numbers (for Swedish sample) and genotypes are shown above and below the photograph, respectively.

The left hand side photo was taken under UV illumination after ethidium bromide staining of the gel. The right hand side photo was taken under visible light illumination after silver staining of the gel (procedure described in Section 2.5.3, pages 162-163).





For right hand side photo in Figure 2.4.5, arrows link up genotypes with correct lanes of gel.

Figure 2.4.5

Photographs of gel showing electrophoresis of fragments obtained after digestion of PCR-amplified DNA with the restriction endonuclease, HhaI, for different combinations of the three common apoE alleles. The rare genotype E2/2 is not shown, as none of the individuals in the Swedish or STARS samples were homozygous for apoE2. The schematic representation, not to scale, shows the PCR products obtained for the apoE2, apoE3 and apoE4 alleles. The variable cutting sites are starred (none in apoE2, one in apoE3 and two in apoE4). Sizes of fragments on photograph and schematic representation are shown as number of base pairs. ID numbers (for Swedish sample on left and STARS sample on right) and genotypes are shown above and below the photograph, respectively.

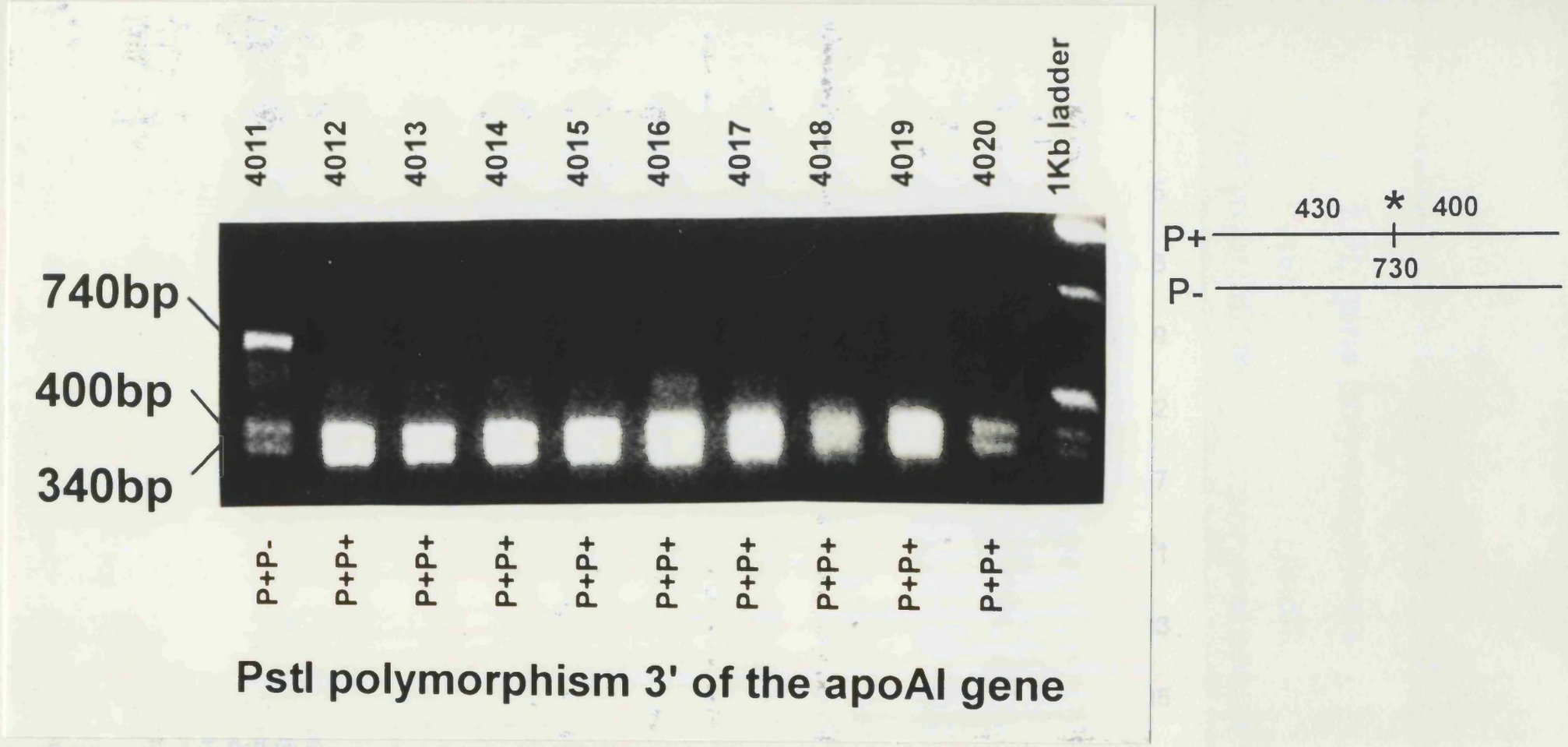
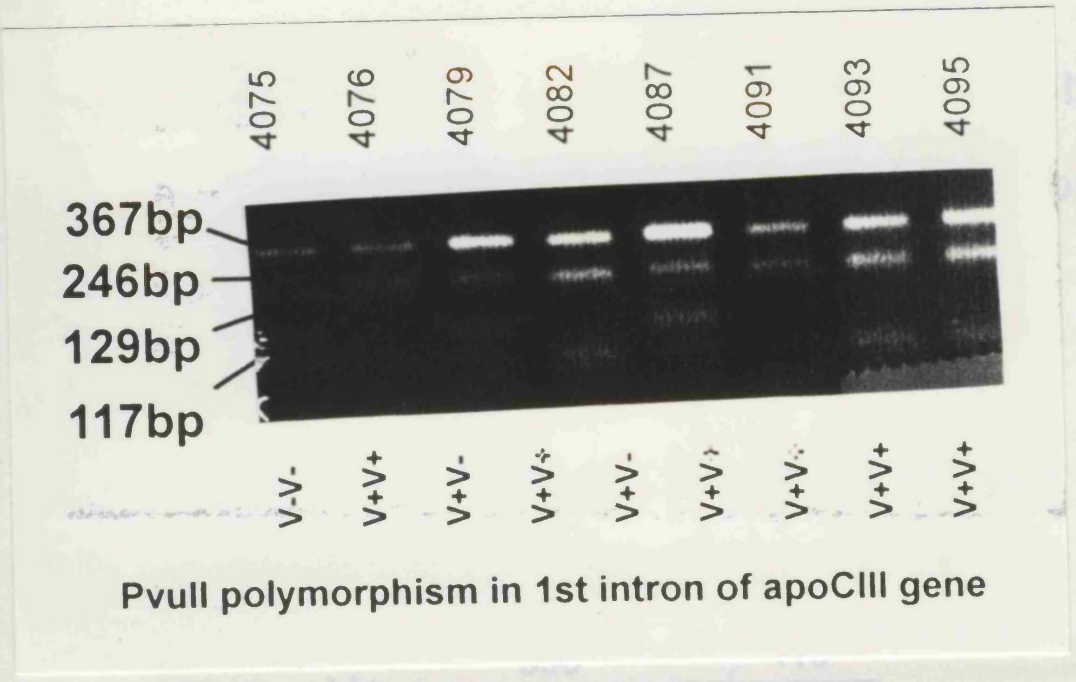


Figure 2.4.6

Photographs of gel showing electrophoresis of fragments obtained after digestion of PCR-amplified DNA with the restriction endonuclease, PstI, for different genotypes of the PstI polymorphism 3' of the apoAI gene. The schematic representation, not to scale, shows the PCR products obtained for the V+ and V- alleles. The variable cutting site is starred. Sizes of fragments on photograph and schematic representation are shown as number of base pairs. ID numbers (for Swedish sample) and genotypes are shown above and below the photograph, respectively.



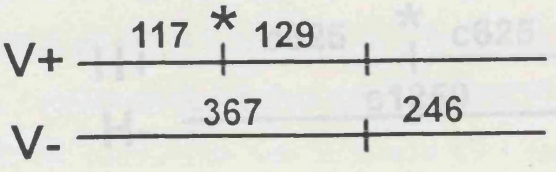
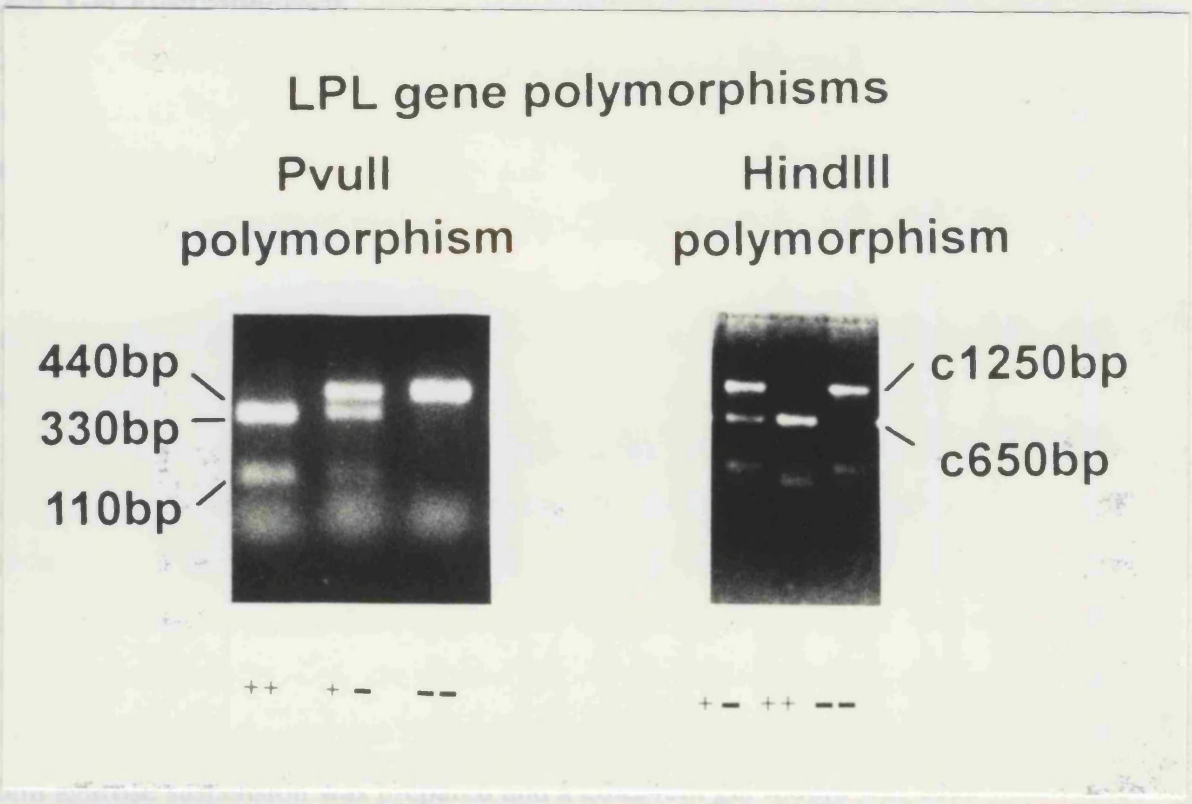


Figure 2.4.7

Photograph of gel showing electrophoresis of fragments obtained after digestion of PCR-amplified DNA with the restriction endonuclease, PvuII, for different genotypes of the PvuII polymorphism in the 1st intron of the apoCIII gene. The schematic representation, not to scale, shows the PCR products obtained for the V+ and V- alleles. The variable cutting site is starred. Sizes of fragments on photograph and schematic representation are shown as number of base pairs. ID numbers (for Swedish sample) and genotypes are shown above and below the photograph, respectively.

This photo was representative of the quality of results obtained with this polymorphism; genotypes were scored from both the gel and photo.



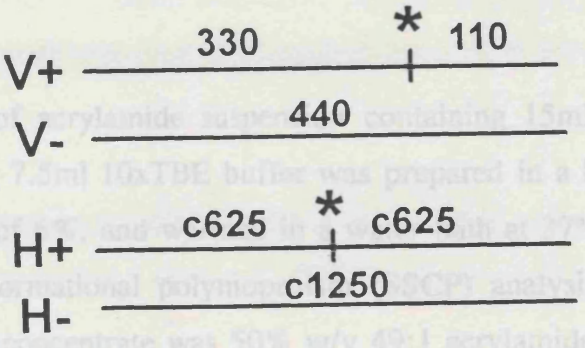


Figure 2.4.8

Photograph of gels showing electrophoresis of fragments obtained after digestion of PCR-amplified DNA with PvuII or HindIII, respectively, for different genotypes of the PvuII and HindIII polymorphisms in the LPL gene. The schematic representation, not to scale, shows the PCR products obtained for the V+ and V- alleles of the PvuII polymorphism and the H+ and H- alleles of the HindIII polymorphism. The variable cutting site is starred. Sizes of fragments on photograph and schematic representation are shown as number of base pairs. Genotypes are shown below the respective lanes.

Section 2.5 Gel Electrophoresis

Section 2.5.1 Agarose

100ml 1.5-3% (w/v) agarose suspension was prepared and boiled in a microwave oven until a homogenous solution was obtained. This was cooled under a running tap to about 60°C, cool enough to hold flask in the hand for several seconds, and poured into a 10x10cm gel mould (midi size), taped at the ends, with two sets of wells. Gelling took 30-60 minutes and gel was ready for transfer to the horizontal bed of a perspex tank filled with 1xTAE with or without ethidium bromide, so that the gel was completely submerged by TAE buffer. 5-10μl of PCR-amplified DNA was mixed with 1ul 6x loading buffer (15% Ficoll 400 (w/v) containing either xylene cyanol or bromophenol blue as a tracking dye) and loaded into each well of the checking gel prior to running for 20-30 minutes at 100 volts. A band of the correct size was obtained if the PCR amplification had worked. If not, new batches of the reagents (particularly water) were used and/or the reaction conditions such as Mg^{2+} concentration, annealing temperature and template-primer ratio were modified. For maxi gels, 250ml agarose suspension was prepared and a 20x24cm gel mould was used with combs each containing 30 wells.

Section 2.5.2 Acrylamide

For sequencing, 75ml of acrylamide suspension containing 15ml 30% w/v 19:1 acrylamide:bis, 31.5g urea and 7.5ml 10xTBE buffer was prepared in a beaker, to give a final acrylamide concentration of 6%, and warmed in a water bath at 37°C to dissolve the urea. For single strand conformational polymoprhism (SSCP) analysis, 5 - 8% non-denaturing gels were used; the concentrate was 50% w/v 49:1 acrylamide:bis and 10% of the final volume was glycerol. For both sequencing and SSCP, 75μl TEMED and 75μl 25% w/v ammonium persulphate were added to allow polymerisation to occur. Two large glass plates, one shorter than the other, were washed with detergent and water, then ethanol until spotless, and the shorter plate was siliconized with Repelcote™ on one side in the fume cupboard. These plates were then taped together, separated by spacers 0.4mm thick. The taped plates were tilted from the horizontal and the acrylamide solution was poured slowly down one side using a 50ml syringe or a 25ml pipette, to fill the plate sandwich up from the bottom without forming air bubbles. Two shark's tooth combs (13cm) with the flat side down were inserted and the plates were clamped using fold-back spring clips. The gel was

allowed to set at room temperature for 1-2 hours before loading. The tape and combs were removed before fixing the gel onto the gel apparatus. The flat loading surface of the gel was washed out and the combs reinserted with the teeth downwards. Just before loading the wells were flushed out again, essential with a denaturing sequencing gel, to remove urea. The loading buffer used was the commerically manufactured 'Stop solution' containing formamide and both tracking dyes (United States Biochemical Corp., Cleveland, USA).

Polyacrylamide gels were electrophorsed in 1xTBE buffer at a constant power of 65W for 2 to 5 hours for sequencing and 12-15W overnight for SSCP. Gels were 40cm x 30cm x 0.4mm. The gel was transferred onto Whatman-3MM chromatographic paper, dried under vacuum and exposed to Hyperfilm β-max (sequencing) or Hyperfilm-MP (SSCP) (Amersham UK) for 16 - 72 hours.

For electrophoresis of PCR products for the apoB signal peptide polymorphism and Hha1-digests of PCR products for the common isoforms of apoE, 10% polyacrylamide gels (80x70x1mm) with a 19:1 acrylamide:bis ratio were used in a 'Mighty Small' II 7cm dual Vertical Slab gel electrophoresis unit (Hoefer Scientific Instruments, USA) run at a constant current of 50mA until the bromophenol blue dye reached the bottom of the gel (about 40 mins).

For both agarose and acrylamide gels, if a DNA size marker was required, the 1Kb size marker (BRL Life Technologies Inc.) was used. This consisted of 23 fragments ranging in size from 75bp to 12,216bp.

Section 2.5.3 Silver staining of small polyacrylamide gels

A modified version of the silver staining protocol (Merril et al. 1981) was used to stain polyacrylamide gels. After completion of electrophoresis, the gel was washed for 3 minutes in (10% ethanol 0.5% acetic acid) twice. Next it was soaked in silver nitrate at a concentration of 1g/l for 10 minutes and subsequently rinsed three times in water. Developing of the gel was done by soaking in a solution containing 3g NaOH, 20mg sodium borohydride and 800μl formaldehyde per 200mls for 20 minutes. Finally fixation was by a

10 minute soak in 7.5g sodium carbonate per litre. The gel was placed on a visible light transilluminator and a photo taken. Gels were also dried onto Whatmann-3M chromatographic paper under vacuum.

Section 2.6 Allele Specific Oligonucleotides (ASOs) - radioactive labelling and separation using Sephadex G-25 columns

ASOs were used to detect single base changes where a restriction endonuclease recognition site was neither created nor destroyed. The melting temperature in °C was empirically calculated from the formula; temperature = 2 (number of A-T pairs) + 4 (number of C-G pairs). ASO's were 5'-phosphorylated with (gamma-^{32}-P) dATP (Amersham, UK) and T4 polynucleotide kinase (GIBCO BRL, UK). This labelling reaction was carried out in a total volume of 100μl containing 1x kinase buffer (made as a 10x stock containing 0.5M Tris HCl pH 7.6, 0.1$MgCl_2$, 50mM DTT, 1mM Spermidine and 1mM EDTA), 10-20 pmole of oligonucleotide, 3-5μl (gamma-^{32}P) dATP (10μCi per μl) and 15 units of T4 kinase. The reaction was incubated at 37°C for 1 hour; 100μl water was added and the ^{32}P-labelled oligo was separated from unincorporated gamma-^{32}-P dATP using a Sephadex G-25 (beads presuspended in TE buffer) column spun at 1500rpm for 3 minutes. 5μl of the eluted ^{32}P-end labelled oligo was counted in a scintillation counter; if the reaction had worked the total activity was approximately 8000 to 10,000 cpm, translating to approximately 1.25μCi of radioactivity per pmole oligonucleotide; assuming that about half of the (gamma-^{32}P) dATP was incorporated onto the end of the oligonucleotide during the T4 kinase-catalysed reaction. Labelled oligo was stored at -20°C until required.

Table 2.6 shows the primer sequences required for PCR-amplification, PCR conditions and sequences of ASOs, hybridization and washing conditions for all the ASOs used in this thesis.

Section 2.7 Southern Blotting and Hybridization

Section 2.7.1 Denaturing and Southern Blotting

After completion of electrophoresis, the gel was soaked in denaturing solution (0.5M NaOH, 1.5M NaCl on a rotary shaker for 30-40 minutes to separate the DNA strands and

Table 2.6 ASO's used in this thesis

Polymorphism	PCR primers and reference	PCR program and conditions	size (bp)	sequences of ASO's and reference	melting point	Hybridiz -ation temp.	Washing temp.
ApoAI G_{-75} to A	AGGGACAGAGCTGATCCTTGAACTCT TAAG TTAGGGGACACCTACCCGTCAGGAAG AGCA Sigurdsson et al. 1992	1 x 95° 5 mins 35 x 95°C 1 min 55°C 1 min 72°C 2 mins Phial mode. Final Mg^{2+} = 1.5mM	433	CAACAGGGCCAGGGCTG (A_{-75}) CAGCCC CGGCCCTGTTG (G_{-75}) Talmud et al. 1994	58 60	54	59
ApoCIII C_{1100} to T	CAATGGGTGGTCAAGCAGGAGC GAGCACCTCCATTCCATTGTTGG Xu et al. 1994	1 x 95°C 5 mins 35 x 95°C 1 min 55°C 1 min 72°C 1.5 mins Phial mode. Final Mg^{2+} = 1.0mM	299	ATGCAGGCTACATGAA (C_{1100}) TTCATGTAACCCTGCAT (T_{1100}) Xu et al. 1994	44	43	49
LPL Asn-291 to Ser	CTGCCGAGATACAATCTTG GCATGATGAAATAGGACTCC Mailly et al. (submitted)	1 x 97°C 5min 75°C 5min 1 x 98°C 1.5min 70°C 1min 72°C 3mins Subsequent cycles - same denaturation and extension temps. but reducing annealing temp; 1 x 65°C 2 x 61°C, 3 x 58°C 32 x 55°C (all cycles 1-1.5mins at 72°C) 1 x 72°C 6mins ('Touchdown' program reported in Oka et al. 1990) Plate mode. Final Mg^{2+}=1.5mM	c.300	TGACTTTATTGATCTCA (A_{1127}) GACTTTACTGATCTCA (G_{1127}) This study	44 44	33	42

subsequently rinsed in distilled water. For each side of the gel, the Hybond N+ membrane (Amersham, UK) was laid on top of the gel and air bubbles were smoothed out. Well positions were marked on membranes using a permanent black marker with well positions. Two sheets of pre-soaked Whatman 3M paper (soaked in 2xSSC) (again with air bubbles excluded) and a pile, 7-8cm high, of dry paper towels were laid flat on top of the membrane. A glass plate and weight (approximately 400g) were put on top of the whole towel-paper-membrane-gel sandwich and blotting took place overnight. The next morning, the PCR-amplified DNA was fixed on to the membranes by soaking them in denaturing solution for 15-30 minutes depending on size of DNA fragments and finally rinsed briefly in 5xSSC. Membranes were stored wrapped in Saran film at 4°C.

Section 2.7.2 Hybridisation of Southern Blots with ASOs, washing and autoradiography

Filters were hybridised for 2-5 hours in 5xSSPE / 0.5%SDS / 5x Denhardt's solution with 20-40μl (1 to 2.5μCi radioactivity) ^{32}P-labelled probe per 3-5 mls hybridisation solution (for 90-180 samples per hybridisation bottle). Hybridisation was performed in tubes in a rotating oven (Bachoffer GmBH, Germany) at 5-8°C below the melting temperature for the oligonucleotide, and then washed in 2xSSPE / 0.1% SDS for 5 - 10 minutes at the melting temperature of the ASO. Autoradiography was carried out for 16 to 72 hours at -70°C on X-ray (Fuji Ltd, UK) using intensifying screens. Figures 2.7.2.1 (apoAI), 2.7.2.2 (apoCIII), 2.7.2.3 and 2.7.2.4 (LPL) show the results of autoradiography after exposure of ^{32}P-labelled filters to X-ray film at -70°C.

Section 2.8 Direct sequencing of PCR-amplified DNA

This method utilised streptavidin coated magnetic beads (Dynal AS, Norway) following the recommended protocol. PCR was carried out using one normal primer and the other 5' labelled with biotin. 40μl of the PCR product was subsequently mixed with an equal volume of Dynabeads (Dynal AS, Norway) in a solution of 10mM Tris-HCl pH 7.5, 1mM EDTA and 2.0M NaCl and incubated at room temperature for 15 mins. The supernatant was then removed whilst holding the beads in the tube with a magnet (Dynal AS, Norway). The beads were washed once in 40μl of 10mM Tris-HCl pH 7.5/ 1mM EDTA / 1.0M NaCl and then resuspended in 8μl 0.1M NaCl and incubated for 10 mins. After removal of this

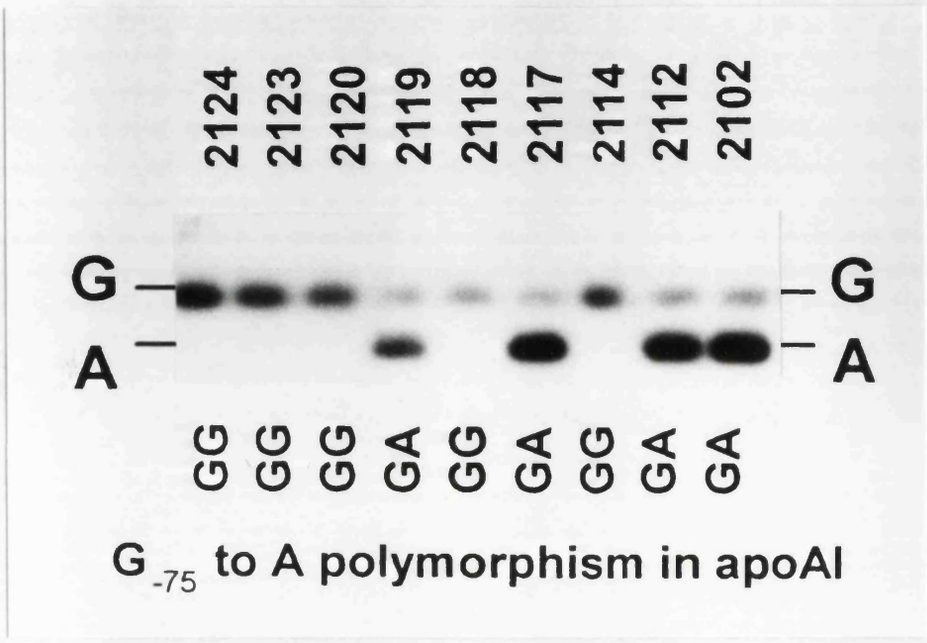


Figure 2.7.2.1

Photograph of autoradiogram showing an example of the results obtained after hybridization of ASOs recognising the alleles for G_{-75} (top) and A_{-75} (bottom) of the G_{-75} to A polymorphism in the apoAI promoter. ID numbers (for Swedish sample) and genotypes are shown above and below the photograph, respectively.

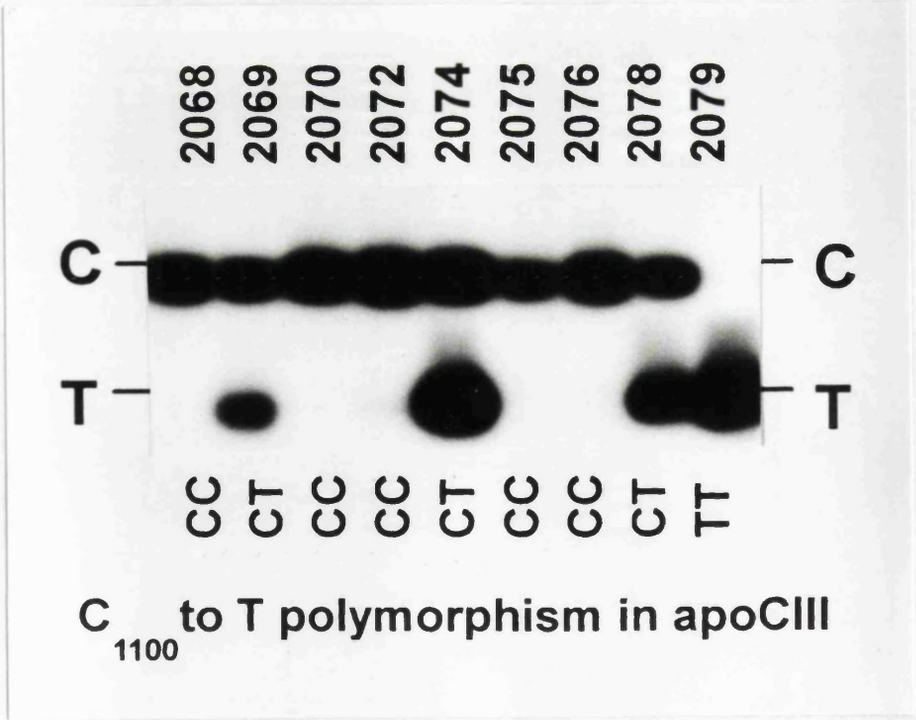


Figure 2.7.2.2

Photograph of autoradiogram showing an example of the results obtained after hybridization of ASOs recognising the alleles for C_{1100} (top) and T_{1100} (bottom) of the C_{1100} to T polymorphism in the apoCIII gene. ID numbers (for Swedish sample) and genotypes are shown above and below the photograph, respectively.

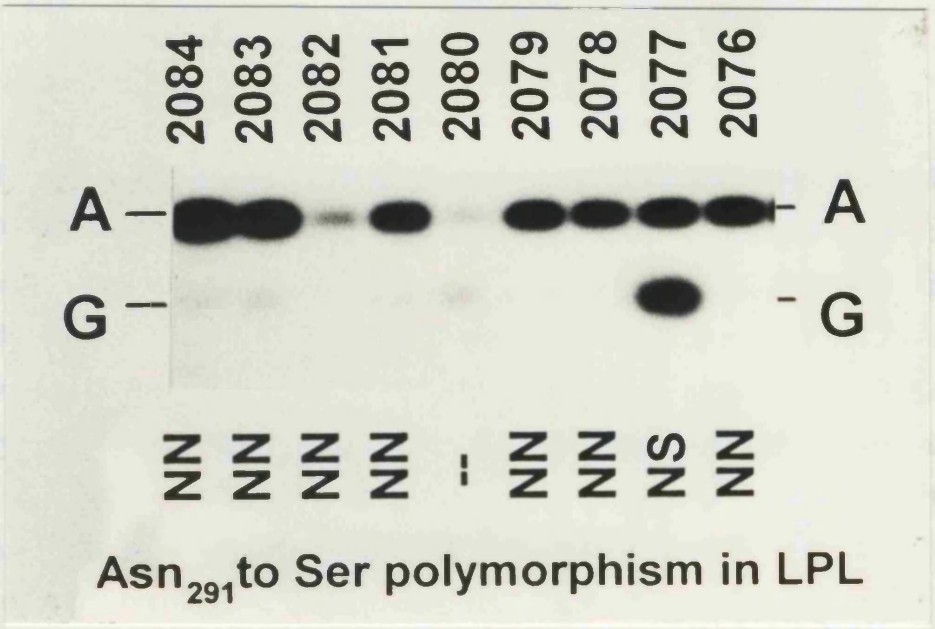


Figure 2.7.2.3

Photograph of autoradiogram showing an example of the results obtained after hybridization of ASOs recognising the alleles for A_{1127} (top) and G_{1127} (bottom) of the Asn (A_{1127}) to Ser (G_{1127}) substitution at residue 291 in exon 6 of the LPL gene. ID numbers (for Swedish sample) and genotypes are shown above and below the photograph, respectively.

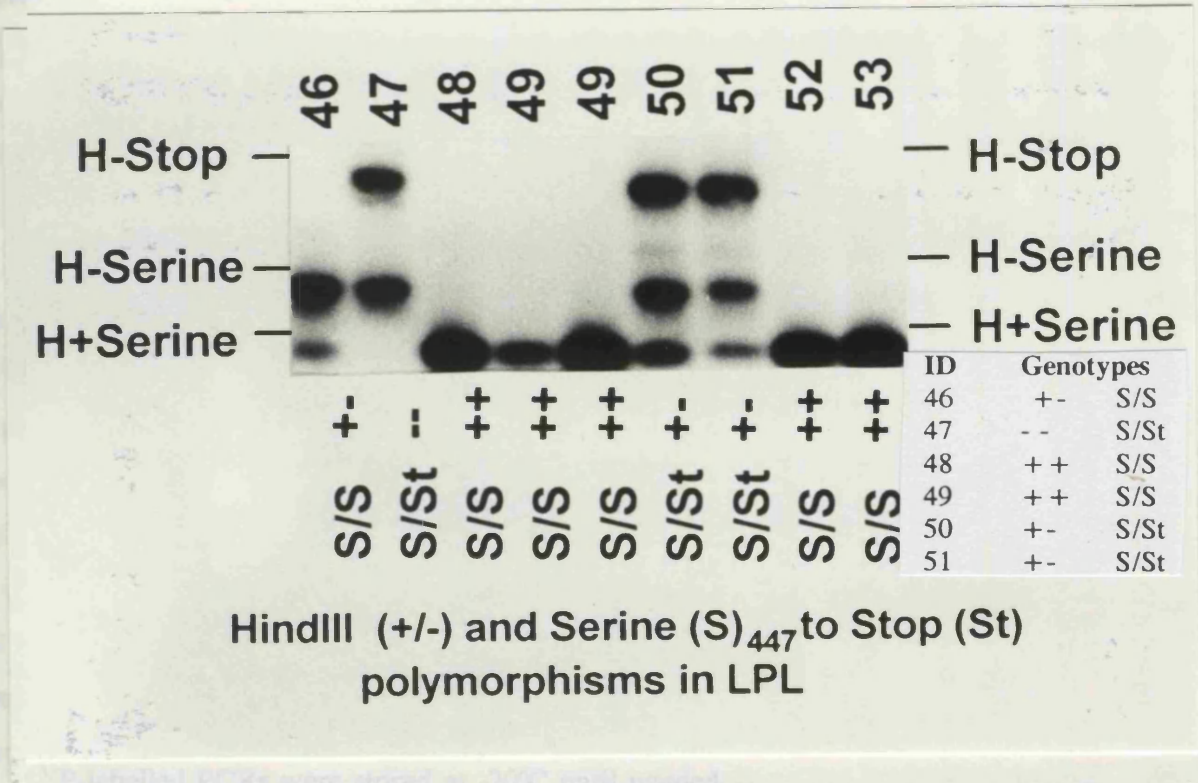


Figure 2.7.2.4

Photograph of autoradiogram showing an example of the results obtained after ASO detection of the Serine to Stop polymorphism at residue 447 in exon 9 of the LPL gene, using Southern Blot of HindIII-digested DNA; the same PCR primers and conditions were used for both the HindIII and Serine to Stop polymorphisms. Four of the six possible haplotype combinations of the HindIII and Serine to Stop polymorphisms are shown; the two not shown are [S/S H-H-] and [St/St H-H-], which were both found to exist unequivocally in the Swedish and STARS samples. ID numbers (for STARS sample) and genotypes are shown above and below the photograph, respectively.

denaturing solution, the beads were washed successively with 50μl each of: 0.1M NaOH, 10mM Tris-HCl pH 7.5 / 1mM EDTA / 1.0M NaCl and TE buffer. After removal of the final wash, beads (attached to the single strand of PCR DNA with the biotin-labelled primer at the 5' end) were resuspended in 7μl TE and sequenced using modified T7 DNA polymerase (Sequenase, USB, Cleveland USA) following the manufacturer's protocol, based on the dideoxy method described by Green et al. (1989). Annealing was carried out in a total volume of 10μl with 200ng amplified fragment (approx 0.5pmol) and 20pmol oligonucleotide primer in Sequenase buffer containing 0.5% NP-40 (Sigma, UK). The annealing mixture was boiled at 65°C for 2 mins and allowed to cool slowly over 30 mins. This was followed by the labelling and termination reactions. 5μCi (0.5μl) $\alpha(^{35}S)$ dATP (Amersham, UK), 1.0μl 0.1M dithiothreitol, 2.0μl diluted labelling mix (containing dNTPs) and 2.0μl 8x diluted sequenase was added to each annealed primer/template. After 5 mins incubation at room temperature, 3.5μl labelling reaction was added to each of four tubes containing 2.5μl of the particular ddNTP. This was followed by 3 - 5 mins incubation at 37°C that was terminated by adding 4μl of stop solution (95% formamide / 4% EDTA / 0.5% bromophenol blue / 0.5% xylene cyanol). Before loading the gels, reactions were boiled for 5 mins at 95°C on an automated thermal cycler, then 4μl was immediately loaded onto gels (section 2.5.2).

Section 2.9 Single Strand Conformation Polymorphism Analysis

The method of Orita et al. (1989) was utilized. A trial PCR was set up first to check that amplification would work. Using the same batches of primer, buffer, water etc., the PCR cocktail was made up in the usual way (see section 2.3). For example, for a total of x PCRs, cocktail was pipetted into 5 eppendorfs and template DNA added to all eppendorfs, prior to adding 1-2μCi α-^{32}P-dCTP (Amersham, UK) per PCR to the remainder of the cocktail, which was pipetted into (x-5) eppendorfs. The usual program was used for both radioactive and non-radioactive PCRs. When amplification was complete, 5-10μl of each of the non-radioactive PCRs were electrophorsed on a 1.5% agarose gel to check that the amplification had worked. If so, the ^{32}P-labelled PCRs were stored at -20°C until needed.

Non-denaturing polyacrylamide gels containing glycerol were prepared as described

in section 2.5.2. 3μl of ^{32}P-labelled PCR was added to 3μl stop solution in a 96-well microtitre plate and PCR product was denatured by heating at 95°C for 5 mins. The plate was immediately cooled on ice and 4μl was loaded into each well at the top of the gel. Undenatured PCR product was mixed with an equal volume of 'stop solution' and loaded on either side of the row of denatured PCRs. This non-denatured PCR ran as a single band as a control both to locate the double stranded DNA in the lanes containing denatured PCRs and to verify that denaturation had occured to produce ssDNA, which migrates more slowly than dsDNA. Gel electrophoresis, drying and autoradiography were as described in Section 2.5.2.

Section 2.10 Statistical Analysis

Analyses described in sections 2.10.4 to 2.10.7 and 2.10.10 were carried out using the SSPC/PC+ Statistical Package. Data was $\log_{10}$-transformed prior to carrying out parametic tests; regression, analysis of variance (ANOVA), the F-test and correlation analysis, if it was not already approximately normally distributed and if it appreciably reduced the kurtosis and skewness of the data.

Section 2.10.1 Allele Frequencies and Hardy-Weinberg Equilibrium

The chromosome-counting method was used for the estimate of allele frequencies. 95% confidence intervals for sample allele frequecies were calculated according to Colton (1974) = $p \pm 1.96 \int p(1-p)/n$; where p = proportion of study sample with given allele and n = number of participants.

Comparison of allele frequencies between population samples was done by X^2 analysis using contingency tables. 2x2 or 2x3 contingency tables were used to compare allele or genotype, respectively, frequencies between two groups of individuals for example patients and healthy individuals. Contingency tables were used to see if there was any association between genotypes at polymorphic loci located on different chromosomes, within a group. Numbers of degrees of freedom were 1 and 2, respectively, for 2x2 and 2x3 tables. Estimation of Hardy-Weinberg equilibrium was done by comparing the expected genotype distribution for a given allele frequency; allele frequencies for a diallelic polymorphism in sample being p and q; ($p^2 + 2pq + q^2 = 1.0$)* with the observed distribution using a X^2

Deviation from Hardy-Weinberg equilibrium in a sample occurs if the sample is not representative, in terms of genotype distributions, of the population from which it is drawn. Selection of sample for disease, that is associated with one allele or genotype of the polymorphism, and presence of ethnic admixture in the sample are two reasons for deviations.

The expected genotype distribution in a sample is given by the formula; $p^2 + 2pq + q^2 = 1.0$, where p^2 = frequency of commoner homozygous genotype, $2pq$ = frequency of heterozygous genotype and q^2 = frequency of rarer homozygous genotype. Each of these is multipled by the observed number of individuals with that genotype to give the expected number of individuals with that genotype.

test. Deviation from equilibrium was presumed if the p-value was less than 0.05. * where p is the frequency of one allele and q is the frequency of the other allele and p + q = 1.0.

The percentage effect of a particular allele on a phenotypic trait was calculated as follows;

%age effect of allele = (Difference between mean level of trait in presence of that allele and mean in absence of that allele) divided by mean in absence of that allele x 100%.

Section 2.10.2 Estimation of extent of linkage disequilibrium between two loci

Linkage disequilibrium between alleles at two different diallelic polymorphic sites on the same chromosome was estimated using the correlation coefficient Δ (= 'delta') (Chakravarti et al. 1984) on the 'Excel' Spreadsheet program.

Δ = (ad - bc) divided by the square root of {(a+b) (c + d) (a + c) (b+ d)}

where a, b, c and d are the calculated numbers of the haplotypes A1B1, A1B2, A2B1 and A2B2 respectively. The haplotypes of the doubly heterozygous individuals were estimated using a maximum likelihood procedure, based on the assumption that these individuals would have the same haplotype distribution as that in those individuals who were homozygous at either or both polymorphic sites. This maximum likelihood prodecure is as follows;

Initially the numbers of unambiguous haplotypes were calculated and then the haplotypes of the doubly heterozygous individuals were assumed to be equally distributed between A1B1/A2B2 and A1B2/A2B1. The probability (P_1) of the double heterozygotes being distributed as A1B1/A2B2 is as follows;

$$P_1 = \frac{\text{number of A1B1/A2B2 haplotypes}}{\text{total number of haplotypes observed (= n)}}$$

$$P_1 = \frac{a/n \quad x \quad d/n}{[a/n \times d/n] + [b/n + c/n]}$$

This value, P_1 was then used to redistribute the haplotypes of the doubly heterozygous individuals in these proportions:-

P_1 (A1B1/A2B2) and (1-P_1) (A1B2/A2B1)

The value P_2 was then calculated using the new distribution of haplotypes and this calculation was reiterated until the values of **P** no longer changed (A. Templeton, personal communication).

The chi-squared of delta (Δ) was calculated on the 'Excel' spreadsheet program as $(\Delta)^2$ multipled by the total number of individuals. The number of degrees of freedom of this chi-squared is one. A standard table of critical chi-squared values (Sokal & Rohlf 1981) is consulted for the significance of the chi-square. The null hypothesis is that the chi-squared is not different from zero; it would not be rejected if there is no significant linkage disequilibrium between alleles at the two diallelic polymorphisms.

Section 2.10.3 Estimation of Haplotype Frequencies

First, haplotypes were assigned where homozygosity occured at all or all except one of the polymorphic loci surveyed. Then frequencies of these unambiguous haplotypes were estimated using the 'chromosome-counting' method.

Section 2.10.4 Calculation of the Polymorphic Information Coefficient (PIC)

The polymorphic information coefficient (PIC) for a given gene polymorphism, which is the proportion of total matings in a population that would be informative for that polymorphism. An informative mating is one in which the parents have different genotypes and thus the inheritance by their offspring, of alleles of this polymorphism, can be traced (Botstein et al. 1980). The formula for calculating the PIC of a diallelic polymorphism is as follows;

$$PIC = 1 - \left(\sum_{i=1}^{n} p_i^2\right) - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2p_i^2 p_j^2$$

where Σ = sum of, n = total number of alleles, p_i = frequency of one allele and p_j = frequency of the other allele.

Section 2.10.5 Estimation of Percentage Variance explained by genotypes

Phenotypic traits were first adjusted by linear regression for age and recorded body mass index (BMI), to remove variation due to age and BMI, and the percentage variance associated with age and BMI (R^2 x 100) was estimated. The impact of genotypes on age and BMI adjusted lipid and apolipoprotein traits were estimated by non-linear regression (using dummy variables; a separate column being created for each genotype and recoding it 0 or 1). This removes the dependence on the existence of a linear relationship between the mean of a trait for each genotype and genotypes of the particular DNA polymorphism.

Section 2.10.6 Comparsion of Differences between group means

ANOVA was performed on the age and BMI adjusted levels to test the null hypothesis that phenotypic variations in lipid and apolipoprotein levels were not associated with genetic variation. The average excess effects of each allele of the C to T (-265) substitution in the healthy individuals on serum apoB levels were estimated using the method previously presented by Sing & Davignon (1985).

Section 2.10.7 Calculation of 95% confidence limits for log_{10}-transformed data

For traits that were not normally distributed, 95% confidence limits were calculated on the log_{10}-transformed data (Sokal & Rohlf 1981). The formula is;

$$95\%\ CIs = \log_{10} \text{ of mean} \pm (t_{0.05\,(n-1)} \times \sqrt{(\log_{10} SD)^2/n})$$

where CI = confidence limit, n = number of individuals, SD = standard deviation

Section 2.10.8 Estimation of the interdependence between two traits - the correlation coefficent

For selected pairs of traits, based on what is known about lipid metabolism in general, the relationship was investigated using correlation analysis. Log_{10}-transformation was carried out if the data was not already approximately normally distributed. The Pearson correlation coefficient (r) was computed, and for groups with fewer than 40 individuals, a correcton factor was applied (Sokal & Rohlf 1981).

$$r^* = r[1 + (1 - r^2/2(n-4))]$$

n = no. of individuals in the sample

Either r or r* was squared to give r^2 - the coefficient of determination which is an estimate of the extent to which variation in the two variables is interdependent (proportion of variation of one variable determined by variation of the other). For all correlation analysis unadjusted data was used, to explore inter-relationships between plasma traits. Prior adjustment of the data for age and BMI would mask the actual in-vivo situation.

Section 2.10.9 F-test for homogeneity of standard deviations

The F-test was used to test the null hypothesis that there is no difference between variances (square of the standard deviation) obtained with different genotypes. The F-value was calculated directly from the ratio of larger variance to smaller variance and then looked up in the table of critical F-values. Degrees of freedom (df) of numerator equalled the number of individuals in the group with the larger variance minus one. Df of the denominator equalled the number of individuals in group with smaller variance minus one. The p-value was found by interpolation. If the data was not already approximately normally distributed, the F-test was carried out on the variances obtained with the $\log_{10}$-transformed data.

Section 2.10.10 T-test for homogeneity of two correlation coefficents

To test the null hypothesis that two correlation coefficients were not significantly different from one another (Sokal & Rohlf 1981), the coefficient r was first z-transformed. To calculate the t-statistic the following formula was used.

$t = |Z_1 - Z_2|$ divided by the square root of $[1/(n_1-3) + 1/(n_2-3)]$

where Z_1 and Z_2 are the two Z-transforms; n_1 and n_2 are the numbers of individuals in each group. The t-statistic was determined, at the infinite number of degrees of freedom, from the table of critical values of Student's t-distribution (Sokal & Rohlf 1981) to obtain the two-tailed p-value.

Section 2.10.11 Non-parametic tests

For baseline and progression of disease scores which could not be normalized by any

means the non-parametric analogue of ANOVA, the Kruskal-Wallis test, was used to find associations between severity of disease and genotypes of polymorphisms. Mean levels of each phenotypic trait for healthy individuals and patients were compared using the non-parametic Mann-Whitney test to remove the dependence of the probability value on the assumption that variances, in the two groups being compared, were approximately equal.

Section 2.10.12 Significance levels employed

Statistical significance was normally at the 0.05 level, except for correlations where the more conservative 0.01 level was adopted. However, since the study groups were small and multiple comparsions were being performed, probability values need to be interpreted with caution. The high degree of inter-relatedness between pairwise combinations of these variables reduced the probability of finding spurious associations in haplotype analysis. The significance level employed in Chapter 6 was 0.1 since it was an exploratory study and to reduce the probability of false negative statements (when the null hypothesis is not rejected), this approach has been previously reported (Rothman et al. 1990).

Section 2.11 Suppliers of Materials and Reagents

Oligonucleotides were purchased from Severn Biotech Ltd, Kidderminister, UK, Oswell Scientific Ltd (Edinburgh, UK), Advanced Biotechnology Centre (Charing Cross and Westminister Medical School, London UK) and Genosys Ltd (Cambridge UK). Unless otherwise stated, all chemicals were obtained from BDH Ltd or Sigma Ltd, Poole, Dorest, UK. Suppliers of enzymes, kits and radionucleotides are given in the text.

The constituents of standard solutions referred to in this thesis: 20x SSC, 20x SSPE, 10x TAE, 10x TBE and TE are given in Maniatis et al. 1982.

Standard solutions (Maniatis et al. 1982);
20x SSC (' Sale Sodium citrate) = 3M NaCl and 0.3M Na_3 citrate adjusted to pH 7.0 with a few drops of 5M or 10M NaOH.
20x SSPE (sodium phosphate - EDTA) = 3M NaCl, 0.18M NaH_2PO_4 and 20mM EDTA. This is adjusted to pH 7.4 with 10M NaOH.
10x TAE (Tris-acetate/EDTA electrophoresis buffer) = 0.4M Tris-acetate and 10mM EDTA. The EDTA is made as a 0.5M solution, pH = 8.0.
10x TBE (Tris-borate/EDTA electrophoresis buffer) = 0.9M Tris-borate and 20mM EDTA The EDTA is made as a 0.5M solution, pH = 8.0.
TE (Tris-EDTA) = 10mM Tris.Cl of the appropriate pH (i.e. 7.4 or 7.6 or 8.0); for DNA, TE of pH 7.4 is used).

CHAPTER 3 - THE SWEDISH STUDY: ASSOCIATION OF GENETIC VARIATION WITH FASTING LIPID AND LIPOPROTEIN TRAITS AND ATHEROSCLEROSIS.

Section 3.1 Summary of Study

Association studies were carried out in a sample of 87 patients from Sweden who had survived a myocardial infarction (MI) before the age of 45, and 93 age matched healthy individuals, to compare the impact of polymorphisms at the apolipoprotein (apo) E, B and AI-CIII-AIV gene loci and lipoprotein lipase (LPL) on among-individual differences in fasting plasma lipid and lipoprotein traits, the five high density lipoprotein (HDL) subclasses (2b to 3c), LPL activity and presence and progression of atherosclerosis. Allele frequencies for all polymorphisms were similar in healthy individuals and patients, with the exception of a higher frequency of the Ag(d) epitope in the patients ($p < 0.03$). In the group of healthy individuals, polymorphisms creating the common apoE isoforms were, as expected, associated with significant differences in total and low density lipoprotein (LDL) cholesterol (11.7% and 11.6% of sample variance). For apoB, the polymorphism with the largest effect on apoB levels (16% of sample variance) was the C to T transition 265bp 5' of the cap site, in the promoter (detectable by MspI). Both this and the XbaI polymorphisms were associated with significant effects on LDL-cholesterol (8.3% and 9.3% of sample variance, respectively). The Ag(x/y) epitopes were associated with a significant effect on HDL cholesterol alone. In the patients, polymorphisms creating the three common apoE isoforms were associated with large effects on cholesterol, apoB and triglyceride levels (19.9%, 20.3% and 23.9% of sample variance, respectively) - of similar magnitude as in the healthy individuals. ApoB polymorphisms were associated with much smaller effects on lipid traits than in the healthy individuals.

In the healthy individuals, levels of LDL-TGs were significantly assocatied with genotypes of the apoCIII-PvuII polymorphism ($p = 0.02$), but no other associations were found between lipids, LPL activity or HDL subclasses and single polymorphisms in the apoAI-CIII-AIV gene cluster. Levels of TGs and very low density lipoprotein (VLDL) trigylcerides were significantly higher in the presence of the haplotype defined by the presence of apoCIII-T_{1100} and common alleles of the other three polymorphisms, explaining 5.8% and 7.8% ($p < 0.05$) respectively, of sample variance. In the patients, no associations were found between lipids or HDL subclasses and variation at the apoAI-CIII-AIV gene cluster. Associations were also

examined between levels of HDL subclasses and variation at other gene loci. No associations were found between HDL subclasses and apoB or apoE gene polymorphisms. In the healthy individuals, genotypes of the LPL-HindIII polymorphism explained 5.3% ($p<0.07$) of sample variance in levels of protein in the HDL2b subclass, and in the patients, genotypes of the LPL-HindIII polymorphism explained 22.1%, 19.3% and 11.4% ($p<0.05$) of sample variance in levels of protein in HDL2b, HDL2a and HDL3a subclasses, respectively. In the healthy individuals, weak associations were found between genotypes of the HindIII polymorphism and TGs and the PvuII polymorphism and HDL cholesterol ($p=0.03$ and 0.09, respectively). No associations were found between these or other lipid traits or lipase activity and genotypes of the Serine447 to Stop polymorphism, and thus it is unlikely to be the cause of the associations seen with the PvuII and HindIII polymorphisms.

Baseline global severity of coronary atherosclerosis was associated with the apoB signal peptide polymorphism ($p=0.008$), haplotype combinations of the LPL HindIII and Serine447 to Stop polymorphisms ($p=0.02$) and with genotypes of the apoCIII C_{1100} to T polymorphism ($p=0.09$). When all three gene loci were considered together, individuals with all the three genotypes, each associated with high median atherosclerosis severity, had the highest median severity (2.4) and those with none of these genotypes had the lowest (0.4) (chi-squared overall = 15.7; $p=0.001$); none of the lipid traits measured showed a similar association with these genotypes. Thus, in this sample of young male post-infarction patients, genetic variation at these three loci is having an additive effect on the development of atherosclerosis, that cannot be explained solely by their observed effect on fasting lipid and lipoprotein traits.

Section 3.2 Rationale for Study

Epidemiological studies have established that elevated plasma levels of LDL and apoB and depressed levels of HDL and apoAI are correlated with increased risk of CHD and premature atherosclerosis. HDL_2, in part regulated by LPL, and, in particular, the HDL2b subclass are implicated in the inverse relation between HDL and CHD. Previous population studies have reported associations between apoE, B, AI-CIII-AIV and LPL gene polymorphisms and CHD or atherosclerosis, and between LPL polymorphisms and hypertriglyceridaemia, manifested as allele frequency differences between patients and healthy individuals or as associations between genotypes and disease severity (see chapter 1 of this

thesis). These and other studies have reported associations between these polymorphisms and fasting serum lipid and lipoprotein levels. However associations between genotypes and HDL subclasses have not previously been investigated.

Thus, in a well-characterized sample of MI survivors and healthy individuals, associations were investigated between polymorphisms in the above-mentioned gene loci and baseline global severity and rate of progression of coronary atherosclerosis and between these gene polymorphisms and fasting plasma levels of the major lipoprotein fractions, HDL subclass distribution and LPL activity. These investigations were followed by eludication of mechanisms underlying the associations between genetic variation and coronary atherosclerosis.

Section 3.3 Gene Polymorphisms in the Swedish sample and frequencies of alleles and haplotypes

Genotypes were determined at the apoE, apoB, apoAI-CIII-AIV and LPL loci and frequencies of the rare alleles for each polymorphism are given in Table 3.3.1. If a particular factor is associated with a greater risk of atherosclerosis, it is possible that prevalence of this factor would be greater in patients compared to a group of healthy matched individuals. However if the effect on risk associated with the factor is small, large samples are required for differences in frequency to reach statistical significance (Humphries et al. 1989). No significant differences in allele frequencies were observed between the healthy individuals and patients, with the exception of the apoB a1/d epitope pair. This is in constrast to previous case-control studies (all on Caucasians) involving patients with arterial disease (Monsalve et al. 1988), CHD (Myant et al. 1989, Genest et al. 1990, Paulweber et al. 1990, Van Bockxmeer & Mamotte 1992), MI (Hegele et al. 1986) or hypertriglyceridaemia (Hata et al. 1990), and may be due to the small sample size or possibly the young age of the patients compared to those from other studies (Hegele et al. 1986, Monsalve et al. 1988, Myant et al. 1989, Genest et al. 1990).

The proportion of current smokers was the same at 45% in both the Swedish patients and healthy individuals, and they were age-matched and all were men (Hamsten et al. 1986). Therefore age, smoking status and male gender, all known risk factors for CAD (section 1.1)

Genotype distributions in the Swedish sample. Numbers below each genotype = no. of individuals with that genotype. Numbers not in brackets refer to the healthy individuals and those in brackets refer to the patients.

Polymorphism	Genotypes				
ApoE isoforms	E2/3 12 (6)	E3/3 48 (47)	E2/4 4 (5)	E4/3 22 (23)	E4/4 4 (5)
C to T_{-265} in apoB promoter	CC 71 (68)		CT 18 (15)		TT 2 (1)
Signal peptide polymorphism	SP27/27 43 (41)		SP27/24 40 (30)		SP24/24 7 (14)
Ag (a1/d) epitope pair	a1a1 27 (15)		a1d 45 (44)		dd 18 (27)
XbaI polymorphism	X-X- 20 (22)		X+X- 42 (38)		X+X+ 28 (26)
Gln4154 to Lys polymorphism	Gln/Gln 58 (61)		Gln/Lys 28 (23)		Lys/Lys 3 (1)
Ag (x/y) epitope pair	yy 61 (56)		xy 23 (28)		xx 5 (2)
G to A_{-75} polymorphism in apoAI promoter	GG 68 (62)		GA 19 (19)		AA 3 (2)
PstI polymorphism 3' of apoAI	P+P+ 82 (78)		P+P- 9 (8)		P-P- 2 (1)
C to T_{1100} in apoCIII	CC 53 (40)		CT 32 (40)		TT 6 (5)
PvuII in apoCIII	V+V+ 66 (56)		V+V- 17 (28)		V-V- 7 (2)
PvuII in LPL	V+V+ 31 (31)		V+V- 43 (37)		V-V- 19 (18)
HindIII in LPL	H+H+ 53 (49)		H+H- 36 (35)		H-H- 3 (1)
Serine447 to Stop in LPL	Ser/Ser 80 (76)		Ser/Stop 8 (8)		Stop/Stop 1 (0)

TABLE 3.3.1
ALLELE FREQUENCIES IN THE SWEDISH SAMPLE

Gene Polymorphism	Rare Allele	Frequency of Rare Allele	
		Healthy Individuals (n= 93)	Patients (n=87)
Apo E isoforms	E2 (Cys158)	0.096	0.063
	E4 (Arg112)	0.181	0.224
Apo B			
C to T_{-265} Promoter (MspI)	T_{-265} no restriction site	0.113	0.100
Signal Peptide (SP) length polymorphism	SP-24	0.307	0.331
Ag (a1/d) epitope pair	Ag (a1)	0.550	0.430
Thr2488 (XbaI) Neutral (C to T) polymorphism	X+(T) with restriction site	0.560	0.517
Gln4154 to Lys4154 (EcoRI)	Lys4154	0.195	0.151
Ag (x/y) epitope pair	Ag (x)	0.187	0.190
ApoAI-CIII-AIV gene cluster			
G to A_{-75} polymorphism in apoAI gene promoter	A (-75)	0.139	0.141
PstI polymorphism in 3' flanking region of apoAI gene	P- no cutting site	0.049	0.046
C to T_{1100} polymorphism in apoCIII gene	T_{1100}	0.242	0.294
PvuII polymorphism in first intron of apoCIII gene	V- no cutting site	0.172	0.184
Lipoprotein Lipase			
PvuII polymorphism in intron 6	V- no cutting site	0.435	0.425
HindIII polymorphism in intron 8	H- no cutting Site	0.228	0.218
Serine447 to Stop polymorphism in exon 9	Stop447	0.056	0.052

Footnote:
Using 2x2 contingency tables, none of the allele frequencies were significant between the healthy individuals and patients, with the exception of the apoB a1/d epitope pair where chi-square = 5.05, $p<0.03$ at 1 degree of freedom.

are not likely to be the causes of the absence of allele frequency differences between the two groups. In contrast, previous studies which reported significant differences in allele frequencies of gene polymorphisms all reported significant differences in key characteristics between patients and healthy individuals. A greater proportion of patients than controls were current smokers (44% versus 21% in Hegele et al. 1986; 52% versus 29% in Genest et al. 1990, 43% versus 25% in Paulweber et al. 1990). The mean age (in years) of patients was considerably higher than that in controls (67 versus 47 in Monsalve et al. 1988; <60 versus >65 in Genest et al. 1990; 30 - 50 versus 20 - 34 in Bockxmeer & Mamotte). Both diabetes mellitus and hypertension were more frequent in patients (Hegele et al. 1986, the only study that did not exclude diabetes mellitus). Approximately 50% and 18% of patients, respectively, presented with hypercholesterolaemia or with hyperapoB (a risk factor for CAD, discussed in Section 1.3) (Myant et al. 1989), however these authors made no reference to any possible differential in smoking habits between patients and controls. Patients were selected for specific perturbations of lipids; high TGs and low HDL-cholesterol (Hata et al. 1990). One study reported a different gender distribution between patients and controls (Monsalve et al. 1988), with 71.7% of patients and 50% of controls being male. Hence differences in allele frequencies between patients and controls could be due, at least in part, to the selection criteria employed in case-control studies and less to a real relationship of specific genetic variation to presence of CAD. All these studies raise the possibility that well-established risk factors for atherosclerosis, such as age, gender, smoking status and dyslipidaemia are interacting with genetic variation and magnifying allele frequency differences between patients and controls.

Compared to the group of healthy individuals, in the patients the frequency of the E2 allele was lower and that of the E4 allele was higher as reported (Davignon et al. 1988). Small differences in allele frequecies between this study and previous studies are likely to be due to differences in the criteria used in selection of individuals, particularly the patients, and differences in ethnic origins of population samples used. Allele frequencies in this Swedish sample were also similar to those in previous studies on healthy Caucasian population samples from North Europe and the USA (Law et al. 1986, Talmud et al. 1987, Davignon et al. 1988, Humphries et al. 1988, 1990, 1992, Lusis et al. 1988, Chamberlain et al. 1989, Myant et al. 1989, Paulweber et al. 1990, Thorn et al. 1990, Xu et al. 1990a, 1994, Heizmann et al. 1991, Houlston et al. 1991, Ordovas et al. 1991, Sigurdsson et al. 1992). For all the polymorphisms

studied, genotype distributions were close to and not significantly different from the Hardy-Weinberg prediction. As expected, for genes on different chromosomes, no significant association was found between genotypes at different gene loci.

Table 3.3.2 shows the polymorphic information coefficients (PIC) for all the diallelic polymorphisms studied at the apoB, apoAI-CIII-AIV and LPL gene loci. As the frequency of the commoner allele (p) was similar between the healthy individuals and patients, PICs are only shown for the combined sample. The apoB-XbaI polymorphism is the most informative, its PIC approaching the maximum that can be obtained for a diallelic polymorphism which is 0.375. The 3'apoAI-PstI polymorphism the least informative. These two PICs for the apoB-XbaI and the 3' apoAI-PstI polymorphisms, can be interpreted as 37.3% and 8.6%, respectively, of matings being informative.

Haplotypes were inferred by visual inspection of the data and were deduced only in those individuals homozygous for all or all but one polymorphic sites at the particular gene locus. Frequencies of these inferred haplotypes observed in the healthy individuals and patients were similar, therefore results from the combined sample are presented in Table 3.3.3. Out of 16 possible haplotypes in the apoAI-CIII-AIV gene cluster, 7 were inferred to be unambiguously present, with the rare allele of the 3'apoAI-PstI polymorphism being only seen on the same chromosome as the common alleles of the other three polymorphisms, and with the 5'apoAI-A_{-75} allele nearly always being seen on the same chromosome as the apoCIII-T_{1100} allele, confirming previous observations (Xu et al. 1994). The rarest haplotype, GP+TV- was observed unambiguously in only one of the individuals who was a patient. The strong linkage disequilibrium between polymorphisms at the apoAI-CIII-AIV gene cluster confirms previous results (Xu et al. 1990b, 1994, Benlain et al. 1991, Paul-Hayase et al. 1992). The lack of significant linkage disequilibrium between alleles at the 3'apoAI-PstI polymorphic site and alleles at any of the three other polymorphic sites is due to the low frequency of the rare allele of the PstI polymorphism. Power to detect linkage disequilibrium is strongly dependent upon allele frequencies (Thompson et al. 1988). The negative value of delta for alleles of the PstI and apoCIII-C_{1100} to T polymorphisms is explained by the rare allele of the PstI polymorphism only being found on the same chromosome as the common allele of the C_{1100} to T polymorphism. The rare allele of the apoCIII-PvuII polymorphism (V-)

Table 3.3.2
Polymorphic Information Coefficients (PICs) for all the diallelic polymorphisms studied in the Swedish sample.

Gene polymorphism	PIC - for combined sample
ApoB	
C_{-265} to T	0.173
Signal Peptide	0.340
Ag (a1/d)	0.371
XbaI	0.373
EcoRI	0.245
Ag (x/y)	0.259
ApoAI-CIII-AIV	
G_{-75} to A	0.212
3'apoAI-PstI	0.086
C_{1100} to T	0.315
apoCIII-PvuII	0.250
LPL	
PvuII	0.370
HindIII	0.286
Serine447 to Stop	0.097

Footnote:
Maximum PIC for a diallelic polymorphism is 0.375 (where frequencies of both alleles are 0.5) i.e. 37.5% of total matings in the population from which the sample is drawn, would be informative.

TABLE 3.3.3

a) HAPLOTYPE FREQUENCIES IN THE APOAI-CIII-AIV GENE CLUSTER AND LPL GENE FOR HEALTHY INDIVIDUALS AND PATIENTS COMBINED (n=number of individuals)

Inferred Haplotype (AI-CIII-AIV)	n	Frequency (as percentage)
G_{-75} P+ C_{1100} V+	146	58.9
G_{-75} P+ C_{1100} V-	40	16.1
G_{-75} P+ T_{1100} V+	40	16.1
A_{-75} P+ T_{1100} V+	11	4.4
G_{-75} P- C_{1100} V+	6	2.4
A_{-75} P+ C_{1100} V+	4	1.6
G_{-75} P+ T_{1100} V-	1	0.4
Inferred Haplotype (LPL)		
V+ H+ Serine447	158	54.5
V- H+ Serine447	83	28.6
V- H- Serine447	19	6.6
V- H- Stop447	11	3.8

Haplotypes at each gene were only inferred if individuals were homozygous at all or all but one of the polymorphic loci studied.

b) Extent of linkage disequilibrium between pairwise combinations of apoAI-CIII-AIV and LPL gene polymorphisms in the combined Swedish sample

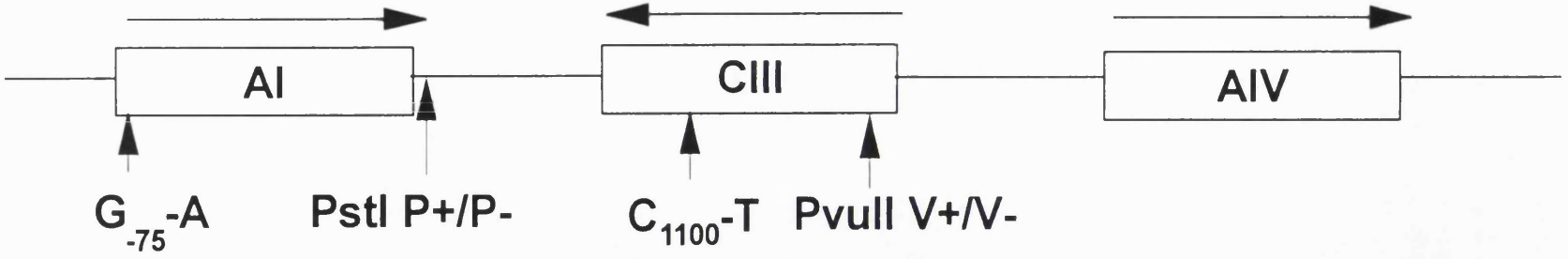
Polymorphism pair	Delta value	chi-square of delta	p-value #
ApoAI-CIII-AIV gene cluster			
G_{-75} to A and PstI	0.01	0.01	N/S
G_{-75} to A and PvuII-CIII	-0.15	3.95	<0.05
G_{-75} to A and C to T_{1100}	0.54	49.38	<0.001
PstI and PvuII-CIII	0	0	N/S
PstI and C_{1100} to T	-0.11	2.29	N/S
PvuII-CIII and C_{1100} to T	-0.25	10.93	<0.001
LPL gene			
PvuII and HindIII	0.40	27.03	<0.001
PvuII and Serine447 to Stop	0.27	12.40	<0.001
HindIII and Serine447 to Stop	0.45	33.16	<0.001

at one degree of freedom N/S = not significantly different from zero

was only found on the same chromosome as the common alleles of the 5'apoAI G/A and the apoCIII C_{1100} to T polymorphisms except in the one instance of the rarest haplotype where the V- allele was on a T_{1100} chromosome.

Figure 3.3.1 shows a simple representation of the apoAI-CIII-AIV gene cluster with all four polymorphic sites marked on it and a postulated phylogenetic tree for the apoAI-CIII-AIV gene cluster. Five of the seven unequivocally determined haplotypes form the most parsimonious tree, consistent with a simple model that all base substitutions arose in a sequential order, and requiring no obligatory recombination events to explain the observed haplotypes. The two rarest (unpredicted) haplotypes, AP+CV+ and GP+TV-, accounting for 2% of the unambiguous haplotypes, do not fit into the most parsimonious tree and it is likely that they arose via either recombination or second generation of the base substitution during evolutionary history within the apoAI-CIII-AIV gene cluster. It is not possible from the haplotype data in this Swedish sample, to determine the possible mechanism of generation of these unpredicted haplotypes. The haplotype AP+CV+, the commner unpredicted one, could have originally arisen by recombination in the apoAI-apoCIII intergenic region between haplotypes GP+CV+ and AP+TV+ to give this unpredicted haplotype plus another common observed haplotype GP+TV+. The rarest unpredicted haplotype might have originally arisen by recombination within the apoCIII gene between haplotypes GP+TV+ and GP+CV- to give this unpredicted haplotype plus the common haplotype GP+CV-. Neither of these possible recombination events give rise to any of the other nine possible haplotypes that could, in theory, occur with these four polymorphisms at the apoAI-CIII-AIV gene cluster.

CpG dinucleotides are known 'hot spots' for mutations in the human genome with the likely direction of mutation being CpG to TpG or CpA (Bird 1980, Svedberg & Bird 1990). The recognition site for the restriction endonucleotide MspI (C↓CGG) includes a CpG dinucleotide (underlined); the likely direction of mutation would be from C↓CGG to CCAG, creating the G to A polymorphism in the apoAI promoter. The C to T sequence change creating the C to T polymorphism in the apoCIII gene does not precede a 'G' nucleotide (Protter et al., 1984, Xu et al. 1994), nor does the recognition site for PvuII (CAG↓CTG) contain a CpG dinucleotide. Therefore second generation of the base substitution during evolutionary history is more likely to have occured in respect of the G_{-75} to A polymorphism



Simple representation of the apoAI-CIII-AIV gene cluster (not to scale) showing relative positions of the four polymorphic sites. Arrows indicate direction of transcription

Postulated Phylogenetic Tree for the apoAI-CIII-AIV gene cluster

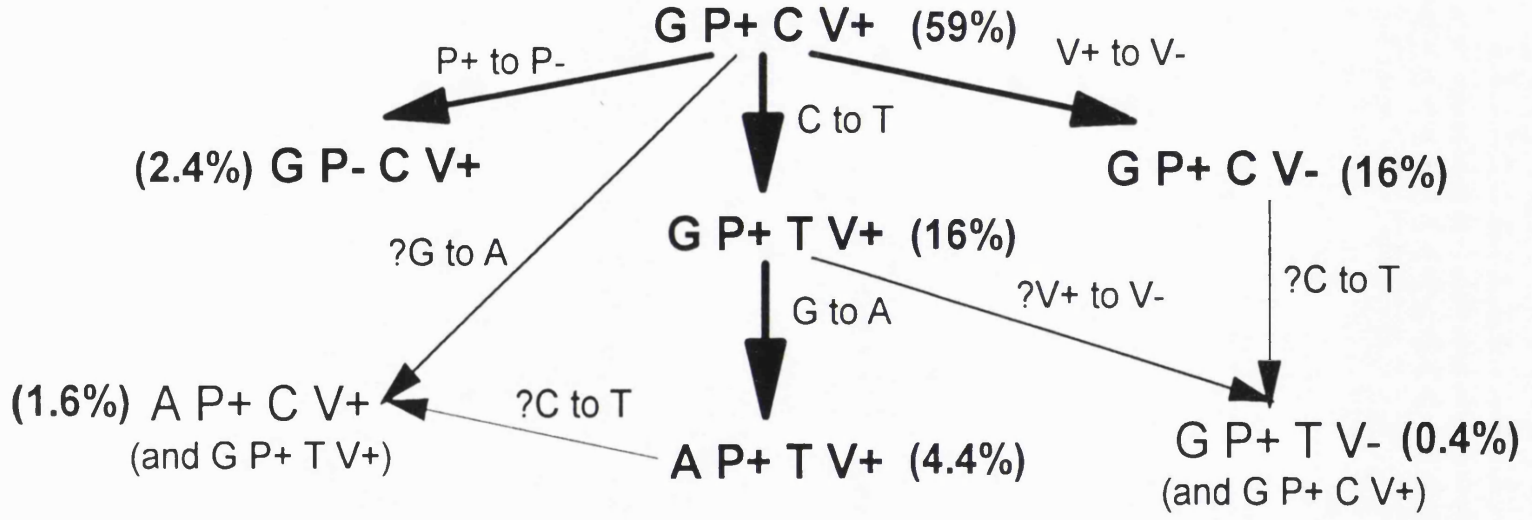


Figure 3.3.1

The most parsimonious tree is indicated by thick arrows, where single base substitutions are assumed to occur in a sequential manner, giving rise to new haplotypes. Thin arrows indicate the possible recombination events that could have given rise to the two unpredicted haplotypes. The other haplotype that is produced from each of these recombination events is shown in brackets below each unpredicted haplotype. Both definite base substitutions, giving rise to predicted haplotypes, and possible base substitutions, giving rise to unpredicted haplotypes, are indicated. Relative frequencies of each haplotype in the combined Swedish sample are given as percentages.

than for either of the two apoCIII polymorphisms (PvuII in the 1st intron and C_{1100} to T in the third exon).

For LPL gene polymorphisms, it was possible to determine haplotypes unequivocally where homozygosity occured at two or all three of the polymorphic loci. The results from the haplotype analysis for the healthy individuals and patients were not different and combined results are presented in Table 3.3.3. Out of eight possible haplotypes, five were found unequivocally. The Stop447 allele was always found on the same chromosome as the rare alleles of both the PvuII and HindIII polymorphisms, showing strong allelic association. Significant allelic association was also detected between the rare (no cutting site) alleles of the PvuII and HindIII polymorphisms. Estimates of the extent of linkage disequilibruim (delta values) for each pairwise combination of loci were similar in the healthy individuals and patients so the sample was combined to obtain the estimates of linkage disequilibrium shown in Table 3.3.3.

The highly significant linkage disequilibrium between any pairwise combination of the three LPL gene polymorphisms studied, suggests that there has been little, if any, recombination in at least the 3' part of the LPL gene; this is not unexpected since distances involved are quite small. Linkage disequilibrium between the rare alleles of the PvuII and HindIII polymorphisms (V- and H- respectively) has been reported in Caucasian and Japanese population studies (Chamberlain et al. 1989, Heizmann et al. 1991). The commonest haplotype in all the Caucasian populations surveyed to date, including this study, is V+H+. The finding that the Stop447 allele was always on the same chromosome as the V- and H- alleles, strongly suggests that the single base substitution, creating this stop codon, occured in an individual with the haplotype V-H-.

Section 3.4 Genotypes of the apoB C_{-265} to T polymorphism and haplotypes of the apoB gene

For individuals carrying the rare allele, T_{-265} (no cutting site) of the apoB C to T polymorphism that destroys a variable MspI site, this allele was assigned to apoB haplotypes. These had been determined by examination of seven polymorphic sites along the apoB gene, from the signal peptide polymorphism to the Ag(x/y) epitope pair (Dunning et al. 1993) in 90

healthy individuals and 143 young myocardial infarction survivors. Firstly, for these 233 individuals, apoB haplotypes carrying the T allele were assigned for those who were homozygous for the T_{-265} allele. All of these individuals had the T allele on haplotypes 1 to 4 (Table 3.4.1), so those individuals heterozygous for the T_{-265} allele had their T-carrying haplotypes assigned on the basis that the T allele would only be found on haplotypes 1 to 4, at least in this Swedish sample, although there are at least nine unequivocally determined haplotypes in this Swedish sample. There were no significant differences between the healthy individuals and patients so data for the combined sample is shown for haplotypes 1 to 4 only in Table 3.4.1.

Significant linkage disequilibrium ($p < 0.05$) between all pairwise combinations of the diallelic polymorphisms in apoB in this sample has been demonstrated (Dunning et al. 1993). Results from linkage disequilibruim determinations that included the C_{-265} to T polymorphism are shown in Table 3.4.2. The same strong disequilibrium was evident between pairwise combinations of the C_{-265} to T polymorphism and other apoB diallelic polymorphisms, with the T allele found only on haplotypes bearing the SP-27 allele of the signal peptide polymorphism and the X- allele of the XbaI polymorphism, in the Swedish sample. The one exception is the lack of significant linkage disequilibrium between alleles of the C_{-265} to T polymorphism and alleles of the Ag(x/y) epitope pair; the T allele being found on both y-bearing and x-bearing chromosomes. Thus, the original base substitution destroying the MspI variable site in the apoB promoter could have occured on haplotype 1, and been propagated through haplotypes 2, 3 and 4 which are all descended from haplotype 1 on the published phylogenetic tree (Dunning et al. 1993), see Figure 3.4.1 for reproduction of this phylogenetic tree. Alternatively, different base substitutions other than C to T at position -265 could be responsible for destroying the MspI variable, explaining the association of absence of the cutting site with different haplotypes. Thirdly, recombination in the 5' part of the apoB gene (3' of the signal peptide) could have generated associations of the rare allele with several haplotypes. Sequencing of the apoB promoter region was therefore carried out on a total of 5 Swedish individuals homozygous for the rare allele plus five heterozygous individuals (as 'controls') to find out which, if any, of these possibilities was most likely or could be ruled out.

Table 3.4.1
Haplotypes in the apoB gene bearing the T(-265) allele in the Swedish combined sample of 90 healthy individuals and 143 myocardial infarction survivors.

Haplotype in apoB gene	Haplotype No. (Dunning et al. 1993)	C (-265)			T (-265)		
		unequivocal	assigned	Total	unequivocal	assigned	Total
SP-27 g d X- yt	1	7	10	17	5	9	14
SP-27 g a1 X- yt	2	2	4	6	1	5	6
SP-27 g a1 X- xt	3	19	56	75	1	1	2
SP-27 g a1 X-yz	4	13	32	44	4	26	30
all others	5-18	133	190	323	0	0	0

SP-27 = common allele of signal peptide polymorphism, g = g allele (Thr-71) of the antigen (c/g) pair, a1/d = Val-591 and Ala-591, respectively, alleles of the antigen (a1/d) pair, X- = common allele of the XbaI polymorphism, y/x = Asn-4311 and Ser-4311, alleles of the antigen (x/y) pair and t/z = Glu-4154 and Lys-4154, alleles R+ and R- of the EcoRI polymorphism.

Table 3.4.2
Extent of linkage disequilibrium between the C(-265) to T polymorphism and selected apoB polymorphisms in the combined Swedish sample.

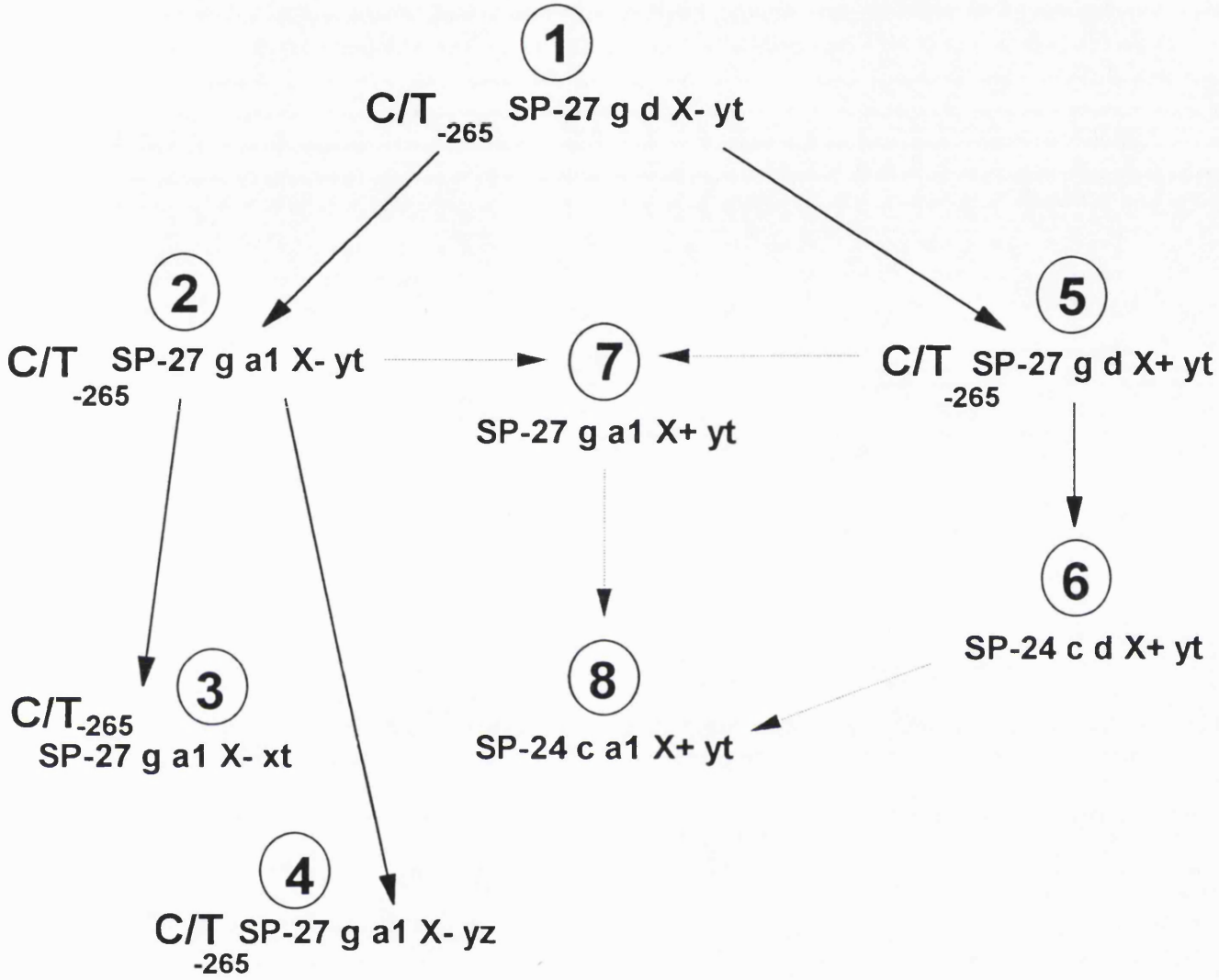
Polymorphism pair	Delta value	chi-square of delta	p-value at 1 degree of freedom
C_{-265} to T and signal peptide	-0.26	15.52	<0.001
C_{-265} to T and Ag(c/g) pair	-0.26	15.80	<0.001
C_{-265} to T and Ag(a1/d) pair	0.14	4.58	<0.05
C_{-265} to T and XbaI polymorphism	-0.36	29.24	<0.001
C_{-265} to T and EcoRI polymorphism	0.40	36.47	<0.001
C_{-265} to T and Ag(x/y) pair	-0.12	3.56	$0.1>p>0.05$

Table 3.4.3 ApoB haplotypes in T_{-265} carriers for whom results of sequencing are shown in Figure 3.4.1.

ID	Classification No. (Dunning et al. 1993 and Table 3.4.1)	
	C_{-265}-bearing haplotype	T_{-265}-bearing haplotype
2042	1	1
NK25 (North Karelia)	-	1 and 5
NK29 "	-	1 and 4
NK43 "	-	5 and 5
2138	4	1
4027	-	1 and 2

Figure 3.4.1

Reproduction of the postulated phytogenetic tree of apoB haplotypes (from Dunning et al. 1993, by courtesy of Alison Dunning).



The most parsimonious tree is shown around the outer edge (haplotypes 1 to 6). Haplotypes 7 and 8 are the two most frequently unequivocally observed unpredicted haplotypes.

——► indicates proposed single mutations

·····► indicates possible recombination events, giving rise to the unpredicted haplotypes.

SP-27 and SP-24 = alleles of the signal peptide polymorphism; c & g = alleles of Ag(c/g) epitope pair; a1 & d = alleles of Ag(a1/d) epitope pair; X+ & X- = alleles of the XbaI polymorphism; x & y = alleles of Ag(x/y) epitope pair; t & z = alleles of Ag(t/z) epitope pair (the EcoRI polymorphism).

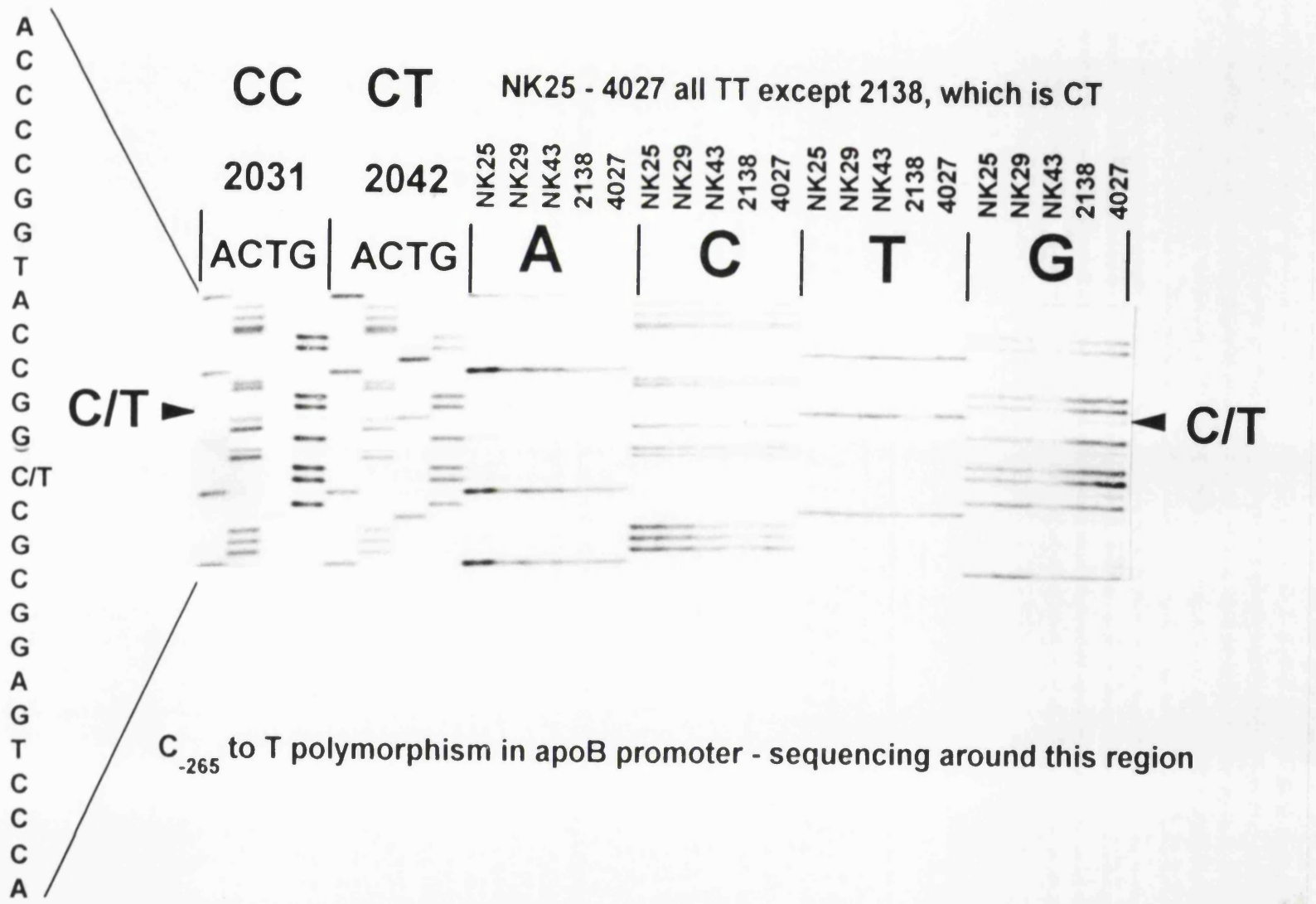


Figure 3.4.2.

Sequencing results for the region around the C_{-265} to T polymorphism in the apoB promoter. Samples with ID numbers 2031, 2042, 2138 and 4027 are from the Swedish sample, while samples with ID numbers NK25, NK29 and NK43 are from the North Karelia sample described in Xu (1990). Samples were run at a constant power of 65W for 1.5 hours.

Results from sequencing are shown in Figure 3.4.2; individuals who had different apoB haplotypes were chosen. As shown in Figure 3.4.2, the only base change that occured was the C to T substitution at position -265, giving the sequence 5'-CTGG-3' (Jones et al. 1989, Xu 1990). This C to T base change was totally concordant with presence/absence of the polymorphic MspI recognition site. The recognition site of MspI, 5'-CCGG-3', is preceded by a GG dinucleotide on the anti-sense strand (Carlsson & Bjursell 1989) and the whole sequence GGCCGG is cleaved only slowly by MspI (Busslinger et al. 1983). The frequency of the rare allele may have been slightly overestimated, sequencing revealed that two Swedish individuals previously typed as TT were actually CT (they had haplotypes 1 + 3 and haplotypes 1 + 4, respectively). Table 3.4.3 shows the haplotype numbers in the individuals sequenced and who had the T allele. The North Karelia sample was genotyped for apoB polymorphisms, and the frequency of the T allele was 0.18; significantly higher than in the combined Swedish sample ($\chi^2 = 3.86$, $p<0.05$ at one degree of freedom). The unequivocal existence of the T_{-265} allele on haplotype 5 (SP-27 X+ R+) is not surprising, given the direct descent of this haplotype from haplotype 1 (Dunning et al. 1993). These sequencing results indicate that the possibility of different base substitutions destroying the MspI recognition site is unlikely. Recombination or independent occurance of the C_{-265} to T substitution on different haplotypes, during evolutionary history, remain possible explanations. Future genotyping of population samples for the 5'apoB MspI polymorphism might be more successfully accomplished using Allele Specific Oligonucleotides.

Section 3.5 Associations between gene polymorphisms and serum lipid traits

Table 3.5.1 shows the contribution to the sample variance explained by age and BMI in the healthy individuals and patients. Age and BMI explained a significant proportion of total variance in VLDL-cholesterol, total TG and VLDL-TG in both groups and HDL-cholesterol in the healthy individuals. The results from regression of age and BMI adjusted data for apoE genotypes and for the apoB polymorphisms are also shown in Table 3.5.1. For example, in the healthy individuals, apoE genotypes explained 11.7% and 11.6%, respectively, of total variance in total and LDL-cholesterol levels. In the patients the associations were stronger explaining 19.9%, 20.3%, 17.0%, 23.9% and 19.2%, respectively, of total variance in cholesterol, TG, LDL-cholesterol, apoB and HDL-TG levels. The significant associations observed between LDL-cholesterol or HDL-TG and genotypes of the

TABLE 3.5.1

a) PROPORTION OF VARIANCE (R^2 X 100) OF EACH TRAIT ATTRIBUTABLE TO AGE AND BMI AND TO GENETIC VARIATION AT THE APOE AND APOB LOCI IN THE HEALTHY INDIVIDUALS
Healthy Individuals

Trait	Age and BMI a	ApoE	Apolipoprotein B					
			C to T (-265)	Signal Peptide	Ag (a1/d)	XbaI	EcoRI	Ag (x/y)
Cholesterol								
Total	2.1	11.7 *	6.6	2.1	2.6	4.3	1.3	1.3
VLDL	9.7 *	2.0	0.6	1.0	4.5	7.0	2.1	3.0
LDL	2.4	11.6 *	8.3 *	1.7	5.4	9.3 *	1.6	0.8
HDL	11.2 **	3.6	1.5	2.0	5.0	7.5 *	2.9	7.9 *
Triglycerides								
Total	8.8 *	1.8	0.4	0.3	2.6	3.9	2.2	1.4
VLDL	8.3 *	1.5	0.5	1.4	4.5	7.2 *	3.5	2.5
LDL	1.2	1.7	0.3	2.1	0.4	2.7	1.6	1.5
HDL	2.2	2.8	1.1	1.2	1.1	0.6	4.6	1.7
Apo AI	5.6	4.9	0.7	1.5	3.6	1.5	0.5	2.2
Apo B	3.1	11.1	15.7 **	1.4	2.4	7.4	3.0	2.4

* = $p<0.05$ and ** = $p<0.01$ The four hyperlipidaemic individuals (cholesterol >9.5mM and/or triglycerides >3.0mM) are excluded.
a Unadjusted data used in this regression
For apoE, the five genotypes (there were no individuals with the E2/2 genotype) were treated as separate groups in the regression.

TABLE 3.5.1

b) PROPORTION OF VARIANCE (R^2 X 100) OF EACH TRAIT ATTRIBUTABLE TO AGE AND BMI AND TO GENETIC VARIATION AT THE APOE AND APOB LOCI IN THE PATIENTS

Trait	Age and BMI a	ApoE	Apolipoprotein B					
			C to T (-265)	Signal Peptide	Ag (a1/d)	XbaI	EcoRI	Ag (x/y)
Cholesterol								
Total	0.3	19.9 **	1.9	3.8	2.6	1.0	3.6	1.3
VLDL	11.2 **	16.6 **	0.7	1.7	1.6	0.4	3.8	6.3
LDL	1.7	17.0 **	3.5	0.8	0.5	0.9	8.7 *#	0.5
HDL	6.2	6.5	2.9	0.5	1.5	1.2	1.6	1.3
Triglycerides								
Total	10.2 *	20.3 **	0.2	2.8	2.0	0.1	5.5	3.5
VLDL	13.0 **	17.1 **	0.4	2.8	2.4	0.4	4.6	6.1
LDL	1.9	6.8	0.5	0.2	1.1	1.7	3.2	0.2
HDL	4.9	19.2 **	3.5	0.7	1.4	0.5	9.1 *#	11.6 **
Apo AI	4.2	10.4	5.0	11.9 *	3.0	0.4	2.7	2.6
Apo B	2.7	23.9 **	2.8	2.5	0.9	4.9	0.8	0.8

* = $p<0.05$ ** = $p<0.01$

a unadjusted data used in this regression

For apoE, the five genotypes (there were no individuals with the E2/2 genotype) were treated as separate groups in the regression.

Association becomes insignificant when the single individual homozygous for the rare allele of the EcoRI polymorphism is excluded from the analysis.

EcoRI polymorphism disappeared when the single patient homozygous for the rare allele was excluded from the analysis. This patient had hypertriglyceridaemia, with elevations of cholesterol and TG in the VLDL fraction and depressed cholesterol levels in LDL and HDL. As shown in Table 3.5.2, the E4 and E2 alleles are associated with higher and lower, respectively, total and LDL-cholesterol levels in both the healthy individuals and patients. However trends are less consistent with lipids in the VLDL fraction. ApoE genotypes have little effect on means for HDL-TGs, with the exception of the high mean observed in those patients with the genotype E4/2; this accounts for the highly significant F-value observed in the patient group. The genotype E2/3 is associated with lower levels of triglyceride and apoB in both groups, reaching statistical significance in the patients. In general, apoE genotypes were associated with higher cholesterol levels in all and LDL fractions and higher triglyceride levels in all and VLDL fractions and higher apoB levels in the patients compared to the healthy individuals. Exceptions involved the rare genotypes E4/2 and E4/4. Within the group of healthy individuals, those with the genotype E4/2 had higher mean LDL-cholesterol levels, while those with the genotype E4/4 had slightly higher mean cholesterol and TGs in VLDL, compared to the patients with the corresponding genotypes. The trends for VLDL-cholesterol and VLDL-TG were reversed upon adjusting data for age and BMI (not shown), but that for LDL-cholesterol remained unchanged. Age and BMI explain a significant proportion of sample variance in VLDL-cholesterol and TG (Table 3.5.1). This, coupled with the small numbers of individuals with the rare apoE genotypes, causes a larger change (but still small in absolute terms) in mean level upon adjustment of data for age & BMI, despite mean ages and BMI in these groups being close to the whole sample mean (data not shown).

For the apoB polymorphisms, in the healthy individuals, genotypes of the apoB C_{-265} to T polymorphism were significantly associated with both LDL-cholesterol and apoB levels. The smaller effects associated with the XbaI polymorphism on LDL- and HDL-cholesterol and VLDL-TGs, and the Ag(x/y) epitope pair on HDL-cholesterol levels were also significant. Individuals with genotypes yy and xy had similar mean HDL-cholesterol levels, but the genotype xx was associated with a higher mean HDL-cholesterol level. Mean levels from the ANOVAs that gave significant results of each trait for each apoB genotype in either the healthy individuals or patients are presented in Table 3.5.3. The T allele (absence of the MspI site) of the C_{-265} to T polymorphism was associated with lower levels of LDL-cholesterol and

TABLE 3.5.2

MEAN LIPID LEVELS (±SE) FOR APOE GENOTYPES THAT HAVE SIGNIFICANT F VALUES FROM ANOVAS IN EITHER HEALTHY INDIVIDUALS OR PATIENTS

(a) **Healthy Individuals** - excluding the four hyperlipidaemic individuals

Trait (mmol/l)	Apo E Genotype (No)					F Value and Significance in ANOVA b
	E3/2 (12)	E3/3 (44)	E4/2 (4)	E4/3 (20)	E4/4 (3)	
Total Cholesterol	5.61± 0.99	5.85± 1.01	7.07± 0.67	6.12± 0.82	6.78± 1.32	F=2.6 p=0.04
VLDL-cholesterol †	0.49 (0.35-0.68)	0.40 (0.28-0.58)	0.44 (0.09-2.07)	0.36 (0.27-0.48)	0.67 (0.27-1.64)	F=0.3 p=0.8
LDL-Cholesterol	3.65± 0.88	3.79± 0.85	4.78± 0.85	4.15± 0.73	4.54± 0.98	F=2.6 p=0.05
Triglycerides †	1.31 (1.02-1.69)	1.22 (1.07-1.39)	1.16 (0.47-2.87)	1.11 (0.94-1.32)	1.73 (0.60-5.00)	F=0.3 p=0.9
VLDL-triglycerides †	0.76 (0.53-1.10)	0.66 (0.54-0.80)	0.59 (0.15-2.35)	0.57 (0.43-0.77)	1.07 (0.38-3.07)	F=0.2 p=0.9
HDL-triglycerides	0.13± 0.01	0.14± 0.01	0.14± 0.03	0.14± 0.01	0.16± 0.01	F=0.6 p=0.6
Apo B (mg/100ml)	102.4 ±19.6	103.4 ±17.0	122.7 ±9.6	110.8 ±16.4	118.3 ±10.7	F=2.2 p=0.07

b Traits were adjusted for age and BMI prior to ANOVA
† antilogs of log_{10}-transformed means and 95% confidence limits
Unadjusted means are shown since adjustment for age and BMI made little difference to the genotype means.

TABLE 3.5.2

MEAN LIPID LEVELS (±SE) FOR APOE GENOTYPES THAT HAVE SIGNIFICANT F VALUES FROM ANOVAS IN EITHER THE HEALTHY INDIVIDUALS OR PATIENTS

(b) Patients

Trait (mmol/l)	Apo E Genotype					F value and Significance in ANOVA b
	E3/2 (6)	E3/3 (47)	E4/2 (5)	E4/3 (23)	E4/4 (5)	
Total Cholesterol	6.06± 1.30	7.11± 1.02	8.71± 1.47	7.22± 1.40	8.36± 0.66	F=5.0 p=0.001
VLDL-cholesterol †	0.72 (0.31-1.66)	0.90 (0.76-1.07)	2.50 (1.05-5.98)	0.77 (0.59-1.00)	0.59 (0.37-0.95)	F=5.0 p=0.001
LDL-Cholesterol	3.79± 0.99	4.91± 0.92	4.33± 1.52	4.94± 1.11	6.16± 0.42	F=4.1 p=0.004
Triglycerides †	1.70 (0.95-3.03)	2.26 (1.97-2.58)	5.45 (2.18-13.61)	2.13 (1.76-2.59)	1.72 (1.20-2.48)	F=5.8 p<0.001
VLDL-Triglycerides †	1.16 (0.54-2.51)	1.50 (1.26-1.80)	4.14 (1.36-12.54)	1.44 (1.14-1.83)	1.00 (0.60-1.68)	F=4.6 p=0.002
HDL-Triglycerides	0.14± 0.05	0.16± 0.04	0.24± 0.10	0.15± 0.04	0.14± 0.02	F=4.7 p=0.002
Apo B (mg/100ml)	104.5 ±25.2	119.9 ±18.4	135.8 ±17.7	135.4 ±16.3	150.0 ±17.2	F=6.4 p=0.0002

b Traits were adjusted for age and BMI prior to ANOVA

† antilogs of $\log_{10}$-transformed means and 95% confidence limits

Unadjusted means are shown since adjustment for age and BMI made little difference to the genotype means.

TABLE 3.5.3
MEAN LIPID LEVELS (±SE) FOR APOB GENOTYPES THAT HAVE SIGNIFICANT F VALUES FROM ANOVAS IN EITHER THE HEALTHY INDIVIDUALS OR PATIENTS OR PREVIOUS STUDIES

(a) **Healthy Individuals** - excluding the four hyperlipidaemic individuals
⊕ n=60

Trait (mmol/l)	ApoB Genotype (No) C_{-265} to T polymorphism			F Value and Significance in ANOVA b
	CC (67)	**CT (15)**	**TT (2)**	
LDL-Cholesterol	4.03±0.10	3.69±0.21	2.63±0.26	F=3.7, p=0.03
Apo B (mg/100ml)	110±2.1⊕	95.6±3.9	84±13.0	F=6.9, p=0.002
	Signal peptide length polymorphism			
	SP27/27 (39)	**SP27/24 (37)**	**SP24/24 (7)**	
Total-triglycerides†	1.24 (1.10-1.39)	1.21 (1.03-1.41)	1.18 (0.80-1.73)	F=0.1, p=0.9
ApoAI (mg/100ml)	123.6±3.33	123.9±2.69	131.2±7.36	F=0.6, p=0.5
	XbaI polymorphism			
	X-X-(17)	**X+X-(39)**	**X+X+(27)**	
Total Cholesterol	5.63±0.22	5.94±0.15	6.25±0.22	F=1.6, p=0.2
LDL-Cholesterol	3.47±0.17	3.92±0.13	4.26±0.17	F=4.1, p=0.02
HDL-Cholesterol	1.54±0.10	1.34±0.05	1.47±0.06	F=3.2, p=0.04
Total-triglycerides	1.22 (0.98-1.53)	1.30 (1.14-1.47)	1.11 (0.93-1.32)	F=1.7, p=0.2
VLDL-triglycerides †	0.71 (0.50-1.01)	0.74 (0.62-0.89)	0.53 (0.40-0.71)	F=3.2, p=0.05
ApoB (mg/100ml)	97.1±3.5	107.0±2.9	111.9±3.5	F=2.7, p=0.07
	Ag(x/y) epitope pair			
	yy (56)	**xy (23)**	**xx (4)**	
HDL-Cholesterol	1.39±0.04	1.43±0.08	1.86±0.23	F=3.4, p=0.04
HDL-triglycerides	0.14±0.01	0.14±0.01	0.16±0.02	F=0.7, p=0.5

b Traits were adjusted for age and BMI prior to ANOVA
† data is presented as antilogs of log_{10}-transformed means and 95% confidence limits.
Unadjusted means are shown since adjustment for age and BMI made little difference to the genotype means.

TABLE 3.5.3
MEAN LIPID LEVELS (±SE) FOR APOB GENOTYPES THAT HAVE SIGNIFICANT F VALUES FROM ANOVAS IN EITHER THE HEALTHY INDIVIDUALS OR PATIENTS OR PREVIOUS STUDIES.
(b) Patients

Trait (mmol/l)	ApoB Genotype (No) C_{-265} to T polymorphism			F Value and Significance b
	CC (68)	CT (15)	TT (1)	
LDL-cholesterol	4.98±0.13	4.61±0.24	6.12	F=1.7, p=0.2
ApoB (mg/100ml)	128.2±2.43	119.8±6.01	141.0	F=1.2, p=0.3
	Signal peptide length polymorphism			
	SP27/27 (29)	SP27/24 (23)	SP24/24 (11)	
Total-triglycerides†	2.39 (2.01-2.85)	1.97 (1.67-2.32)	2.56 (1.85-3.56)	F=1.3, p=0.3
ApoAI (mg/100ml) ¥	105.6±3.2	113.5±4.1	122.3±4.6	F=4.0, p=0.02
	XbaI polymorphism			
	X-X- (22)	X-X+ (38)	X+X+ (26)	
Total-cholesterol	7.46±0.24	7.14±0.21	7.34±0.27	F=0.4, p=0.6
LDL-cholesterol	5.05±0.23	4.80±0.16	5.00±0.23	F=0.8, p=0.4
HDL-cholesterol	1.10±0.05	1.11±0.04	1.14±0.04	F=0.3, p=0.7
Total-triglycerides†	2.38 (1.89-3.00)	2.19 (1.88-2.55)	2.26 (1.77-2.88)	F=0.1, p=0.9
VLDL-triglycerides †	1.59 (1.16-2.18)	1.46 (1.21-1.76)	1.53 (1.13-2.07)	F=0.03, p=1.0
ApoB (mg/100ml)	131.9±2.9	121.9±3.8	129.6±4.4	F=2.3, p=0.1
	Ag(x/y) epitope pair			
	yy (55)	xy (28)	xx (2)	
HDL-cholesterol	1.09±0.03	1.16±0.05	1.16±0.13	F=0.8, p=0.5
HDL-Triglyerides	0.17±0.05	0.14±0.03	0.15±0.02	F=5.3 p=0.007

b Traits were adjusted for age and BMI prior to ANOVA
† data is presented as antilogs of $\log_{10}$-transformed means and 95% confidence limits.
¥ Data on apoAI levels was only available on 63 of the patients.

Unadjusted means are shown since adjustment for age and BMI made little difference to the genotype means.

apoB in both the healthy individuals and patients with the exception of the patient homozygous for the T_{-265} allele who had high levels of both traits. Dividing those healthy individuals who had data on apoB levels into three approximately equal groups, the frequency of the T allele was 0.25 in the group with the lowest apoB levels (lower than 98mg/100ml), whereas it was only 0.02 in the group with the highest apoB levels (higher than 113mg/100ml). Using 2x2 contingency tables, this difference in frequency was statistically significant ($X^2 = 11.4$, $0.01 > p > 0.001$). For the XbaI polymorphism there was a co-dominant effect with respect to LDL-cholesterol levels, the X+ allele being associated with significantly higher LDL-cholesterol levels and non-significantly higher apoB levels, this co-dominant effect was absent in the patient group.

In the patients, a significant association was found between apoAI levels and genotypes of the apoB signal peptide polymorphism, with homozygosity for the SP-27 isoform associated with higher apoAI levels, the same trend was observed in the healthy individuals. Genotypes of the Ag(x/y) epitope pair were significantly associated with HDL-TG levels in the patients. In the healthy individuals, mean levels of HDL-cholesterol and HDL-TG were similar for genotypes yy and xy, but clearly higher for genotype xx. This is consistent with previous reports, which also showed no clear co-dominant effect of the two alleles (Dunning et al. 1992, Moreel et al. 1992). The effect of the Ag(x) epitope is thought to be recessive (Berg et al. 1976, Dunning et al. 1992), with genotypes yy and xy being associated with similar mean levels of plasma lipids/lipoproteins and genotype xx being different (Moreel et al. 1992). In both the healthy individuals and patients, the xy genotype was associated with the lowest mean HDL-triglyceride levels, although this was not statistically significant in the healthy individuals. Genotypes of the Ag(a1/d) epitope pair (Val/Ala-591) were not associated with any significant effects in either the healthy individuals or patients, despite the occurance of an amino acid change in the apoB protein and the strong linkage disequilibrium with the XbaI polymorphism. However, in those homozygous for Ag(a1) [Val-591], a trend towards a more atherogenic lipoprotein profile was observed in the patients, and to a lesser extent, the healthy individuals (Table 3.5.4), with higher mean levels of TGs and apoB observed in these individuals.

Combining data from the XbaI polymorphism and both Ag(a1/d) and Ag(x/y) epitope

TABLE 3.5.4

MEAN LIPID LEVELS (±SE) FOR GENOTYPES OF THE Ag (a1/d) EPITOPE PAIR IN THE SWEDISH SAMPLE

a) Healthy Individuals - excluding the four hyperlipidaemic individuals

Trait (mmol/l)	a1/a1 (25)	a1/d (42)	d/d (16)	F-value and Significance in ANOVA b
Cholesterol	6.04 ± 0.18	5.82 ± 0.15	6.28 ± 0.31	F = 0.7 p = 0.5
LDL-cholesterol	3.93 ± 0.18	3.82 ± 0.11	4.24 ± 0.26	F = 1.3 p = 0.3
HDL-cholesterol	1.48 ± 0.08	1.37 ± 0.04	1.47 ±0.08	F = 1.1 p = 0.3
Triglycerides†	1.26 (1.08 - 1.47)	1.22 (1.07 - 1.40)	1.15 (0.91 - 1.45)	F = 1.4 p = 0.3
ApoB (mg/100ml)	106.3 ± 4.34	104.8 ± 2.28	111.6 ± 5.55	F = 0.5 p = 0.6

b) In patients

Trait (mmol/l)	a1/a1 (15)	a1/d (44)	d/d (27)	F-value and Significance in ANOVA b
Cholesterol	7.68 ± 0.29	7.12 ± 0.19	7.32 ± 0.24	F = 0.9 p = 0.4
LDL-cholesterol	5.05 ± 0.25	4.86 ± 0.18	4.96 ± 0.17	F = 0.1 p = 0.9
HDL-cholesterol	1.07 ± 0.04	1.14 ± 0.04	1.10 ± 0.03	F = 0.6 p = 0.5
Triglycerides†	2.53 (1.82 - 3.50)	2.12 (1.82 - 2.46)	2.36 (1.92 - 2.90)	F = 0.7 p = 0.5
ApoB (mg/100ml)	130.3 ± 5.97	126.2 ± 3.25	125.8 ± 3.94	F = 0.5 p = 0.6

b Traits were adjusted for age and BMI prior to ANOVAs
† data is presented as antilogs of log_{10}-transformed means and 95% confidence limits.

Unadjusted means are shown since adjustment for age and BMI made little difference to the genotype means.

pairs both in the healthy individuals and in the patients, the haplotype with the Ag(x) epitope was associated with reduced levels of cholesterol, TG, LDL-cholesterol and apoB, with a lower LDL/HDL ratio and with raised levels of HDL-cholesterol, while, of the other haplotypes, the one defined by the presence of Ag(a1) in combination with the XbaI X+ allele was associated with higher LDL-cholesterol and lower HDL-cholesterol levels (Table 3.5.5). It was noticeable that there were significant effects associated with these haplotypes both in the healthy individuals and patients, with similar trends in all the lipid traits studied, although the effects were of different magnitude in the two groups. The fact that the effect on cholesterol, total TG, HDL-TG, apoB and apoAI, associated with the haplotypes was greater in the patient sample than in the sample of healthy individuals is unexplained, but, if confirmed is of obvious clinical significance. The association of lower cholesterol levels with Ag(x) is in agreement with the original findings of Berg et al. (1976), and has recently been strongly confirmed in samples of male MI survivors and matched healthy controls from three centres in Europe (Moreel et al. 1992). Figure 3.5.1 shows a cartoon of postulated functional haplotypes of the apoB gene as identified in the Swedish sample.

None of the lipids (cholesterol and TGs in plasma, VLDL, LDL or HDL fractions were significantly associated with genotypes at the apoAI-CIII-AIV gene cluster in either healthy individuals or patients (Table 3.5.6) with the exception of LDL-TGs with genotypes of the 3'apoCIII-PvuII polymorphism in the healthy individuals. Levels of LDL TGs were 21% higher in healthy individuals carrying the V- allele of the 3'apoCIII-PvuII polymorphism compared to those without this allele (Table 3.5.7). Exclusion of the four hyperlipidaemic individuals (with cholesterol levels >9.5mM and/or triglyceride levels >3.0mM) made little difference to associations between lipids or lipoproteins and single polymorphisms at this gene cluster, so they were included in the analysis.

In the 29 healthy individuals who had never smoked compared to the whole group, a weak association was found between HDL cholesterol or apoAI levels and genotypes of the 5'apoAI-G_{-75} to A polymorphism. In these non-smokers, carriers of the 5'apoAI-A_{-75} allele had higher mean HDL-cholesterol (1.59 versus 1.45mM) and apoAI levels (133 versus 123mg/100ml) compared to those lacking this allele in agreement with previous observations on non-smokers (Gudnason et al. 1992). These differences did not reach statistical

TABLE 3.5.5 COMPARISON OF MEAN LEVELS OF LIPID TRAITS BY APOB HAPLOTYPES

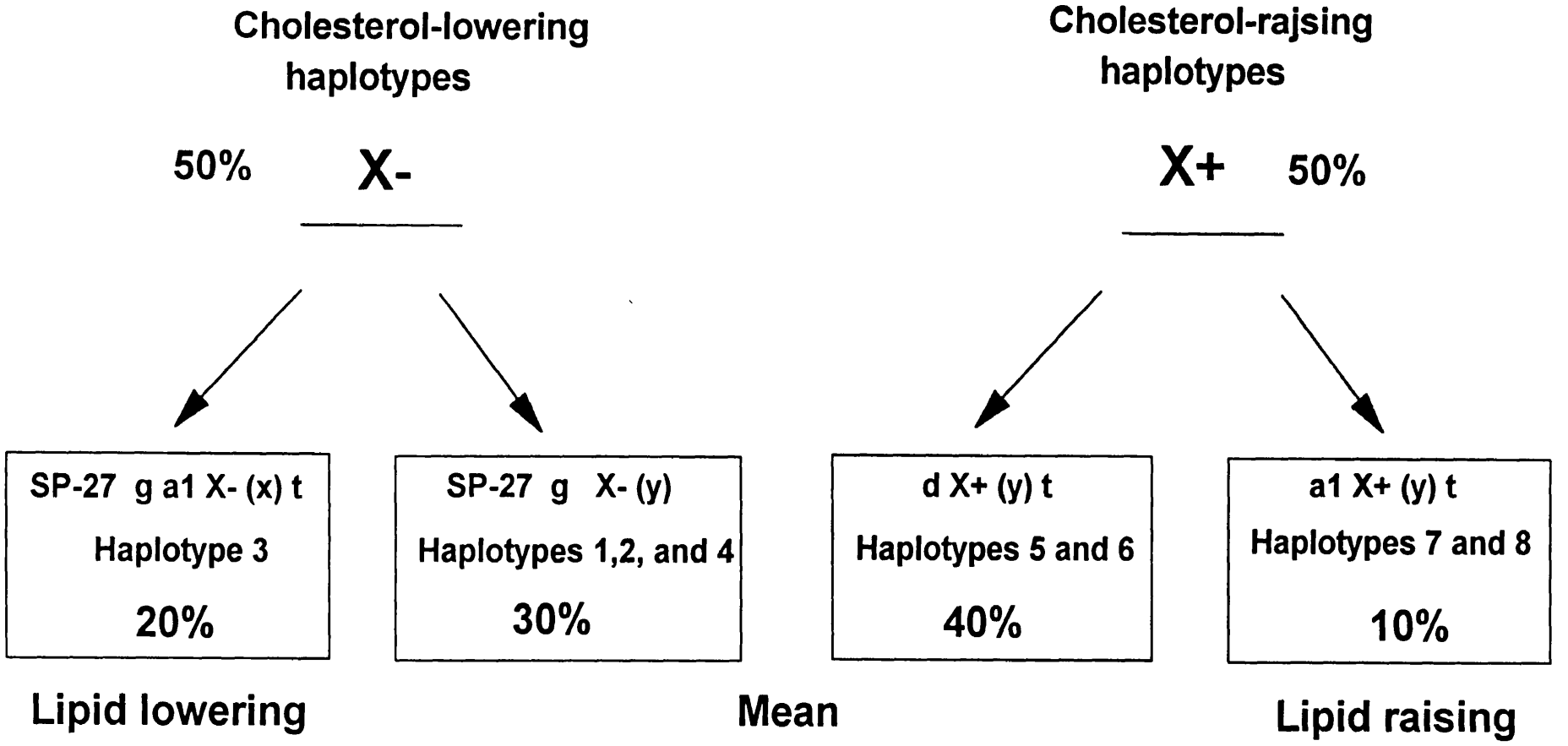
Trait (mmol/l)	Healthy individuals (n = 87) Ag(x) (n=23)	All others (n=41)	a1X+ (n=23)	R^2 x 100 (%), F and significance from regression*	Patients (n = 86) Ag(x) (n=28)	All others (n=43)	a1X+ (n=15)	R^2 x 100, F and significance from regression*
Cholesterol	5.78 ± 0.17	6.17 ± 0.20	6.34 ± 0.25	3.7% F=1.5, p=0.2	6.99 ±0.24	7.32±0.18	7.71±0.36	11.8% F=3.8, p=0.03
LDL-cholesterol	3.68 ± 0.12	4.09 ± 0.17	4.14 ± 0.19	5.0% F=2.0, p=0.1	4.80±0.20	4.97±0.16	5.03±0.31	2.0% F=0.6, p=0.6
HDL-cholesterol	1.51 ± 0.08	1.37 ± 0.08	1.40 ± 0.07	7.1% F=2.9, p=0.06	1.17±0.05	1.10±0.03	1.06±0.05	3.3% F=1.0, p=0.4
LDL-chol/ HDL-chol	2.59 ± 0.16	3.07 ± 0.13	3.13 ± 0.22	10.0% F=4.3, p=0.02	4.25±0.21	4.60±0.17	4.95±0.28	5.2% F=1.6, p=0.2
Total triglycerides	1.24 (1.03 -1.48)	1.29 (1.12 - 1.49)	1.32 (1.00 - 1.76)	0.8% F=0.3, p=0.7	1.91 (1.62 - 2.25)	2.37 (2.03 - 2.76)	2.71 (1.88 - 3.90)	11.1% F=3.6, p=0.04
LDL-triglycerides	0.33 ± 0.02	0.35 ± 0.02	0.37 ± 0.03	2.1% F=0.8, p=0.4	0.45±0.0.02	0.46±0.03	0.48±0.03	1.1% F=0.3, p=0.7
HDL-triglycerides	0.14 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.7% F=0.3, p=0.8	0.14±0.01	0.17±0.01	0.17±0.01	14.6% F=4.9, p=0.01
apoB (mg/dl)	100.0 ± 2.91	110.2 ± 3.11	113.3 ± 4.48	5.2% F=2.1, p=0.1	123.0±3.56	125.1±3.09	138.6±6.60	8.1% F=2.5, p=0.09
apoAI (mg/dl)	123.2 ± 3.92	126.1 ± 2.41	123.0 ± 4.88	2.4% F=1.0, p=0.4	116.8±5.20	110.6±2.56	103.2±4.12	5.3% F=1.6, p=0.2

Means ± S.E.s shown for traits (unadjusted since adjustment for age and BMI made little difference to the means) except for triglycerides for which antilogs of $\log_{10}$-transformed means and 95% confidence limits are shown.

For the healthy individuals, the 4 hyperlipidaemic individuals were included since they did not make any difference to the trends observed between apoB haplotypes and lipid traits.

* regression carried out on data adjusted for age and BMI.

Figure 3.5.1



Representation of postulated functional haplotypes of the apoB gene as identified in the Swedish sample.

Data is taken from Dunning et al. (1993). Where any one of the six diallelic polymorphisms is not shown, either allele is present on that haplotype or group of haplotypes.

SP-27 and SP-24 = alleles of the signal peptide polymorphism; g = Thr71; a1 = Val591; d = Ala591; X- and X+ = alleles of the XbaI polymorphism; (y) = Pro2712 + Asn4311; (x) = Leu2712 + Ser4311; t = Glu4154.

TABLE 3.5.6

PROPORTION OF VARIANCE (R^2x100) OF EACH TRAIT ADJUSTED FOR AGE AND BMI ATTRIBUTABLE TO GENETIC VARIATION AT THE APOAI-CIII-AIV GENE CLUSTER

a) healthy individuals - no exclusion

Trait	5'apoAI G_{-75} to A	3'apoAI PstI	apoCIII C_{1100} to T	apoCIII PvuII
Cholesterol				
Total	1.5	0.8	1.2	1.9
VLDL	0.9	0.2	0.5	3.0
LDL	2.6	1.6	1.5	0.8
HDL	4.5	0.3	2.2	3.2
Triglycerides				
Total	1.8	0.6	0	2.6
VLDL	1.2	0.7	0.3	1.7
LDL	3.5	0.1	4.2	7.4*
HDL	3.5	0.1	6.1	0.8

* $p < 0.05$

b) patients

Trait	5'apoAI G_{-75} to A	3'apoAI PstI	apoCIII C_{1100} to T	apoCIII PvuII
Cholesterol				
Total	2.2	1.5	4.3	2.8
VLDL	3.4	1.0	1.8	0.7
LDL	3.7	0	2.1	0.6
HDL	1.5	0.6	0.2	0.1
Triglycerides				
Total	2.6	2.0	2.0	1.9
VLDL	2.4	1.6	2.6	1.3
LDL	0.8	1.0	2.0	0.5
HDL	2.3	0.1	0.5	1.0

Only major lipoprotein fractions ie. VLDL, LDL and HDL were analysed for effects of genotypes.

TABLE 3.5.7
GENOTYPE MEANS (±S.E.) FOR SELECTED LIPOPROTEIN AND HDL SUBCLASS VARIABLES

Adjustment for age and BMI made little difference to the genotype means, so unadjusted values are shown here.

a) In the healthy individuals

Trait (mmol/l)	**Genotypes (n)** ApoCIII-PvuII RFLP		R^2x100, F-value and Significance from ANOVAS **b**
	V+V+ (64)	V+V- and V-V- (23)	
LDL-TGs	0.33 ± 0.01	0.40 ± 0.04	7.4, F = 6.2, p = 0.02
	Inferred haplotype G_{-75} /P+/T_{1100} /V+		
	Presence (18)	Absence (68)	
Triglycerides† ⊖	1.53 (1.27-1.85)	1.13 (1.02-1.24)	5.8, F = 5.1, p = 0.03
VLDL-TGs † ⊖	0.98 (0.74-1.29)	0.58 (0.50-0.68)	7.8, F = 7.0, p = 0.01
mg/protein/dl plasma	LPL genotypes		
	H+H+ (35)	H+H- and H-H- (26)	
HDL2b	0.20 ± 0.02	0.26 ± 0.03	5.3, F = 3.4, p = 0.07
HDL2a	0.41 ± 0.02	0.40 ± 0.02	0.1, F = 0.1, p = 0.8
HDL3b	0.34 ± 0.01	0.31 ± 0.01	3.1, F = 1.9, p = 0.2
LPL activity §	88.9 ± 4.55	95.8 ± 6.13	1.7, F = 1.0, p = 0.3

b) In the patients

Trait (mmol/l)	**Genotypes (n)** ApoCIII-PvuII polymorphism		R^2x100, F-value and Significance from ANOVAS **b**
	V+V+ (57)	V+V- and V-V- (29)	
LDL-TGs	0.45 ± 0.02	0.46 ± 0.02	0.5, F = 0.09, p = 0.7
	Inferred haplotype G_{-75} /P+/T_{1100} /V+		
	Presence (19)	Absence (67)	
Triglycerides†	1.94 (1.63 - 2.32)	2.33 (2.03 - 2.67)	1.4, F = 1.2, p = 0.3
VLDL-TGs †	1.25 (0.99 - 1.58)	1.58 (1.33 - 1.87)	1.3, F = 1.1, p = 0.3
mg/protein/dl plasma	LPL genotypes		
	H+H+ (20)	H+H- and H-H- (16)	
HDL2b	0.15 ± 0.02	0.08 ± 0.01	22.1, F = 9.7, p = 0.004
HDL2a	0.35 ± 0.02	0.25 ± 0.02	19.3, F = 8.1, p = 0.007
HDL3b	0.35 ± 0.02	0.42 ± 0.02	11.4, F = 4.4, p = 0.04
LPL activity §	77.4 ± 5.36	70.3 ± 6.61	0.8, F = 0.3, p = 0.6

b age and BMI adjusted data used.
† data is shown as antilogs of $\log_{10}$-transformed mean and 95% confidence limits.
⊖ Excludes the four hyperlipidaemic individuals (healthy individuals only)
§ LPL activity is measured in milliunits which is nmoles of fatty acid released per minute per ml of plasma.

significance due to the small sample size. Data from all four polymorphisms in the apoAI-CIII-AIV gene cluster was combined by determining 'haplotypes' and in the healthy individuals (with the exclusion of the three hyperlipidaemic individuals who had levels of cholesterol >9.5mM and TG >3mM), plasma and VLDL-TG levels, but not other traits, were significantly associated with presence/absence of the inferred haplotype 5'apoAI-G_{-75} 3'apoAI-P+ apoCIII-T_{1100}apoCIII-V+ (rare allele of the C to T_{1100} polymorphism but the common alleles of the other three polymorphisms, designated G/P+/T/V+) explaining 5.8 and 7.8%, respectively, of sample variance (Table 3.5.7). In the healthy individuals, carriers of the haplotype G/P+/T/V+ had 35.4% higher mean triglyceride levels and 69% higher mean VLDL-triglyceride levels than did those lacking this haplotype and those in which haplotypes could not be inferred (Table 3.5.7). In the patients, the reverse trend was observed which was not statistically significant. ANOVA on TGs versus presence/absence of this inferred haplotype with healthy individual/patient (group) status as covariate revealed a highly significant interaction between presence/absence of this inferred haplotype and group status ($F=17.03$, $p<0.001$) in modulating TG levels.

For the LPL gene, in the healthy individuals, triglyceride levels were significantly associated with genotypes of the HindIII polymorphism, and three other associations were nearly significant; levels of VLDL-cholesterol ($p = 0.05$) and VLDL-TG ($p = 0.05$) with genotypes of the HindIII polymorphism, and HDL-cholesterol levels ($p = 0.09$) with genotypes of the PvuII polymorphism. After adjustment for age and BMI, each of these associations accounted for at least 5% of total sample variance (Table 3.5.8). When the four hyperlipidaemic individuals (all homozygous for the H+ allele) were excluded, genotypes explained 5.3% of sample variance in TGs ($p=0.1$) and 4.2% of sample variance in cholesterol and TGs in the VLDL fraction ($p=0.2$). There were no significant associations between genotypes of the Serine447 to Stop polymorphism and serum lipid traits. None of the LPL gene polymorphisms used here were associated with a significant effect on any trait in the patient group.

Genotype means and standard errors or 95% confidence limits are presented in Table 3.5.9 for the above four associations both for healthy individuals and patients. For the PvuII polymorphism, heterozygous individuals had mean levels of HDL-cholesterol roughly 12%

Table 3.5.8

Proportion of variance (R^2x100) in traits adjusted for age + BMI that is explained by genotypes of polymorphisms in the LPL gene.

a) **Healthy Individuals** - no exclusion

Trait	Age + BMI a	PvuII	HindIII	Serine447 to Stop
Cholesterol				
Total	1.9	4.3	2.1	0.4
VLDL	12.6**	3.7	6.2	0.6
HDL	12.3**	5.6	2.8	1.4
Triglycerides				
Total	12.6**	2.7	7.4*	0.3
VLDL	11.8**	2.8	6.2	0.2
HDL	2.3	0.6	2.4	2.0
LPL Activity	2.0	0.7	2.2	0.9

Footnote; when the four hyperlipidaemic individuals were excluded (all had the genotype H+H+) genotypes of the HindIII polymorphism explained 5.3% (p=0.1), 4.2% and 4.2% (p=0.2) of sample variance in total- and VLDL triglycerides and VLDL-cholesterol, respectively.

b) **Patients**

Trait	Age + BMI a	PvuII	HindIII	$Serine_{447}$ to Stop
Cholesterol				
Total	0.3	0.1	0.8	0
VLDL	11.2**	2.5	0.1	0
HDL	6.2	1.9	0.2	0.1
Triglycerides				
Total	10.2	2.4	0.5	0
VLDL	13.0	2.0	0	0.1
HDL	4.9	3.7	2.2	0.3
LPL Activity	5.9	4.3	1.5	3.7

a = unadjusted data, * = $p < 0.05$, ** = $p < 0.01$

TABLE 3.5.9 MEANS ± STANDARD ERRORS, FOR SELECTED TRAITS, BY GENOTYPES OF POLYMORPHISMS IN THE LPL GENE.

	HEALTHY INDIVIDUALS				PATIENTS			
Trait (mmol/l)	V+V+ (29)	V+V- (42)	V-V- (19)	F-value and signif. from ANOVAS **b**	V+V+ (29)	V+V- (38)	V-V- (18)	F-value and signif. from ANOVAS **b**
HDL-cholesterol	1.32 ± 0.05	1.51 ± 0.05	1.38 ± 0.08	F=2.5, p=0.09	1.11 ± 0.04	1.10 ± 0.04	1.18 ± 0.04	F=0.8, p=0.5
	H+H+ (52)	H+H- (34)	H-H- (3)		H+H+ (49)	H+H- (35)	H-H- (1)	
HDL-cholesterol	1.39 ± 0.05	1.50 ± 0.05	1.18 ± 0.12	F=1.2, p=0.3	1.13 ± 0.03	1.08 ± 0.03	1.23	F=0.3, p=0.8
VLDL-cholesterol †	0.50 (0.41-0.61)	0.34 (0.28-0.41)	0.50 (0.20-1.22)	F=2.8, p=0.07	0.83 (0.68-1.02)	0.95 (0.78-1.17)	0.67	F=0.1, p=0.9
TGs †	1.42 (1.22-1.66)	1.06 (0.92-1.21)	1.26 (0.85-1.89)	F=3.4, p=0.04	2.24 (1.90-2.42)	2.31 (1.97-2.70)	1.48	F=0.2, p=0.8
VLDL-TGs †	0.80 (0.65-0.99)	0.54 (0.44-0.67)	0.72 (0.47-1.10)	F=2.8, p=0.07	1.45 (1.18-1.78)	1.61 (1.31-1.97)	1.21	F=0, p=1.0
	H+H+ Ser/Ser (52)	H+H- and H-H- combined Ser/Ser (26)	Pres. of Stop(9)	F-value and signif. from ANOVAS **b**	H+H+ Ser/Ser (49)	H+H- and H-H- combined Ser/Ser (28)	Pres. of Stop(9)	F-value and signif. from ANOVAS **b**
VLDL-cholesterol †	0.50 (0.41-0.61)	0.33 (0.26-0.41)	0.40 (0.29-0.56)	F=3.8, p=0.05	0.83 (0.68-1.02)	0.95 (0.75-1.20)	0.84 (0.52-1.37)	F=0.1, p=0.8
TGs †	1.42 (1.22-1.66)	1.02 (0.87-1.18)	1.18 (1.07-1.50)	F=4.7, p=0.03	2.24 (1.90-2.42)	2.28 (1.90-2.74)	2.13 (1.51-3.00)	F=0.1, p=0.8
VLDL-TGs †	0.80 (0.65-0.99)	0.52 (0.41-0.65)	0.63 (0.44-0.91)	F=3.8, p=0.05	1.45 (1.18-1.78)	1.60 (1.27-2.03)	1.47 (0.99-2.17)	F=0.1, p=0.9
LPL activity (milliunits)	(36) 88.9 ± 4.6	(20) 96.9 ± 8.0	(6) 91.8 ± 8.7	F=0.5, p=0.5	(20) 77.4 ± 5.4	(12) 74.4 ± 7.8	(4) 58.0 ± 11.9	F=0.7, p=0.4

b on age and BMI adjusted data. † data is shown as antilogs of log-transformed means and 95% confidence limits

Unadjusted means are shown since adjustment for age and BMI made little difference to the genotype means

In the healthy individuals, when the four hyperlipidaemic individuals, who are all homozygous for the H+ allele are excluded, means for VLDL-cholesterol, TGs and VLDL-triglycerides are 0.45, 1.32 and 0.73 mmol/l, respectively.

higher than either of the homozygous genotype groups. This effect was not seen in the patients. The H+ allele of the HindIII polymorphism was associated with a small elevating effect on TGs in the healthy individuals, regardless of inclusion or exclusion of the four hyperlipidaemic individuals. However trends were not consistent between the healthy individuals and patients, in the former group, H+H- heterozygotes had the lowest mean levels and in the patients, the single individual homozygous for the H- allele had the lowest levels. To ascertain whether haplotypes of the HindIII and the Serine447 to Stop polymorphisms might confer any influence on lipids in addition to that seen with genotypes of the HindIII polymorphism, individuals were grouped as follows. Individuals homozygous for the haplotype H+ Serine447 were combined into one group and those carrying the rare allele, H- were sub-divided into groups carrying and lacking the Stop allele, respectively. This allowed simultaneous comparison of haplotypes of both polymorphisms. In the healthy individuals, the group carrying the H- allele but lacking the Stop447 allele had the lowest mean levels of TG, VLDL-cholesterol and VLDL-TG (72%, 66% and 65% respectively of the mean levels seen with genotype H+H+) while the group homozygous for the H+Serine447 haplotype had the highest means for all of these traits. When the four hyperlipidaemic healthy individuals were excluded the association with triglyceride levels remained statistically significant.

Section 3.6 Associations between gene polymorphisms and lipase activity or HDL subclasses

In 64 of the healthy individuals and 36 of the patients measurement of LPL activity and HDL subclasses had been carried out. The characteristics of this subgroup were not significantly different from the whole population (data not shown). None of the apoB, apoAI-CIII-AIV or LPL polymorphisms were associated with a significant effect on levels of LPL activity, with the exception of genotypes of the apoB XbaI polymorphism in the patient group (33.4% of sample variance explained by genotypes, $F=8.3$, $p=0.001$, due to individuals homozygous for the X- allele having 33% lower LPL activity (mean $\pm$ S.E = 59.3 ± 4.7) compared to those with other genotypes (mean $\pm$ S.E. = 85.0 ± 5.2). A similar trend was observed in the healthy individuals with homozygosity for the X- allele associated with 8.4% lower mean activity (mean $\pm$ S.E. = 84.9 ± 7.3) than in the presence of the X+ allele (mean $\pm$ S.E. = 92.7 ± 4.4). This needs to be confirmed in an independent sample before the speculation that common variation in the apoB gene does have an effect on LPL activity is

TABLE 3.6.1

PROPORTION OF VARIANCE (R^2 x 100) IN HDL AND RELATED TRAITS ADJUSTED FOR AGE AND BMI THAT IS EXPLAINED BY GENOTYPES OF POLYMORPHISMS AT THE APOAI-CIII-AIV AND APOE GENE LOCI.

In healthy Individuals (n = 90; 63 for HDL subclasses)

Trait **b**	Age+BMI **a**	5'apoAI G_{-75} to A polymorphism	3'apoAI-PstI	apoCIII C_{1100} to T	PvuII RFLP in apoCIII	ApoE	HindIII polymorphism in LPL gene
HDL 2b	11.4 *	4.9	0.6	0.3	4.4	1.9	5.3
2a	4.2	1.5	1.2	0.3	1.0	5.6	0.1
3a	0.9	0.5	0.2	1.2	5.0	5.0	0
3b	15.9 **	0.3	0.8	0.5	0.7	1.2	3.1
3c	8.7	1.9	1.2	0.3	0.5	0.1	1.6
ApoAI	6.8 *	3.3	0	0.6	3.2	3.8	0
ApoAII	7.4 *	2.6	0	0.7	2.6	0.7	0.2

In patients (n = 86; 36 for HDL subclasses)

Trait **b**	Age+BMI **a**	5'apoAI- G_{-75} to A polymorphism	3'apoAI-PstI	apoCIII C_{1100} to T	PvuII RFLP in apoCIII	ApoE	HindIII polymorphism in LPL gene
HDL 2b	4.7	2.4	6.6	0.4	1.5	1.0	22.1 **
2a	13.2	1.2	10.5	1.9	13.5	2.6	19.3 **
3a	1.0	4.4	8.3	0.7	1.2	4.0	0.4
3b	26.9 **	7.2	0.8	0.8	4.3	6.9	11.4 *
3c	20.5 *	16.5	0.1	5.3	3.9	2.6	4.2
ApoAI	4.2	0.8	1.9	0.5	1.0	3.1	0.5
ApoAII	0.7	0.3	1.1	6.6	1.2	0.4	0

Footnote; for apoE, genotypes were grouped as follows; E3/2 vs. E3/3 vs. (E4/3 and E4/4 combined) leaving out the rare genotype E4/2

a unadjusted data used **b** data was adjusted for age and BMI prior to regression for genotypes

* $p < 0.05$ ** $p < 0.01$

warranted. For the LPL gene, in both patients and healthy individuals, those carrying the Stop447 allele, had lower mean LPL activities though the effect was noticeably stronger in the patients (24% lower than the others not carrying this allele; Table 3.5.9).

Associations between variation at other gene loci and HDL subclasses were examined. For the common apoE isoforms, genotypes were grouped as follows, E3/2 versus E3/3 versus (E4/3 and E4/4 combined) leaving out the rare genotype E4/2. No associations were found between apoE genotypes and levels of any of the HDL subclasses in either the healthy individuals or patients. In both groups, no associations were found between levels of the HDL subclasses and either the signal peptide or XbaI polymorphisms in the apoB gene, chosen for their high rare allele frequencies. In the patients, the HDL2b, HDL2a and HDL3b subclasses were significantly associated with genotypes of the HindIII polymorphisms in the LPL gene explaining 22.1%, 19.3% and 11.4%, respectively, of sample variance (Table 3.6.1). In the healthy individuals, the proportion of sample variance explained by the PvuII polymorphism in the LPL gene was larger for HDL2b and HDL2a (1.5 and 5.5%: N/S) than for HDL3a and HDL3b (0.4 and 0.7%). The association between HDL cholesterol levels and genotypes of the LPL-PvuII polymorphism ($p = 0.09$) in this sample is mainly explained by the HDL_2 subclasses rather than the HDL_3 subclasses. The significant association between levels of HDL2b, HDL2a and HDL3b and variation in the LPL gene observed in the patients was not seen in the healthy individuals. In the patients means of HDL2b and HDL2a were 47% and 29% lower in the presence of the H- allele and means of HDL3b were 20% higher in the presence of the H- allele (Table 3.5.7). The LPL-HindIII polymorphism did not have a significant impact on LPL activities in either the healthy individuals or patients, however in both groups, both HDL2b levels and LPL activities followed the same trend (Table 3.5.7).

Section 3.7 Associations between Gene polymorphisms and severity of atherosclerosis

The possibility was examined that genotypes at the apoE, apoB, apoAI-CIII-AIV or LPL loci were associated with baseline atherosclerosis or progression scores. Seventy-one of the MI survivors had data on both baseline severity and progression of atherosclerosis. For genotypes of the apoB signal peptide length polymorphism, haplotype combinations of the LPL HindIII and Serine447 to Stop polymorphisms and genotypes of the apoCIII C_{1100} to T polymorphism, effects were observed on development of atherosclerosis. Box plots showing

Descriptive data for atherosclerosis scores (no units)	Genotypes of apoB signal peptide polymorphism		
	SP27/27	SP27/24	SP24/24
Mean	1.8	0.9	1.1
Median	2.0	0.8	0.9
Minimum	0	0	0
Maximum	3.6	2.9	2.9
Skewness	-0.15	0.90	0.70
Standard deviation	1.10	0.81	0.95
Interquartile range	2.00	1.33	1.55

In the Swedish patient sample, both baseline and progression of atherosclerosis were scored on a scale from 0, which means no baseline disease or no progression, to 4.0 which means severe baseline disease or severe progression.

For figures 3.7.1 to 3.7.4 inclusive, baseline scores for coronary atherosclerosis are given on the vertical axis. Numbers of individuals (in brackets) and genotypes are given above the boxes on the horizontal axis. Values of medians are shown below the respective boxes.
50% of individuals within each subgroup have values within the box, which represents the interquartile range. Range of scores for each genotype is indicated by the lines perpendicular to the box. * = median.

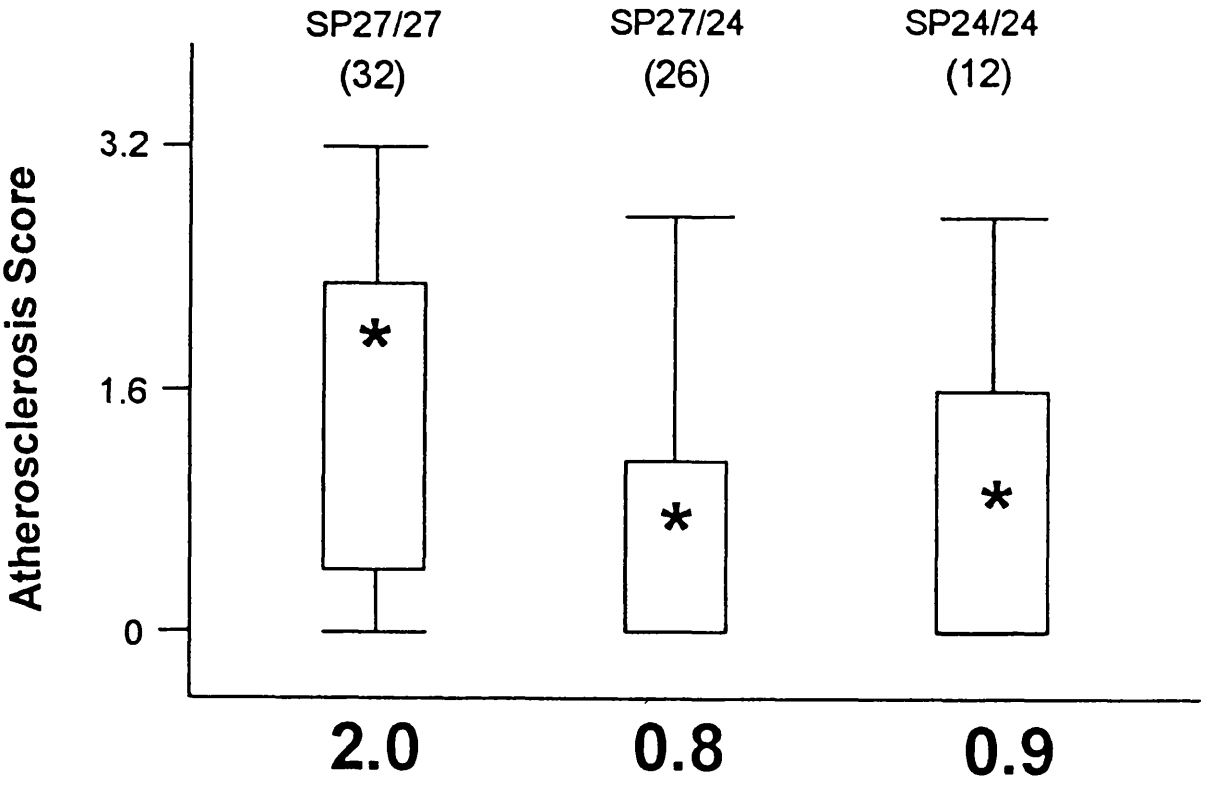


Figure 3.7.1; apoB
Box plot showing the distribution of baseline scores for coronary atherosclerosis for each of the three genotypes of the signal peptide (SP) polymorphism; SP27/27, SP27/24 and SP24/24. Chi-square = 9.6, p = 0.008 across all three genotypes.

The median is the mid-point of the range (the 50th percentile). The lower and upper boundaries of the boxes are the 25th and 75th percentiles, respectively. 50% of cases have values within the box and the length of the box corresponds to the interquartile range, which is the difference between the 25th and 75th percentiles.

The length of the box gives an idea of the spread and variability of observations. Where the median is not in the centre of the box, the distribution is skewed. If the median is closer to the bottom of the box than the top, this means the data is positively skewed, with a tail of large values. Negatively skewed data is denoted by the median being closer to the bottom of the box than the top.

Descriptive data for atherosclerosis scores (no units)	Haplotypes of HindIII and Ser447/Stop polymorphisms in the LPL gene		
	H+H+ Ser/Ser	H+H- Ser/Ser	H+H- Ser/Stop
Mean	1.3	1.1	2.1
Median	1.0	1.0	2.8
Minimum	0	0	0.2
Maximum	3.0	3.5	3.6
Skewness	0.37	0.70	-0.70
Standard deviation	0.98	1.00	1.16
Interquartile range	1.78	1.63	1.85

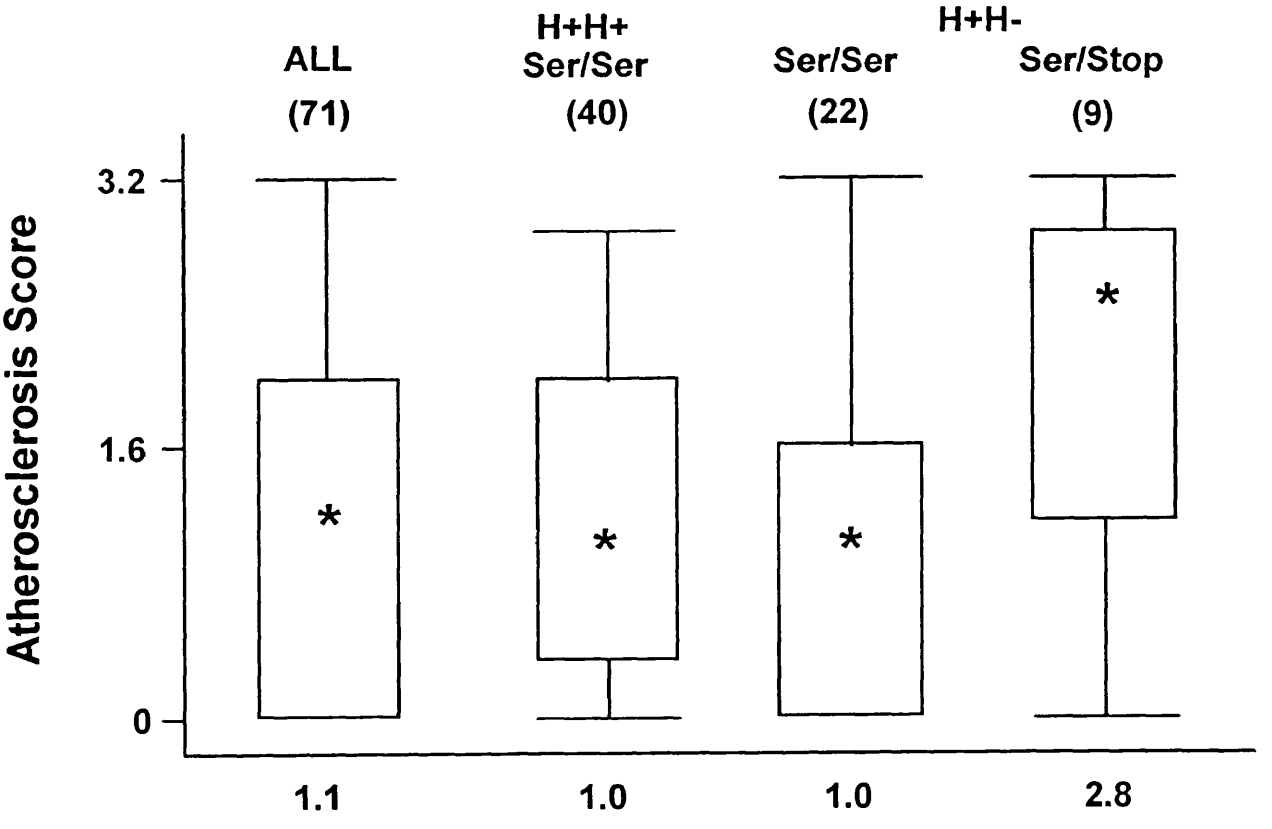


Figure 3.7.2; LPL
Box plot showing the distribution of baseline scores for coronary atherosclerosis for the whole patient sample and in the absence and presence (with and without the Stop allele of the Ser447 to Stop polymorphism) of the H- allele of the HindIII polymorphism. Chi-square = 5.5, p = 0.07 across all three groups; p = 0.02 between those with the H- Ser haplotype and those with the H- Stop haplotype and p = 0.05 between individuals with the H+ Ser haplotype and those with the H- Stop haplotype.

Descriptive data for atherosclerosis score (no units)	Genotypes of the C_{1100} to T polymorphism in apoCIII	
	CC	CT and TT
Mean	1.6	1.2
Median	1.6	0.8
Minimum	0	0
Maximum	3.6	3.5
Skewness	0.03	0.70
Standard deviation	0.98	1.08
Interquartile range	1.65	1.93

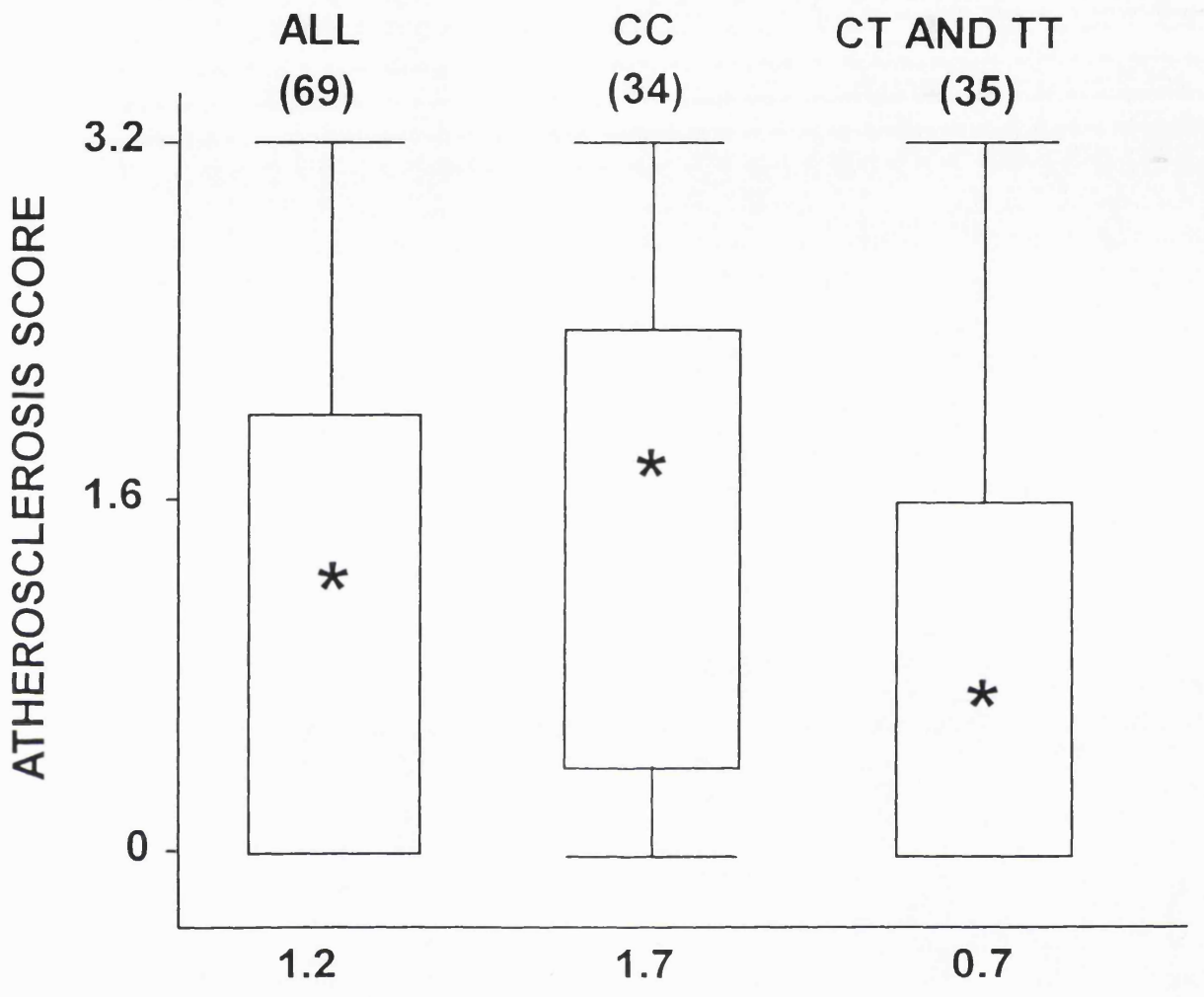


Figure 3.7.3; apoCIII
Box plot showing the distribution of baseline scores for coronary atherosclerosis for the whole patient sample and in the absence and presence of the T allele of the C_{1100} to T polymorphism. p = 0.09 between absence and presence of the T allele, using the Mann-Whitney test.

the distribution of global coronary atherosclerosis at the time of the first coronary angiography in connection with the acute event in patients with different genotypes are presented in Figure 3.7.1 (apoB signal peptide genotypes), Figure 3.7.2 (LPL haplotypes) and Figure 3.7.3 (apoCIII genotypes). Since the distributions of these parameters could not be normalized, the non-parametic Kruskal-Wallis test was used to test the null hypothesis that there was no association between genetic variation and atherosclerosis score. No significant associations were observed between genotypes of the apoB Ag(a1/d) epitope pair and severity or progression of atherosclerosis, though a trend towards worse baseline coronary atherosclerosis was observed in those homozygous for the Ag(a1) epitope compared to those with Ag(d). The correlation between baseline and progression scores was stronger in the subgroup of individuals homozygous for the SP-24 allele than in the other genotypes of the signal peptide length polymorphism or in the patient sample as a whole (values of Pearson correlation coefficients were 0.54 for homozygosity for the SP-24 allele, 0.29 for the heterozygotes, -0.008 for homozygosity for the SP-27 allele and 0.25 for the whole patient sample; none were significantly different from one another). Means and standard errors of selected lipid/lipoprotein traits with genotypes, which were associated with severity of coronary atherosclerosis at the first angiography, are displayed in Table 3.7.1. 'Atherogenic' genotypes are defined as those which were associated with the highest median baseline coronary atherosclerosis score. For the apoB gene, the 'atherogenic' genotype, homozygosity for the SP-27 allele, was associated with about 10% lower mean apoAI levels compared to presence of the SP-24 allele, for the apoCIII gene, the 'atherogenic' genotype, CC, was associated with the highest mean triglyceride levels, about 14% higher than for presence of the T allele, and for the LPL gene, the 'atherogenic' haplotype, H- Stop447 was associated with the lowest mean levels of the HDL2b subclass, as measured by the amount of protein in that subclass (approximately 50% lower than for the other two haplotypes, H+ Serine447 and H- Serine447 combined). This haplotype, H-Stop447, was also associated with 24% lower LPL activity, and smaller lowering effects on HDL2a and HDL3b levels (Table 3.7.1).

The combined association between baseline atherosclerosis scores and genotypes at all three loci was also examined. An 'atherogenic genotype' is defined as the genotype at the locus associated with the highest atherosclerosis score. In the Swedish patient sample, for apoB, this is homozygosity for the common allele of the signal peptide polymorphism, for

Table 3.7.1

MEANS (± S.E.s) FOR PLASMA TRAITS WHICH SHOWED THE STRONGEST ASSOCIATIONS WITH GENOTYPES THAT SHOWED A SIGNIFICANT ASSOCIATION WITH BASELINE CORONARY ATHEROSCLEROSIS SCORE IN THE SWEDISH PATIENTS.

Trait (unadjusted)	Genotypes of apoB signal peptide length polymorphism			F-value and significance from ANOVAs b
ApoAI (mg/100ml) ¥	SP27/27 (29) ¶ 105.6 ± 3.2	SP27/24 (23) 113.5 ± 4.1	SP24/24 (11) 122.3 ± 4.6	F = 4.0, P =0.02
	Genotypes of apoCIII C_{1100} to T polymorphism			
Triglycerides (mmol/l) †	CC (39) ¶ 2.43 (2.06 - 2.87)	CT (40) 2.18 (1.84 - 2.59)	TT (5) 1.74 (0.99 - 3.07)	F = 1.0, p = 0.4
	Haplotypes of LPL HindIII and Serine447 to Stop polymorphisms			
HDL subclass (mg/protein/100ml plasma)	H+H+	H+H- and H-H- combined		
	Serine/Serine (20)	Serine/Serine (12)	Presence of Stop (4) ¶	
HDL 2b	0.15 ± 0.02	0.09 ± 0.01	0.06 ± 0.02	F = 5.6, p = 0.008
HDL 2a	0.35 ± 0.02	0.25 ± 0.02	0.27 ± 0.02	F = 4.0, p = 0.03
LPL activity	77.4 ± 5.4	74.4 ± 7.8	58.0 ± 11.9	F = 0.7, p = 0.4

¥ Data on apoAI levels was only available on 63 of the patients.
¶ Genotype associated with higest median atherosclerosis score at the first angiography (most severe disease)
† Data is shown as antilogs of $\log_{10}$-transformed means and 95% confidence limits.

Descriptive data for atherosclerosis score (no units)	Number of 'atherogenic' genotypes			
	0	1	2	3
Mean	0.6	1.5	1.9	2.3
Median	0.4	1.3	2.3	2.4
Minimum	0	0	0	0.6
Maximum	2.0	3.5	3.0	3.6
Skewness	1.47	0.29	-0.85	-0.60
Standard deviation	0.57	0.99	1.06	1.28
Interquartile range	0.65	1.73	1.80	2.45

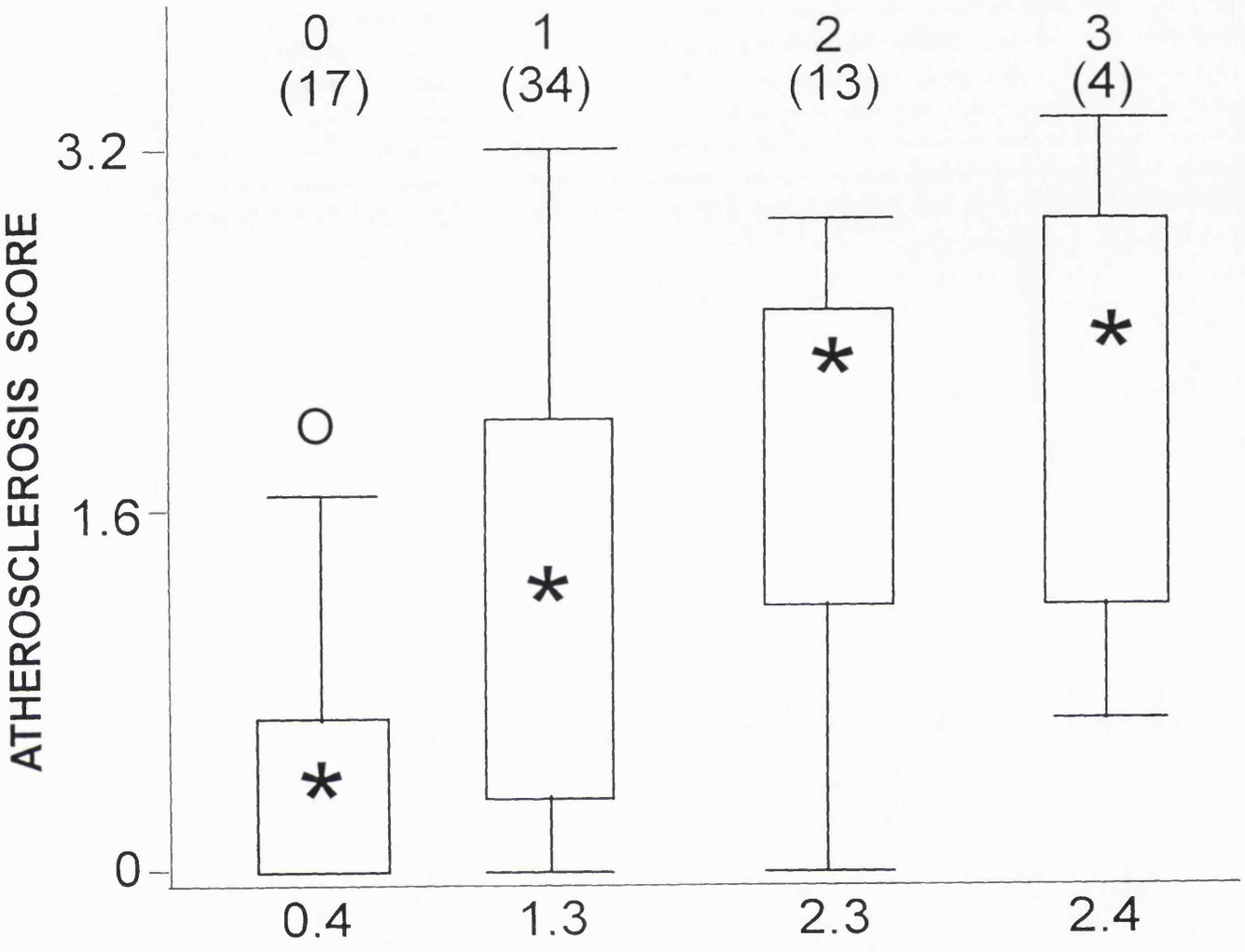


Figure 3.7.4; apoB, LPL and apoCIII.
Box plot showing the distribution of baseline scores for coronary atherosclerosis for the subgroups of the patient sample, having, respectively, 0, 1, 2 or 3 'atherogenic' genotypes - these genotypes being 1) homozygosity for SP-27 (apoB); 2) presence of both the H- and Stop alleles (LPL) and 3) homozygosity for the C allele (apoCIII). The 'atherogenic' genotype of each of these polymorphisms is defined as the one associated with the highest median score. Chi-square = 16.2, p = 0.001 across all four groups. Distribution of individuals among the four groups was as expected for random association of all three gene loci.
o = outli er (= individual who has a score that is at least two standard deviations away from the mean score for that subgroup).

LPL, the H- Stop447 haplotype (presence of the rare alleles of the HindIII and Serine-Stop447 polymorphism) and for the apoCIII gene, homozygosity for the C allele of the C_{1100} to T polymorphism. Individuals can be distinguished as having 3, 2, 1 or none of the 'atherogenic' genotypes and the baseline atherosclerosis score in these groups is shown in Fig. 3.7.4. The median score is significantly different among all four groups ($p = 0.001$); being six times higher in individuals with all three 'atherogenic' genotypes than in those with none of these genotypes. The number of 'atherogenic' genotypes was not significantly associated with lipoprotein levels or other characteristics such as fibrinogen or plasminogen activator inhibitor-1 (PAI-1) (Table 3.7.2). These two non-lipid traits were chosen because elevated levels of fibrinogen were associated with IHD in healthy men (Meade et al. 1986) and with severity of atherosclerosis in the MI survivors (Hamsten et al. 1986). Elevated levels of PAI-1 underlied reduced fibrinolytic capacity in MI survivors (Hamsten et al. 1985) and were associated with risk of re-infarction in the Swedish patients (Hamsten et al 1987). Level of PAI-1 is a candidate risk factor for thrombosis (reviewed in Dawson & Henney 1992). There was no clear trend observed in most of these traits across all four groups, with the exception of fibrinogen activity which showed an increase in means from zero to three 'atherogenic' genotypes. PAI-1 which shows a strong postive correlation with TG levels (Hoffman et al. 1992) explaining 25% of sample variance in PAI-1 levels in the Swedish patient sample, when adjusted for TGs, showed a linear decrease as the number of 'atherogenic' genotypes increased from 1 to 3. Excluding the four individuals with all three 'atherogenic' genotypes, mean levels of total and LDL-cholesterol increased as the number of 'atherogenic' genotypes increased from zero to 2. When the individuals with two or three 'atherogenic' genotypes were combined into one group, the trend became clearer, with an increase in mean total-cholesterol, LDL-cholesterol, TGs and fibrinogen levels as the number of 'atherogenic' genotypes increased from zero to two.

Section 3.8.1 Discussion of associations between genotypes and fasting lipids, LPL activity and HDL subclasses

In agreement with previous population studies (reviewed in Davignon et al 1988), there was a significant association between apoE genotypes and total and LDL-cholesterol and apoB in both the healthy individuals and patients. In both healthy individuals and patients, the E2 allele was associated with lower mean total and LDL-cholesterol and apoB levels, as

Table 3.7.2
MEANS ± S.Es FOR SELECTED PLASMA TRAITS VERSUS NUMBER OF 'ATHEROGENIC GENOTYPES' IN THE SWEDISH PATIENTS

Trait (adjusted for age + BMI) mmol/l	**Number of 'atherogenic' genotypes** (number of individuals in brackets)				**F-value and significance** from ANOVAs
	0 (17)	1 (34)	2 (13)	3 (4)	
Median atherosclerosis score	0.40	1.30	2.30	2.40	From Kruskal-Wallis test; chi-squared = 15.68, p = 0.001 (3 d.f.)
Total cholesterol	6.79 ± 0.28	7.40 ± 0.18	7.62 ± 0.31	7.42 ± 1.21	F = 1.0, p = 0.4
LDL-cholesterol	4.61 ± 0.22	5.10 ± 0.20	5.23 ± 0.27	4.81 ± 0.82	F = 0.7, p = 0.6
HDL-cholesterol	1.19 ± 0.60	1.11 ± 0.03	1.20 ± 0.07	0.96 ± 0.07	F = 1.8, p = 0.2
Triglycerides†	2.03 (1.59 - 2.59)	2.19 (1.87 - 2.57)	2.08 (1.59 -2.74)	2.66 (1.21 - 5.87)	F = 0.3, p = 0.8
Fibrinogen g/l ⊕	3.42 ± 0.16	3.56 ± 0.18	3.63 ± 0.35	4.10 ± 0.50	F = 0.5, p = 0.7
PAI-1 §	17.24 ± 2.63	20.99 ± 1.97	15.97 ± 2.09	8.92 ± 5.53	F = 2.1, p = 0.1

† antilog of $\log_{10}$-transformed mean and 95% confidence limits. d.f. = degrees of freedom

⊕ Levels are unadjusted as age and BMI only explained 1.8% of sample variance in fibrinogen levels.

§ units of PAI-1 (Plasminogen activator inhibitor-1) which are arbitrary. One arbitrary unit corresponds to inhibiton of one international unit of tissue plasminogen activator (t-PA) as compared with the first international t-PA standard (Hamsten et al. 1986). Levels are adjusted for $\log_{10}$ of triglycerides, as triglycerides explained 25% of sample variance in PAI-1 levels.

'Atherogenic' genotype = genotype associated with the highest median global coronary atherosclerosis score at baseline. For the apoB gene, this is the SP27/27 genotype of the signal peptide length polymorphism; for the apoCIII gene, this is homozygosity for the C_{1100} allele of the C_{1100} to T polymorphism and for the LPL gene, this is presence of both the H- allele of the HindIII polymorphism and the Stop allele of the $Serine_{447}$ to Stop polymorphism.

68 of the patients had data for extent of atherosclerosis plus data for genotypes at all three loci - it is on this subgroup of patients that the above data on plasma traits is shown.

compared to the E4 allele, as expected from previous population studies (Davignon et al. 1988). The lowering and elevating effects of the apoE2 and apoE4 alleles, respectively, on total and LDL-cholesterol and apoB are well documented in the literature. No other gene product has yet been identified with a larger contribution to the determination of inter-individual differences in cholesterol levels in the normal range of variability (Davignon et al. 1988).

The healthy individuals and patients exhibited quite different results for the effect of genetic variation at the apoE locus on TGs, HDL-cholesterol and HDL-TGs. This is in concordance with the lack of a consistent relationship between apoE genotype and TGs in previous population studies (Davignon et al. 1988). Any effects of apoE genotype on TGs and the HDL fraction may be small and population-specific (Davignon et al. 1988), as seen in the healthy individuals. In the patients, the effect of apoE genotype on total and HDL-TGs was largely due to inclusion of the individuals with the rare genotype E4/2. The large variability of triglyceride levels amongst and within individuals (Austin 1990) could mask a clear effect of apoE phenotype on triglyceride levels (Dallongeville et al. 1992). These authors, by pooling the results from 45 population sample studies, demonstrated that, in general, TGs were higher in individuals carrying the apoE2 allele and those with the E4/3 genotype than in individuals homozygous for the apoE3 allele. This was observed in the healthy Swedish individuals for total and VLDL-TGs, with genotypes E2/3 and E4/4 being associated with the highest levels. This trend was absent in the patients, this may be due to interaction between genetic or environmental factors that mask the effect of apoE genotype. Thus in the Swedish sample, apoE genotypes exerted their strongest and most consistent effects on total and LDL-cholesterol and apoB levels; and weaker and less consistent effects on TGs and the HDL fraction. The mechanism underlying this effect of apoE genotypes on lipids and lipoproteins is discussed in the introduction of this thesis (Section 1.4.6); it is a combination of the greatly diminished receptor-binding affinity of the apoE2 isoform compared to the apoE3 and apoE4 isoforms and the differential distribution, among lipoprotein fractions, of the apoE4 isoform compared to apoE2 and apoE3.

In the healthy individuals and patients, apoB genotypes had a smaller impact on lipid traits than that observed with apoE genotypes. For the apoB gene polymorphisms, the largest

effect seen was the association between the C_{-265} to T polymorphism and apoB and LDL-cholesterol in the healthy individuals. Genotype CC was associated with the highest levels and genotype TT with the lowest levels of both apoB and LDL-cholesterol. A similar effect was observed in the patients i.e. individuals with genotype CT had lower mean apoB levels than did those with genotype CC but this was not statistically significant. This confirms a previous finding (Xu 1990). The high apoB and LDL-cholesterol levels in the single patient with the TT genotype is puzzling in the light of the proposed lowering effect of the T allele on these two traits. The most plausible reason is genetic or environmental factors underlying the severe baseline atherosclerosis (score = 2.8) in this individual interacting to override the normal protective effect of the T allele. In the Swedish healthy individuals the effect associated with the T allele on apoB levels was to lower by 13.3mg/dl and that of the C allele to raise by 1.9mg/dl. The mechanism of the association is unclear at present. The C to T substitution at position -265 relative to the transcriptional start site is found within a region of the apoB gene spanning from positions -900 to -129 and known to contain both positive and negative regulatory elements (Carlsson & Bjursell 1989, Scott 1990). The positive and negative elements interact to produce a high level of apoB expression restricted to two tissues, liver and small intestine (Carlsson & Bjursell 1989). Hypomethylation of CCGG and CGCG sequences correlates with gene activity (Levy-Wilson & Fortier 1989). The cytosine at position -265 is within one such sequence and is unmethylated in HepG2 and Caco2 cells, both apoB expressing cells (Levy-Wilson & Fortier 1989). A number of proteins have been identified which bind to the 5' flanking region of the apoB gene and modulate transcription (Scott 1990) and more recently, Metzger et al (1993) demonstrated an synergistic effect of two distinct DNA-binding proteins which bind to the proximal promoter region (-81 to -52 relative to the transcription start site) on apoB transcription. The C to T substitution at position -265 may change affinity of binding of any one of these proteins or combinations of these, leading to changes in rate of transcription. Alternatively it may be in linkage disequilibrium with sequence differences in other parts of the -900 to -129 region, or with sequence differences within the proximal region spanning positions -111 to -33, which is a strong positive element active only in apoB expressing cells (Carlsson & Bjursell 1989) or with sequence changes in the general positve element in the non-coding part of exon 1 (Carlsson & Bjursell 1989). Previous studies on the beta-globin gene promoter (Superti-Furga et al. 1988) and the LDL-receptor gene promoter (Dawson et al. 1988) have demonstrated that single base substitutions

can enhance as well as decrease promoter function. This hypothesis was tested for the apoB C_{-265} to T substitution (Xu 1990) using a mobility shift assay with synthetic 20 base oligonucleotides containing either C or T at position -265 and nuclear extracts from Hep3B cells (a liver cell line, Aden et al. 1979). Both oligonucleotides bound to nuclear protein B (NPB) but the affinity of the T_{-265} oligonucleotide for NPB was much weaker, suggesting that this C_{-265} to T substitution may be functional (Xu 1990). No further experiments to examine this possibility have been carried out.

In a number of published studies, the X+ allele of the XbaI polymorphism has been associated with higher total- and LDL-cholesterol levels, this is discussed in the Introduction of this thesis. This was also observed in the group of Swedish healthy individuals with the difference in LDL-, but not total-, cholesterol reaching statistical significance. In this group, genotype X+X+ was associated with the highest mean apoB and LDL-cholesterol levels and genotype X-X- with lowest mean levels ($p < 0.05$). The same trend was observed for total cholesterol, but weaker. The observations are compatible with a co-dominant effect which is not surprising since each LDL particle contains only one apoB molecule (Talmud et al. 1987). This co-dominant effect was absent in the patient group, with the X+X- heterozygotes having the lowest mean LDL-cholesterol and apoB levels. The X+ allele has been associated with raised TGs in four studies (Law et al. 1986, Talmud et al. 1987, Jenner et al. 1988, Saha et al. 1992), but this effect was not seen in the healthy individuals. TG levels were associated with genotypes of the XbaI polymorphism ($p=0.02$) in a sample of fifty-two Swedish male myocardial infarction survivors (Wiklund et al. 1989), with the X-X- genotype associated with a mean triglyceride level roughly 70% higher than the mean obtained for presence of the X+ allele. The same trend was observed in the Swedish patients, but was not statistically significant. This could partly be due to differences in selection of the patients, Wiklund et al. (1992) did not exclude three diabetic patients and did not adjust lipid traits for age and BMI prior to statistical analysis.

The XbaI polymorphism, which does not change the amino acid sequence of apoB (Carlsson et al. 1986), must be in linkage disequilibrium with variation elsewhere in the apoB gene that itself affects the rate of apoB production or catabolism - this is discussed further in Chapter 5 of this thesis (Section 5.6). The small and non-significant associations observed

between traits and genotypes of the Ag(a1/d) epitope pair make it unlikely that this amino acid change, alone, could explain all of the associations seen with genotypes of the XbaI polymorphism, despite the strong linkage disequilibrium between the 'a1' and X- alleles. The Ag(x) of the Ag(x/y) epitope pair was associated with raised levels of TGs and cholesterol in the HDL fraction and with lower levels of LDL-cholesterol in the healthy individuals, but these differences were of smaller effect than those associated with the XbaI polymorphism (Dunning et al. 1992). In the patients, Ag(x) was also associated with lower LDL- and higher HDL-cholesterol levels but with lower HDL-TGs. This amino acid substitution explains only some of the differences seen in association with the XbaI polymorphism. Recently evidence was presented in this Swedish sample, that individuals homozygous for Ag(x) have 10-15% lower apoB levels explaining much of the lowering effect of the X- allele, and that carriers of the haplotype a1/X+ have 5-15% higher apoB levels, explaining much of the raising effect of the X+ allele. These two haplotypes in combination may account for most of the difference in apoB levels, and hence LDL-cholesterol levels, frequently observed in association with genotypes of the XbaI polymorphism, in population samples (Dunning 1993).

The data from the apoB haplotype analysis imply that the Ag(a1) X+ and Ag(x) haplotypes encode common variant forms of apoB protein with a mild functional impact on the individual but a significant effect at the population level. The mechanism of this effect could be due to the presence of a novel mutation, unique to the Ag(a1) X+ haplotype, that either, alone or in combination with the Ala591 to Val change, alters the metabolism of the LDL particle or affects the production of apoB protein (Humphries 1992). Alternatively, if this haplotype arose by recombination between other haplotypes, the functional effect might be due to the new combinations of existing amino-acid polymorphisms in the apoB protein (Humphries 1992). Novel combinations of individual amino-acid substitutions in apoB might have a greater effect on the function of the protein and thus on plasma levels of apoB and lipids if the apoB protein has a significant tertiary structure, for which there is growing evidence (Chatterton et al. 1991).

Cladistics, which is a statistical tool used in the search to find 'causative' DNA sequences is used to identify a sub-set of haplotypes that carry functional mutations that are reponsible for phenotypic differences in quantitative risk factors in a population (Sing et al.

1992). The first step involves the construction of a cladogram which is a graphical representation of the evolutionary relationships amongst haplotypes. For apoB haplotypes, the cladogram has already been constructed, using the method of maximum parsimony, linking the observed haplotypes into a network that involves the fewest mutational steps (Dunning et al. 1993). This cladogram is then used as a tool for study of associations between haplotype variation and phenotypic variability, with the aim of finding two haplotypes that have the fewest number of allelic differences yet are associated with a significant phenotypic difference in the trait being examined, such as cholesterol or apoB. The final step is the screening for possible causative variation in the apoB gene, after selecting individuals homozygous for the above two haplotypes. However for haplotype 3, uniquely distinguished by presence of the Ag(x) epitope, the sequence difference does result in an amino acid substitution; and a number of population studies have consistently found associations between the Ag(x) epitope and lower cholesterol and TG levels (e.g. Berg et al. 1986, Dunning et al. 1992, Moreel et al. 1992). It is thus possible that these associations are mediated by the Ag(x/y) epitope pair itself and not other yet-to-be discovered variation. However, the lipid-raising haplotype, which is defined by Ag(a1) in combination with X+, has no unique base substitutions so far detected. Therefore it is likely that some other mutation, that is in linkage disequilibrium with both the Ag(a1) epitope and X+ allele, would be causative, perhaps by altering the tertiary structure of apoB (Dunning et al. 1993).

For the apoAI-CIII-AIV gene cluster, the significant association between higher triglyceride levels and the presence of the inferred haplotype GP+TV+, in the healthy individuals cannot be explained by an association between TGs and any one of the four apoAI-CIII-AIV gene cluster polymorphisms alone. The association with higher triglyceride levels is most likely to be due to this haplotype being a marker for a functionally important sequence change somewhere in the cluster, for example in the promoter region of the apoCIII gene or in an enhancer-like sequence within the cluster. A recent study (Walsh et al. 1993) suggested, using transgenic mouse lines, that the apoAI and apoCIII genes share an intestinal control region, the actual control element in the -1.4 to -0.2Kb region of the apoCIII gene as yet unidentified, and Dammerman et al. (1993) reported that haplotypes involving DNA polymorphisms in the apoCIII gene promoter region and the 3'apoCIII-SstI polymorphism affect susceptibility to severe hypertriglyceridaemia. The rare allele of the SstI polymorphism,

in the 3' non-coding region of the apoCIII gene, which was however not determined in the Swedish sample, has been associated with hypertriglyceridaemia in a number of studies (eg. Rees et al. 1985, Shoulders et al. 1986, Aalto-Setala et al. 1987, Henderson et al. 1987) and is found on the same chromosome as the T_{1100} allele of the apoCIII- C_{1100} to T polymorphism (delta = 0.54, C-F. Xu, unpublished data). The effect of genetic variation in the apoCIII gene on lipid levels in population samples is most likely modulated through variation in apoCIII levels; plasma apoCIII levels correlate with plasma TG and cholesterol levels (Marz et al. 1987, Le et al. 1988, Shoulders et al. 1991) and elevated apoCIII levels have been found in hypertriglyceridaemic individuals (Chivot et al. 1990) and in patients with CAD (Wiseman et al. 1991). There is evidence that apoCIII may regulate TG metabolism by inhibiting the hydrolysis of TG by LPL (Brown & Baginsky 1972, Wang et al. 1985, Ginsberg et al. 1986) and inhibiting the uptake of TG-rich lipoproteins (Quarfordt et al. 1982, Windler & Havel 1985). Recently apoCIII transgenic mice were found to have severe hypertriglyceridaemia, confirming an important role of apoCIII in modulating TG metabolism.

For the LPL gene polymorphisms, in the healthy individuals, a significant association was found between TGs and the HindIII polymorphism, due in part to the inclusion of the four hyperlipidaemic individuals who were all homozygous for the common allele, with weaker associations observed between this polymorphism and VLDL-cholesterol and VLDL-TGs, and between the PvuII polymorphism and HDL-cholesterol. However no consistent trend could be discerned, with those healthy individuals with the genotype H+H- and those patients with the genotype H-H- having the lowest levels in the respective groups. The trend of higher triglyceride levels in carriers of the H+ allele of the HindIII polymorphism in healthy individuals is in agreement with previous findings (Chamberlain et al. 1989, Thorn et al. 1990). In both Caucasian and Japanese populations, analyzed separately (Chamberlain et al. 1989), the H+ allele was significantly more frequent in hypertriglyceridaemic as compared to normotriglyceridaemic individuals. Our findings support the hypothesis previously put forward (Chamberlain et al. 1989, Thorn et al. 1990) that the H+ allele is associated with a variant in or near the LPL gene that predisposes to hypertriglyceridaemia. Screening, using Single Stranded Conformation Polymorphism and sequencing, of the LPL gene for possible causative variation in linkage disequilibrium with the H+ allele of the HindIII polymorphism is described in Chapter 6 of this thesis.

The association between lower HDL-cholesterol levels and the V+ allele of the PvuII polymorphism in the healthy individuals is consistent with the non-significant association reported (Chamberlain et al. 1989), although no significant association between HDL-cholesterol and the PvuII polymorphism was found in another study (Heizmann et al. 1991). Associations between serum HDL-cholesterol levels and genotypes of the HindIII polymorphism have also not been consistent, since in one study (Heizmann et al. 1991) the H- allele was significantly associated with lower HDL-cholesterol levels, while in two others (Chamberlain et al. 1989, Thorn et al. 1990), the H- allele was associated with higher HDL-cholesterol levels. In this study, the H+H- genotype was associated with non-significantly lower and higher means compared with the H+H+ genotype in the patients and healthy individuals, respectively. The most plausible reasons for these inconsistencies are the differences in grouping for statistical treatment, differences in sample and patient selection eg. combining patients with coronary artery disease with healthy individuals into a single group for association studies with lipids and lipoproteins (Heizmann et al. 1991) and errors in measurement of HDL-cholesterol. The effect of LPL on HDL particles is indirect, via transfer to the HDL fraction of surface components from triglyceride-rich lipoproteins that have been acted upon by LPL (Patsch et al. 1987). It is likely that the influence of genetic variation at the LPL locus on HDL levels will be modified by other genetic or environmental factors. Larger population studies will be needed to confirm any association between the LPL PvuII and HindIII polymorphisms and HDL-cholesterol levels, and to ascertain a possible association between the V-H- haplotype and an anti-atherogenic lipid profile. The hypothesis is that the H- allele, particularly when also on a V- chromosome, encodes a LPL enzyme protein that remains as a dimer bound to the endothelium in muscle or adipose tissue, and thus retains activity for a longer period. This would result in greater metabolism of TG-rich lipoproteins such as VLDL, and a drop in VLDL-TG, with more transfer to HDL and a consequent rise in HDL levels. Alternatively the LPL enzyme encoded by the H- allele might have a higher affinity for substrate lipoproteins, and thus remain attached to them for longer. This would have the same outcome as above.

It is plausible that the truncation of the two carboxyl-terminal amino acids caused by the Serine447 to Stop polymorphism might have a direct functional effect on LPL activity, since the primary sequence of LPL is changed. The association between LPL activity and

genotypes of the Serine447 to Stop polymorphism was therefore examined. In the healthy individuals, mean LPL activity was similar in individuals with and without the Stop allele. This observation is consistent with the recent findings of Faustinella et al. (1991a), who concluded, from expression experiments using Cos-1 cells, that the Serine447 to Stop polymorphism had little effect on LPL activity. In contrast, Kobayashi et al. (1992) reported that LPL activity expressed from the LPL-Stop447 construct was lower than that expressed from the wild type LPL-Serine447 construct; experiments with water-soluble and emulsified substrates indicated that the functional defect was due to an impaired lipid-binding ability of the LPL-Stop447 enzyme. The lower LPL activities recorded among the patients carrying the Stop447 substitution may reflect this phenomenon; the assay system used would most likely reveal defects not only in catalytic activity but also in lipid binding ability. This is because this assay system, which uses an emulsified substrate (trioleoyl glycerol emulsion stabilized by dioleoylphosphatidyl choline, Nilsson-Ehle & Ekman 1977) measures both lipid-binding ability and catalytic activity of LPL, and thus variation in lipid-binding ability might mask the effect of genetic variation on LPL activity. Further studies, using water-soluble and emulsified substrates, might be helpful to determine whether the lower enzyme activity in the patients carrying the Stop allele is an artefact of small group size or whether it does indeed represent an impairment of the lipid interface recognition ability in this group.

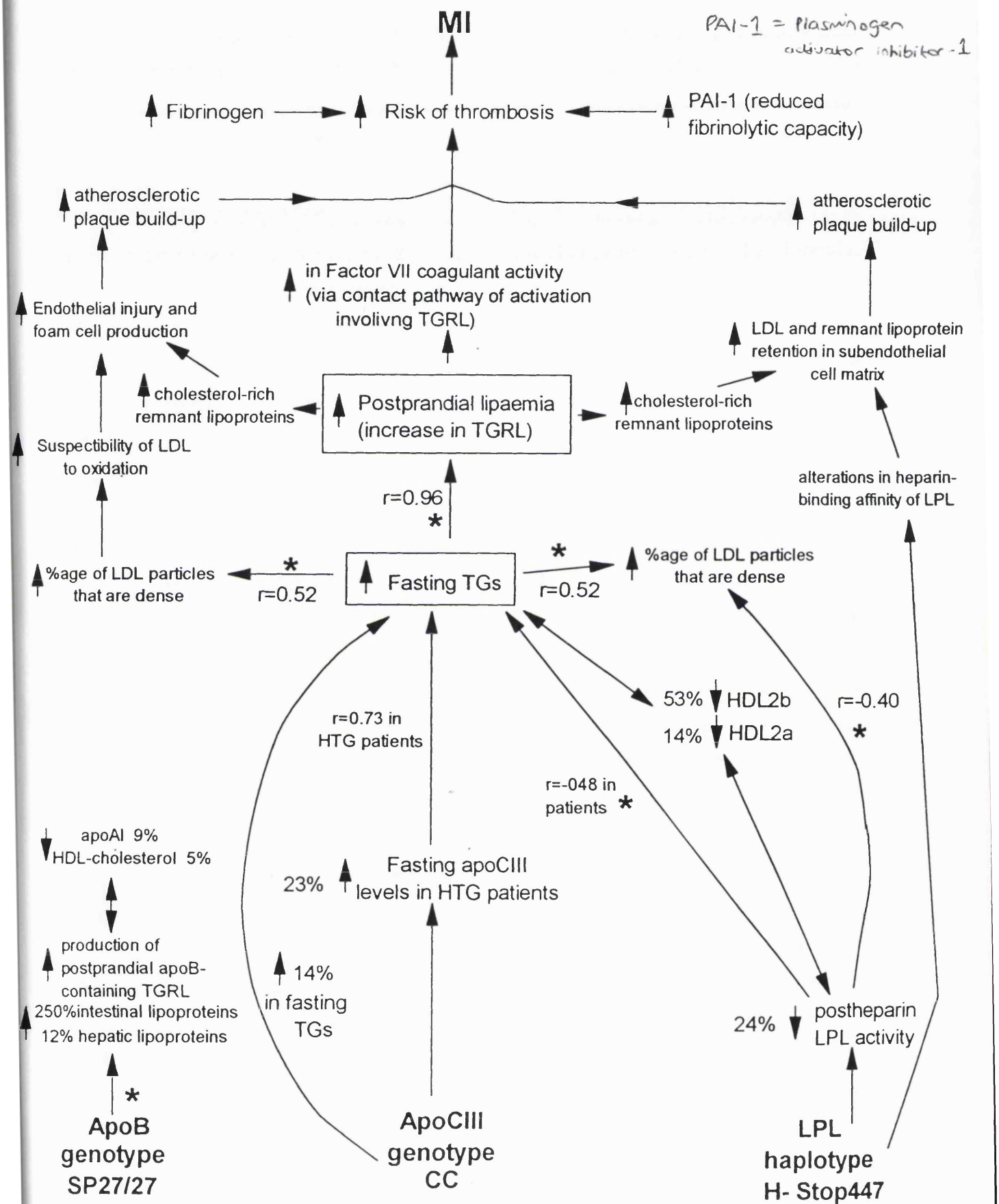
Of the gene loci examined, the gene with the largest effect on HDL subclasses was the LPL gene, with effects reaching statistical significance in the patients but not the healthy individuals. This is probably explained by variation in LPL activity, as the associations between HDL2b or LPL activity and genotypes of the HindIII polymorphism follow the same trend. This would be expected given that the generation of HDL_2 is due to LPL action on triglyceride-rich lipoproteins and subsequent transfer of surface components to HDL_3 (Taskinen & Kussi 1987). The effect of genotypes of the HindIII polymorphism on LPL activity was not significant, possibly due to the design of the assay which measures variation in lipid-binding ability and activity stimultaneously (also discussed in the preceding paragraph). In almost all cases, the associations observed between genotypes and HDL subclasses or LPL activity were in opposite directions in the healthy individuals and patients. This may be due to interaction between unmeasured genetic variation or environmental factors that were different between the healthy individuals and patients.

Section 3.8.2 Possible Mechanisms underlying association of severity of atherosclerosis with genotypes at the apolipoprotein and lipoprotein lipase genes in the Swedish sample.

Genotypes at each of the apoB (Figure 3.7.1), apoCIII (Figure 3.7.2) and LPL (Figure 3.7.3) gene loci had a stronger effect on baseline severity of coronary atherosclerosis in the MI survivors than on levels or activity of the respective gene product, or on levels of fasting lipids or lipoproteins. One exception for the latter is the strong association between haplotypes of the LPL gene and levels of the HDL2b subclass (mean HDL2b level being 53% lower in the presence of the H- Stop447 haplotype than in the absence of this haplotype, $p = 0.008$). This is concordant with the fact that atherosclerosis is multifactorial, a result of numerous genetic and environmental factors interacting additively, therefore small changes in each risk factor can add up to bring about a large increase in severity of atherosclerosis at baseline. Logically, the effect of variation in each gene on modulating severity of atherosclerosis acts via the protein encoded by the gene, and thus on 'intermediate' risk factors such as magnitude of postprandial lipaemia, levels of circulating 'atherogenic' remnant lipoproteins and hypercholesterolaemia. Some of these 'intermediate' risk factors are chronic sources of injury to the arterial endothelium (reviewed in Ross 1993). This, in turn, precipitates the atherosclerotic process and subsequent formation of lesions called atherosclerotic plaques. When the plaque becomes fragile and rupture, this initiates the clotting cascade and causes a MI if the resultant thrombus occludes the artery.

A proposed 'flow-chart' linking variation at the apoB, apoCIII and LPL gene loci (at the bottom of the figure) with manifestation of MI (at the top of the figure), as the final clinical end-point, via their gene products and 'intermediate' traits is illustrated in Figure 3.8.2. As discussed later, in Chapter 5, genotypes of the apoB signal peptide length polymorphism have a strong effect on total levels of Sf 60-400 lipoproteins in the postprandial period, via different production rates of these lipoproteins from both liver and intestine. The 'atherogenic' genotype, homozygosity for the SP-27 allele, is associated with 38.4% more apoB-48 containing particles in the Sf 60-400 range (large chylomicron remnants), and a 2.5 fold higher initial production rate of these lipoproteins. This genotype is also associated with 29.4% more apoB-100 containing particles in the Sf 60-400 range (large VLDL), and a 12% higher initial production rate of these lipoproteins. However, the composition of these

Figure 3.8.2



Proposed Flow Chart linking variation at the apoB, apoCIII and LPL genes (at the bottom) with manifestation of MI (at the top), as the final clinical end-point, via their gene products and 'intermediate' traits

* in postprandial sample (n=42, described in Chapter 5). ⟷ indicates marker

lipoproteins does not seem to be influenced by genotypes of the signal peptide polymorphism; the total level of TG (which accounts for 55 to 68% of the non-protein component by weight in Sf 20-400 lipoproteins; Table 1.2.1), in lipoproteins in the Sf 60-400 range over the postprandial period, is also higher (approximately 15%) for homozygosity for the SP-27 allele, than in the presence of the SP-24 allele.

It is conceivable that the increased number of apoB-containing TG-rich lipoproteins in individuals homozygous for the SP-27 allele, will lead to greater postprandial lipaemia. Overproduction of TG-rich lipoproteins is one cause of excessive postprandial lipaemia (Slyper 1992). Recently Karpe et al (1993c) demonstrated that the magnitude of response of TG-rich lipoproteins was positively correlated with level of apoB in dense LDL, which in turn is a known characteristic of patients with manifest CHD (Sniderman et al. 1982, Swinkels et al. 1989, and discussed in more detail in Section 1.3.7. Small dense protein-rich LDL have an increased susceptibility to oxidation (Tribble et al. 1992 and discussed in Section 1.3.7). Oxidised LDL, in turn, plays a key part in endothelial injury; via direct injury of the endothelium and internalization of these LDL particles by macrophages, which become foam cells. This aids development of the atherogenic lesion through the fatty streak and intermediate stages and eventually the advanced fibrous plaque stage (reviewed in Ross 1993). About 50% of the variability in the distribution of LDL particles between light and dense subfractions was explained by the combined effect of postheparin LPL activity and magnitude of postprandial lipaemia (Karpe et al. 1993c). The percentage of LDL particles that were dense was postively correlated with magnitude of postprandial lipaemia ($r = 0.52$, $p<0.001$) and negatively correlated with postheparin LPL activity ($r = -0.40$, $p<0.05$).

Severity of postprandial lipaemia and severity of atherosclerosis might also be linked via proteins that are not directly involved in lipid metabolism, e.g. proteins involved in coagulability such as the gene for Factor VII. Factor VII is one of the proteins involved in the initiation of the coagulation cascade (reviewed in Rapaport & Rao 1992), and elevated levels were associated with IHD in healthy middle-aged men (Meade et al. 1986). Factor VII and triglyceride levels are directly correlated (Hoffman et al. 1992) and there is good evidence that TGs have an effect on both Factor VII coagulant activity and Factor VII activiation (Mitropoulous et al. 1987, 1989). Previous studies (Simpson et al. 1983, Miller et al. 1985)

have shown an association between hypertriglyceridaemia and increased coagulability, which, in turn, is a known risk factor for post-prandial atherosclerosis (Simpson et al. 1983, Miller et al. 1985). The large, negatively-charged postprandial triglyceride-rich lipoprotein particles (VLDLs and chylomicrons), are thought to activate the intrinsic coagulation pathway via contact surface activation of Factor XII, and therefore ultimately to catalyse Factor VII activation (Mitropoulous et al. 1992). Binding of Factor VII to these lipoproteins also reduces its fractional catabolic rate, resulting in raised coagulant activity (Mitropoulous et al. 1992). This raised coagulant activity would increase the likelihood of atherosclerotic plaques becoming fragile and rupture, initiating the clotting cascade and causing myocardial infarction (MI) if the resultant thrombus occludes the artery (reviewed in Ross 1993). However data on Factor VII was not available in the Swedish MI survivors, so the hypothesis that associations between genotypes and severity of atherosclerosis are, in part, mediated via Factor VII, cannot be directly tested.

An additional route by which elevated numbers of apoB-containing TG-rich lipoproteins could lead to more severe atherosclerosis is via their lipolytic products, remnant lipoproteins. This is embodied in the 'atherogenic remnant' hypothesis first proposed by Zilversmit (1979). Recently the underlying mechanism was proposed by Hennig et al. (1992) in that these lipolytic remnants may be important in the development of atherosclerosis by decreasing the barrier function of the vascular endothelium; permitting the entry of cholesterol-rich lipolytic remnants, as well as LDL into the arterial wall (discussed in more detail in Section 1.8.3).

The significant association between apoB signal peptide genotypes and apoAI levels in the MI survivors (Table 3.7.1), with the 'atherogenic' genotype being associated with the lowest apoAI levels and non-significantly lower HDL-cholesterol levels can be explained by low apoAI and HDL-cholesterol levels being a marker for elevated postprandial lipaemia in these individuals. HDL has been thought to be directly protective via the mechanism of reverse cholesterol transport (Tall 1990); however, as discussed in Section 1.7.4, the evidence for this hypothesis is contradictory. Support for the alternative hypothesis, that low HDL is a marker for increased development of atherosclerosis via postprandial accumulation of chylomicron or VLDL remnants (Patsch et al. 1987, Tall 1990), is growing. Briefly, low

levels of HDL, and hence apoAI, result from both inefficient lipolytic transfer of lipids into HDL and accelerated CE-TG interchange due to the accumulation of TG-rich lipoproteins (Tall 1990).

The 'atherogenic' apoCIII genotype (CC), of the C to T polymorphism in apoCIII, was associated with a 22.9% higher mean fasting apoCIII level (after adjustment for fasting TGs) in the hypertriglyceridaemic (HTG) patients, and with a 14.9% lower mean in the normotriglyceridaemic (NTG) patients in the postprandial sample described in Chapter 5. The MI survivors were divided according to their median TG level (2.2mM). In those with TG levels below the median, the median baseline atherosclerosis score was 1.2 (14% higher) for genotype CC and 1.05 for presence of the T_{1100} allele. However for those with TG levels above the median, the difference was more striking; the median score was 1.8 (157% higher) for genotype CC and 0.7 for presence of the T_{1100} allele ($p=0.1$). This is despite identical median baseline scores (1.15) in the NTG and HTG patients divided according to median TG level. Frequency of individuals with the CC genotype was similar in both the NTG and HTG patient groups. Genotype CC was associated with 14% higher TG levels, compared to presence of the T allele, in the whole patient group (Table 3.7.1), and with 9.8% higher TG in patients with TG levels below 2.2mM and with 2.5% higher TG levels in patients with TG levels above 2.2mM. Within the postprandial sub-sample (described in Chapter 5), fasting apoCIII and TG levels were positively correlated in both the NTG and HTG patients. The correlation coefficient (0.733) in the HTG patients was significantly greater than that (0.348) in the NTG patients ($t = 3.23$ at ∞ degrees of freedom; $p<0.01$). This suggests that the different magnitude of elevating effect of the 'atherogenic' genotype, CC, on baseline atherosclerosis score, in the NTG and HTG patients, is due both to the divergent effect of genotypes on total apoCIII levels, and the different strength of correlation between fasting apoCIII and TG levels, in these two groups of patients. But it does appear that, at least in the HTG patients, total apoCIII levels are a direct modulator of severity of baseline atherosclerosis. This contention is supported by the inducement of HTG by high apoCIII levels in transgenic mice (Aalto-Setala et al. 1992) and the finding of significantly higher mean total apoCIII levels in patients with coronary atherosclerosis, who were not treated with lipid-lowering drugs, and showed progression of disease, than those who were non-progressors (Blankenhorn et al. 1990).

Unfortunately data was not available on the distribution of apoCIII between HDL and non-HDL on the Swedish individuals, so the hypothesis put forward by Blankenhorn et al. (1990) that the distribution of apoCIII between HDL and non-HDL, rather than total apoCIII levels predicts progression of CAD, via perturbations in TG-rich lipoproteins and probability of contact of these lipoproteins with the arterial wall (discussed in Section 1.5.2), cannot be tested here. As mentioned in Section 1.5.2, apoCIII in VLDL is believed to be associated with denser, smaller and more atherogenic VLDL subclasses, with a longer half-life. This would be due to the apoCIII on these particles inhibiting lipolysis and receptor-mediated uptake. Transfer of apoCIII from VLDL to HDL, with a resultant increase in HDL-apoCIII levels, would have a double effect; increased FCR of atherogenic VLDLs and decreased FCR of HDL (Blankenhorn et al. 1990); the latter due to apoCIII-mediated inhibition of the action of hepatic lipase on HDL (Kuusi et al. 1980).

The 'atherogenic' LPL genotype, presence of both the H- and Stop alleles (H- Stop447 haplotype) was associated with 24% lower postheparin LPL activity (N/S), compared to absence of this haplotype in the patients. This same haplotype was associated with 53% lower levels of HDL2b ($p=0.008$, Table 3.7.1) and with 13.6% lower HDL2a levels ($p=0.03$, Table 3.7.1). As discussed in Section 1.7.1, HDL2b and HDL2a are the two subpopulations in the HDL_2 density range; and as mentioned in Section 1.7.3, the well-known inverse relationship between HDL and CAD is due mainly to HDL_2. Thus, variation in the relative abundance and distribution of the HDL subclasses could explain, at least in part, the association between haplotypes of the LPL gene and atherosclerosis. Additionally, studies have shown inverse correlations between magnitude of postprandial lipaemia and HDL levels, particularly HDL_2 (Patsch et al. 1983, 1987). Thus, accumulation of postprandial TG-rich lipoproteins (high postprandial lipaemia) may underlie the association of increased coronary risk with low HDL levels (Karpe et al. 1993). Previously, in this Swedish sample, LPL activity was proposed to be a major determinant of HDL2b levels in HTG individuals (Johansson et al. 1991a, 1991b) and reduced LPL activity was proposed to contribute to the evolution of coronary atherosclerosis in young post-infarction patients (Johansson et al. 1991b).

However, haplotypes of the LPL gene were not associated with either fasting TG levels

or magnitude of postprandial lipaemia in the Swedish patients (data not shown). Thus, alternative mechanisms are likely to be more important in mediating the associations between LPL haplotypes and LPL activities or HDL subclasses. These could be mediated via alterations in heparin-binding affinity of LPL. The Ser447 to Stop polymorphism is just 3' to one of three proposed heparin-binding sites, which spans residues 441 to 446 (Dichek et al. 1993), so it is possible that removal of the two carboxy-terminal amino acids (Serine447 and Glycine448), neither of which are basic, might increase affinity of LPL for heparan proteoglycans. However the effect of truncating the LPL molecule by 2 amino acids on binding to heparan proteoglycans has not been studied. If the LPL446 molecule does, indeed, have an increased heparin-binding affinity compared to normal LPL, one consequence of this greater avidity for heparin would be the increased retention of LDL in the subendothelial matrix, via LPL acting as a 'biochemical bridge' between LDL on the one hand and heparan sulphate glycosaminoglycans on the other hand (Saxena et al. 1992). This would increase LDL retention within the arterial wall, which, in turn, would have a stimulating effect on LPL-mediated foam cell formation from macrophages (this is discussed in more detail in Section 1.6.8). This would have a knock-on effect on the build-up of atherosclerotic plaques and increase the risk of thrombosis and thus the risk of a MI.

The most likely explanation for the additive effect of genotypes at the apoB, LPL and apoCIII gene loci on atherosclerosis score, when all these three gene loci are considered stimultaneously (Figure 3.7.4 and Table 3.7.2), is that the different mechanisms underlying the association of each 'atherogenic' genotype with high median atherosclerosis score, are acting in concert, all leading to a greater risk of thrombosis (Figure 3.8.2). This would underlie the increasing median score with increasing number of 'atherogenic' genotypes. That several mechanisms are likely to be involved is supported by the fact that no single measured lipoprotein variable showed as large a gradient with the combination of 'atherogenic' genotypes as atherosclerosis score (Table 3.7.2). The difference in atherosclerosis score with combined genotypes is large enough to be biologically significant, and if confirmed in a larger patient sample, will be of relevance in screening and identification of at-risk individuals.

Chapter 4 - St. Thomas' Atherosclerosis Regression Study (STARS).

Section 4.1 Summary

Association studies were carried out on a sample of 74 middle-aged male patients with coronary artery disease who completed the St Thomas' Atherosclerosis Regression Study (STARS). With the exception of apoE, allele frequencies for all polymorphisms studied were similar to those reported in healthy individuals. The apoE4 allele frequency (0.21) in the STARS sample was higher (chi-square=4.5;$p<0.05$, 1df) than reported for a sample of healthy individuals from the UK (E4=0.14), this over-representation of the apoE4 allele is likely to be due to CAD-positive status. Genotypes of the apoB Ag(a1/d) epitope pair and XbaI polymorphism were significantly associated ($p<0.05$) with severity of coronary atherosclerosis at baseline, homozygosity for Ag(a1) or the X- allele being associated with greater severity. Genotypes of the apoAI G_{-75} to A substitution were associated with change of coronary atherosclerosis; genotype GG was associated with progression and GA with regression; $p= 0.0002$ by unpaired t-test. No significant associations were found between genotypes and baseline or in-trial lipids and lipoproteins. Thus, the data suggest that variation in the apoB and apoAI-CIII-AIV gene cluster may predict established and progression of coronary atherosclerosis respectively, through effects independent of plasma lipid or lipoprotein levels. In addition, the trend between change in coronary atherosclerosis and treatment regimen was the same for all genotypes of the G_{-75} to A substitution. This suggests that variation at the apoAI-CIII-AIV gene cluster may be influencing prognosis independently of treatment regimen.

Section 4.2 Introduction to the St. Thomas' Atherosclerosis Regression Study

The St. Thomas' Atherosclerosis Regression Study, STARS, (Watts et al. 1992) was designed to assess the effect of a practicable lipid-lowering diet on the progression of atherosclerosis in the coronary arteries of patients with coronary heart disease (CHD), over a treatment period of about three years. 90 men with CHD (age < 66 years, plasma cholesterol level above 6mM but not above 10mM and who had not been previously treated with lipid lowering drugs) were recruited. They underwent baseline coronary angiography and were tested for tolerance to cholestyramine, a lipid lowering drug. Patients were included in the study with their consent and if they responded to and were tolerant of cholestyramine. They were randomised into three groups receiving, respectively, usual care

only (control group), dietary intervention with a low fat diet (fat accounting for 27% of dietary energy) and dietary intervention plus cholestyramine. Patients were seen at 3 month intervals for clinical assessment and repeat measurements of lipids. At the end of the three year study period, 74 of the patients underwent repeat angiography (mean $\pm$ SD for time elapsed between randomisation into the three study groups and second angiography = 39 $\pm$ 3.5 months). Percentages of smokers, regular alcohol takers and patients with history of hypertension or family history of early CHD did not differ amongst the three treatment groups (Watts et al. 1992).

The proportion of patients showing overall progression of coronary narrowing was significantly reduced by both interventions (diet only = 15% and diet plus cholestyramine = 12% relative to the control group = 46%). Proportions of patients showing luminal widening rose significantly in the intervention groups (control group = 4%, diet = 38% and diet plus cholestyramine = 33%). The mean absolute width of the coronary segments (MAWS) studied decreased by 0.201 mm in the controls, increased by 0.003mm in the diet group and by 0.103mm in the diet + cholestyramine group. An decrease in MAWS indicated progression and increase in MAWS indicated regression of atherosclerosis. Change in MAWS was strongly correlated with in-trial LDL-cholesterol levels ($r=-0.40$, $p<0.001$) and with in-trial LDL/HDL cholesterol ratio ($r=-0.42$, $p<0.001$). These results showed that lipid-lowering dietary intervention, supplemented if necessary by drug treatment, is useful in men with symptomatic CHD and serum cholesterol > 6mM to reduce progression of atherosclerosis.

More recently, associations between plasma lipoprotein subfractions and changes in CHD, measured as changes in MAWS were examined in the same sample of men (Watts et al. 1993). For the whole sample, regardless of treatment regimen, changes in MAWS were significantly correlated with in-trial concentrations of cholesterol in intermediate density lipoproteins (IDL) [$r = -0.25$, $p=0.03$] in the LDL_3 fraction [$r= -0.29$, $p=0.01$] and in the HDL_3 fraction [$r=0.27$, $p=0.02$]. In muliple linear regression analysis, LDL_3 was a significant negative predictor ($p<0.05$) of changes in MAWS. Age, smoking, weight and blood pressure had no effect on correlations between changes in MAWS and LDL_3 cholesterol level. Most of the patients showing regression of atherosclerosis during the study

period had LDL_3 cholesterol levels less than 1.8mmol/l. These findings suggest that LDL_3 is the plasma lipoprotein subfraction exerting the most powerful influence on the course of CAD in middle aged men without hypercholesterolaemia, and that in these individuals regression of atherosclerosis may be achieved by maintaining the LDL_3 cholesterol concentration below 1.8mmol/l.

Section 4.3 Rationale for Study

A number of studies have reported different allele frequencies for apoB and apoAI-CIII-AIV gene polymorphisms between patients with arterial disease or myocardial infarction and controls. Other studies have also shown associations between polymorphisms and severity of atherosclerosis, such as the Pathological Determinants of Atherosclerosis in Youth (PDAY) reports (Hixson et al. 1991, 1992), and in the Swedish myocardial infarction survivors (Chapter 3 of this thesis). The aim of this study was to confirm the results of associations between selected genotypes at the apoE, apoB, apoAI-CIII-AIV and LPL genes and severity of prevalent coronary atherosclerosis and prognosis, observed in the Swedish myocardial infarction survivors (Chapter 3 of this thesis). Our collaborators at St. Thomas' Hospital, London, had taken measurements on prevalance and progression of atherosclerosis, as well as baseline and in-trial lipids, on patients who participated in the St. Thomas' Atherosclerosis Regression Study (Watts et al. 1992, 1993). A second aim was to attempt to eludicate the mechanisms underlying the influence of genotypes on the pathology underlying the prevalance and progression of atherosclerosis, by examining the impact of polymorphisms at candidate genes on baseline and in-trial levels of lipids and lipoproteins.

Section 4.4 Gene polymorphisms in STARS, freqencies of alleles and haplotypes

Seventy-four patients from STARS were typed for common DNA polymorphisms at the apoE, apoB, apoAI-CIII-AIV and LPL genes. Allele frequencies with 95% confidence intervals of these polymorphisms are shown in Table 4.4.1. Complete genotype data for the 74 STARS patients is presented in the appendix. In this group of patients, allele frequencies for the polymorphisms studied in the apoB, apoAI-CIII-AIV and LPL genes were not significantly different from those found by our laboratory in healthy UK (where data was available) or in European population samples (Houlston et al. 1991, Dunning et al. 1993, Chapter 3 of this thesis) where the rare allele frequencies for these samples were within the

Table 4.4.1

Observed Allele frequencies in the St Thomas' Atherosclerosis Regression Study.

Polymorphism	Rare Allele	Rare Allele Frequency	95% confidence intervals	Frequency in healthy European population samples	Reference
ApoE isoforms	E2	0.02 *	0.00 to 0.04	0.09	Houlston et al 1991
	E4	0.21 *	0.15 to 0.28	0.14	Houlston et al 1991
ApoB-C/T_{-265}	T_{-265}	0.12	0.07 to 0.18	0.11	Chapter 3 of this thesis
ApoB; signal peptide polymorphism	SP-24	0.29	0.22 to 0.36	0.30	Chapter 3 of this thesis
ApoB; Ag(a1/d epitope pair	a1 (Val591)	0.49	0.42 to 0.57	0.45	Dunning et al 1993
ApoB; XbaI polymorphism	X+	0.49	0.42 to 0.57	0.56	Chapter 3 of this thesis
ApoAI-G/A_{-75}	A_{-75}	0.16	0.14 to 0.19	0.14	Chapter 3 of this thesis
ApoCIII-C/T_{1100}	T_{1100}	0.28	0.25 to 0.31	0.24	Chapter 3 of this thesis
LPL-Serine/Stop447	Stop447	0.11	0.06 to 0.16	0.06	Chapter 3 of this thesis

* significantly different from the general European population ($p<0.05$)

95% confidence intervals for those in the St Thomas' Atherosclerosis Regression Study. Allele frequencies for polymorphisms in the apoB, apoAI-CIII-AIV and LPL gene loci in the STARS patients were similar to those previously reported in population samples of European origin (Paulweber et al. 1990, Thorn et al. 1990, Heizmann et al. 1991, Myant et al. 1991, Tybjærg-Hansen et al. 1991, Xu et al. 1994) and reviewed in Humphries et al. 1988, 1990, 1992, Lusis 1988). Small differences in allele frequencies between the STARS patients and those in groups of patients in previous studies (eg. Monsalve et al. 1988, Hata et al. 1990, Visvikis et al. 1993) are likely to be due to chance effects or differences in the criteria used in patient selection. For all the diallelic polymorphisms studied, genotype distributions were close to, and not significantly different from the Hardy-Weinberg prediction. Genotype distributions and allele frequencies for all the polymorphisms were similar between the three treatment groups (data not shown).

However for the common apoE alleles, compared to a group of 150 healthy British individuals (Houlston et al. 1991) the frequency of the apoE2 and apoE4 alleles in these patients were, respectively, lower [0.02 versus 0.09] and higher [0.21 versus 0.14], and these differences were statistically significantly different [chi-square = 11.43, 2 degrees of freedom, $p<0.01$]. The frequency of the common apoE alleles in this study were also significantly different from those in studies on healthy population samples of Caucasian origin (reviewed in Davignon et al. 1988, Gerdes et al. 1992). The apoE4 allele frequency has been found to be elevated in patients with CAD (Cumming et al. 1984, Kuusi et al. 1989, Laakso et al. 1991, Bockxmeer et al. 1992, Nieminen et al. 1992, and discussed in Section 1.4.9) and in individuals selected for hypercholesterolaemia (Davignon et al. 1988). The STARS patient sample was enriched with individuals with genotypes E4/3 and E4/4 compared to the healthy British sample in the study by Houlston et al. (1991) [28/74 versus 37/150, chi-square = 4.18, 1 degree of freedom, $p<0.05$]. The apoE4 allele has a well-known cholesterol elevating effect (Davignon et al. 1988) and in studies on population samples of different ethnic origins, individuals with genotypes E4/3 or E4/4 have consistently been found to have elevated mean cholesterol levels compared to those with genotype E3/3 and E2/3 (Davignon et al. 1988). Patients for STARS were not selected for hypercholesterolaemia; the inclusion criteria specified a pretreatment mean serum cholesterol of between 6 and 10mM, the over-representation of the apoE4 allele in the STARS patient

sample is likely to be due to CAD-positive status.

The previously published phylogenetic tree based on seven apoB polymorphic sites (Dunning et al. 1993) was utilised so that all individuals had their haplotypes determined at the apoB gene, including those who were heterozygous at more than one locus. The genotypes of all the individuals were assigned to haplotypes, commencing with those that could be determined unambiguously in the individuals who were homozygous at either all the polymorphic sites or all except one. The haplotypes assigned in this manner fell into six groups corresponding to haplotypes 1, (2,3,4), 5, 6, 7 and 8. The haplotypes of the remaining individuals were assigned by fitting their genotypes firstly to haplotypes 1, (2,3,4), 5 or 6 and secondly to haplotypes 7 or 8. This left three individuals whose haplotypes could not be assigned to groups that had already been demonstrated to occur unequivocally. For each of these 3 individuals, one of the chromosomes was assigned to a known haplotype leaving another previously unobserved haplotype (Del Ala591 X-). For homozygosity of the C_{-265} allele, haplotypes were unequivocal if homozygosity occured at all or all except one of the polymorphic loci. None of the STARS patients were homozygous for the T_{-265} allele of the C_{-265} to T polymorphism in the apoB promoter. For individuals carrying one T_{-265} allele and 2 identical haplotypes, the T_{-265} allele was unequivocally present on one of these haplotypes. On the basis of frequencies of unequivocal T_{-265} haplotypes, those individuals carrying the T_{-265} allele and two different haplotypes had their haplotypes assigned.

Distributions of apoB haplotypes in the STARS sample were similar to those in the Swedish sample (healthy individuals, patients and total sample) reported previously (Dunning et al. 1993); when unequivocal and assigned haplotypes were combined and the six groups as in Table 4.4.2 compared between the STARS sample and the Swedish sample, results from chi-square analysis gave a value of 8.6 to 8.7 (5 d.f), $p > 0.1$ (not significant). However in the STARS sample, compared to the Swedish sample, there was a lower than expected frequency of haplotypes defined by presence of Val591 in combination with the X+ allele (STARS vs. Swedish healthy individuals: 9/148 vs. 28/180, chi-square = 7.29, $p<0.01$ at 1 df and STARS vs. Swedish patients: 9/148 vs. 30/286, chi-square = 2.32, not significant at 1d.f.) and a small enrichment of 'unpredicted' haplotypes, all defined by presence of the deletion allele together with Ala591 and X- alleles (STARS vs. Swedish

Table 4.4.2 Haplotypes in the apoB gene in STARS (total number of haplotypes = 148)

Haplotype (classification no. in Dunning et al. 1993)	C_{-265} unequivocal	C_{-265} assigned	C_{-265} Total	T_{-265} unequivocal	T_{-265} assigned	T_{-265} Total
SP-27 d X- (1,18)	4	1	5	0	3	3
SP-27 a1 X- (2,3,4)	23	27	50	1	13	14
SP-27 d X+ (5, 17)	9	13	22	0	0	0
SP-24 d X+ (6, 9, 13, 15)	17	25	42	0	0	0
SP-27/24 a1 X+ (7, 8, 10, 14)	4	5	9	0	0	0
SP-24 d X- (11, 12, 16)	0	3	3	0	0	0

The underlined haplotypes are not predicted to occur in the most simple model phytogenetic tree which assumes that new haplotypes are generated by base changes and that no recombination events between polymorphic sites have occured (Dunning et al. 1993).

Table 4.4.3 Haplotypes in the apoAI-CIII-AIV gene cluster in STARS (total number of unequivocal haplotypes = 114)

Haplotype	Number of unequivocal chromosomes	Number expected based on allele frequencies (if no linkage disequilibrium)	Frequency (as percentage)	Frequency (as percentage) in Swedish sample
G_{-75} C_{1100}	87	69	76.3	77.3
G_{-75} T_{1100}	18	27	15.8	15.5
A_{-75} T_{1100}	6	5	5.3	5.5
A_{-75} C_{1100}	3	13	2.6	1.8

NB. 16 individuals were heterozygous at the G_{-75} to A and C_{1100} to T polymorphisms, so phase could not be determined in these cases. These 16 individuals are excluded from the above table, due to the unequivocal existence of the rare haplotype A-75 C_{1100}. Another individual did not have his genotype determined at the apoAI G_{-75} to A polymorphism.

Table 4.4.4 Haplotypes in the Lipoprotein lipase (LPL) gene in STARS (total number of unequivocal haplotypes = 126)

Haplotype of LPL gene	Number of unequivocal chromosomes	Number of unequivocal chromosomes in the Swedish sample	Frequency (as %age) in STARS	Frequency (as %age) in the Swedish combined sample
V+ H+ Serine447	59	158	46.8	54.5
V- H+ Serine447	33	83	26.2	28.6
V+ H- Serine447	10	19	7.9	6.6
V- H- Serine447	8	11	6.3	3.8
V- H- Stop447	16	19	12.7	6.6

NB. 11 STARS individuals were heterozygous at both the PvuII and HindIII polymorphisms so are excluded from the calculations of haplotype frequencies.

Table 4.4.5 Estimates of Linkage Disequilibrium for each pairwise combination of polymorphisms studied in STARS.

Pairwise combination	Delta value (Δ)	chi-square of Δ	significance of chi-square of Δ; 1d.f. *
ApoB gene			
C/T_{-265} and SP27/24	-0.15	1.72	$0.5>p>0.1$
C/T_{-265} and Ag(a1/d)	-0.02	0.02	$p>0.9$
C/T_{-265} and XbaI	-0.36	9.5	$0.005>p>0.001$
SP-27/24 and Ag(a1/d)	0.65	31.5	$p<0.001$
SP27/24 and XbaI	0.55	22.7	$p<0.001$
Ag(a1/d) and XbaI	0.70	36.6	$p<0.001$
ApoAI-CIII-AIV gene cluster			
G_{-75} to A and C_{1100} to T	0.59	25.1	$p<0.001$
Lipoprotein lipase gene			
PvuII and HindIII	0.34	8.5	$0.005>p>0.001$
PvuII and Serine447 to Stop	0.38	10.5	$0.005>p>0.001$
HindIII and Serine447 to Stop	0.53	20.5	$p<0.001$

* $p<0.05$ indicates significant linkage disequilibrium, where observed haplotype frequencies deviate significantly from expected haplotype frequencies obtained if independent assortment of alleles had occured and haplotype frequencies only depended on allele frequencies.

healthy individuals: 3/148 vs. 1/180, chi-square = 1.47, not significant at 1 df and STARS vs. Swedish patients: 3/148 vs. 4/286, chi-square = 0.24, not significant at 1 df).

Haplotypes of the apoAI-CIII-AIV gene cluster, as defined by the apoAI G_{-75} to A and apoCIII C_{1100} to T polymorphisms showed a distribution in STARS (presented in Table 4.4.3) similar to that in the combined Swedish sample (Table 3.3.3 in Chapter 3 of this thesis, also in right-hand column of Table 4.4.3); chi-square = 0.24, not significant at 3 degrees of freedom). There were three individuals in the STARS sample, who had the unpredicted haplotype A_{-75} C_{1100} which occurs at a low frequency in an independent population sample (Xu et al. 1994).

LPL haplotypes, defined by the PvuII, HindIII and Serine447 to Stop polymorphisms were similarly distributed in STARS (presented in Table 4.4.4) compared to the Swedish sample (Table 3.3.3 in Chapter 3 of this thesis), chi-square = 7.6 (d.f. = 4), not significant, but with an enrichment of haplotypes defined by the rare alleles of the HindIII and Serine447-Stop polymorphisms. Estimates of linkage disequilibrium for pairwise combinations of polymorphisms at each of the three gene loci; apoB, apoAI-CIII-AIV and LPL in STARS are presented in Table 4.4.5. Significant linkage disequilibrium occured at all pairwise combinations with the exception of the apoB C_{-265} to T and either the signal peptide or Ag(a1/d) polymorphisms in the apoB gene. The lack of significant linkage disequilibrium with these pairwise combinations in STARS is in contrast to the results in the combined Swedish sample (see Table 3.4.2). This is due to those individuals who were heterozygous for T_{-265} (there were no individuals homozygous for T_{-265} in STARS) being approximately equally distributed between the genotypes SP27/27 and SP27/24 or between the genotypes a1/a1 and d/d of the Ag(a1/d) epitope pair (data not shown).

Section 4.5 Associations between gene polymorphisms and baseline and progression of atherosclerosis, and comparsions with the Swedish patient sample.

Per-patient and segmental analyses were carried out to look for associations between genotypes and haplotypes at the four gene loci and both severity of coronary atherosclerosis at baseline and the change in severity over three years. Segmental analysis was used, in addition to conventional per-patient analysis, to test for associations between variation at

candidate gene loci and extent and change in extent of coronary atherosclerosis. Segmental analysis increases the statistical power of the study compared to per-patient analysis; although pooling different observations from the same individual into one analysis is not strictly valid because of the potential co-correlation of variables within-individuals. Our decision to carry out segmental analysis (Armitage 1986) was supported by the low intraclass correlation coefficient (ICC) for ΔMAWS (0.12) and ΔMinAWS (0.18). These low ICCs showed that the extent of within-segment differences was greater than the extent of between-segment differences and suggested that ΔMAWS and ΔMinAWS in individual patients might have been behaving as different variables.

Where ICC = $SD_B^2 / (SD_B^2 + SD_W^2)$

SD_B^2 = variance between segments within one individual

SD_W^2 = variance within segment.

A low ICC indicates that the sum of variability of change in diameter within segments is large compared to the sum of variability of change in diameter between segments within one patient. There was no weighing in favour of one genotype in either type of analysis due to similar genotype distributions [shown in Table 4.5.1] when patients and segments were considered (results from contingency tables in Table 4.5.1).

For each of the 74 patients who completed the study period, a maximum of ten coronary artery segment pairs were available for analysis (mean $\pm$ S.D. = 6.4 $\pm$ 2.0). Table 4.5.2 shows the mean values for MAWS and MinAWS at the first angiography for genotypes of the Ag(a1/d) epitope pair and the XbaI polymorphism in the apoB gene. Data for both per-patient and segmental analyses is presented. For both analyses, those individuals with genotypes a1/d and d/d or X+X- and X+X+ had similar means while genotypes a1/a1 or X-X- were associated with significantly lower means ($p<0.05$ for MAWS and $p<0.02$ for MinAWS in segmental analysis). Genotypes a1/a1 or X-X- were associated with a mean lumen 10% smaller compared to presence of the Ag(d) epitope or X+ allele, and therefore with worse baseline coronary atherosclerosis. The associations of baseline MAWS and MinAWS with the XbaI polymorphism became non-significant when adjusting for genotypes of the Ag(a1/d) epitope pair, since genotypes at both polymorphisms were significantly

Table 4.5.1 **Genotype distributions in per-patient and segmental analyses**

	a1/a1	a1/d	d/d	% age with a1/a1 genotype	2x3 contingency test; results
No. of patients	15	43	15	20.5	$\chi^2 = 0.15$ 2 d.f.
No. of segments	106	279	97	22.0	p >0.5
	X-X-	**X+X-**	**X+X+**	**% age with X-X- genotype**	
No. of patients	16	42	15	21.9	$\chi^2 = 0.72$ 2 d.f.
No. of segments	116	277	89	24.1	p >0.1
	ApoAI genotypes				
	GG	**GA**	**%age with 'A' allele**		
No. of patients	48	25	34.2		$\chi^2 = 0.21$ 1 d.f.
No. of segments	310	172	35.7		p >0.5

Table 4.5.2
PER-PATIENT AND SEGMENTAL ANALYSIS.
Data is shown as means ± standard errors

a) Atherosclerosis score at first angiography (baseline) versus genotypes of the a1/d epitope pair and the XbaI polymorphism in the apoB gene.

Trait (μm)	Genotypes (number of patients analyzed)		Genotypes (number of segments analyzed)		
	a1/a1 (15)	**a1/d and d/d (58)**	**a1/a1 (106)**	**a1/d and d/d (376)**	
MAWS	251.5 ± 12.7	276.7 ± 7.0	247.7 ± 9.5	275.0 ± 4.8	*
MinAWS	205.4 ± 13.2	228.5 ± 6.5	202.3 ± 9.4	226.6 ± 4.7	*
	X-X- (16)	**X+X- and X+X+ (57)**	**X-X- (116)**	**X+X- and X+X+ (366)**	
MAWS	256.1 ±18.2	275.8 ± 6.2	249.3 ± 9.5	275.2 ± 4.8	*
MinAWS	213.1 ± 17.8	226.8 ± 5.7	206.6 ± 9.2	225.9 ± 4.7	

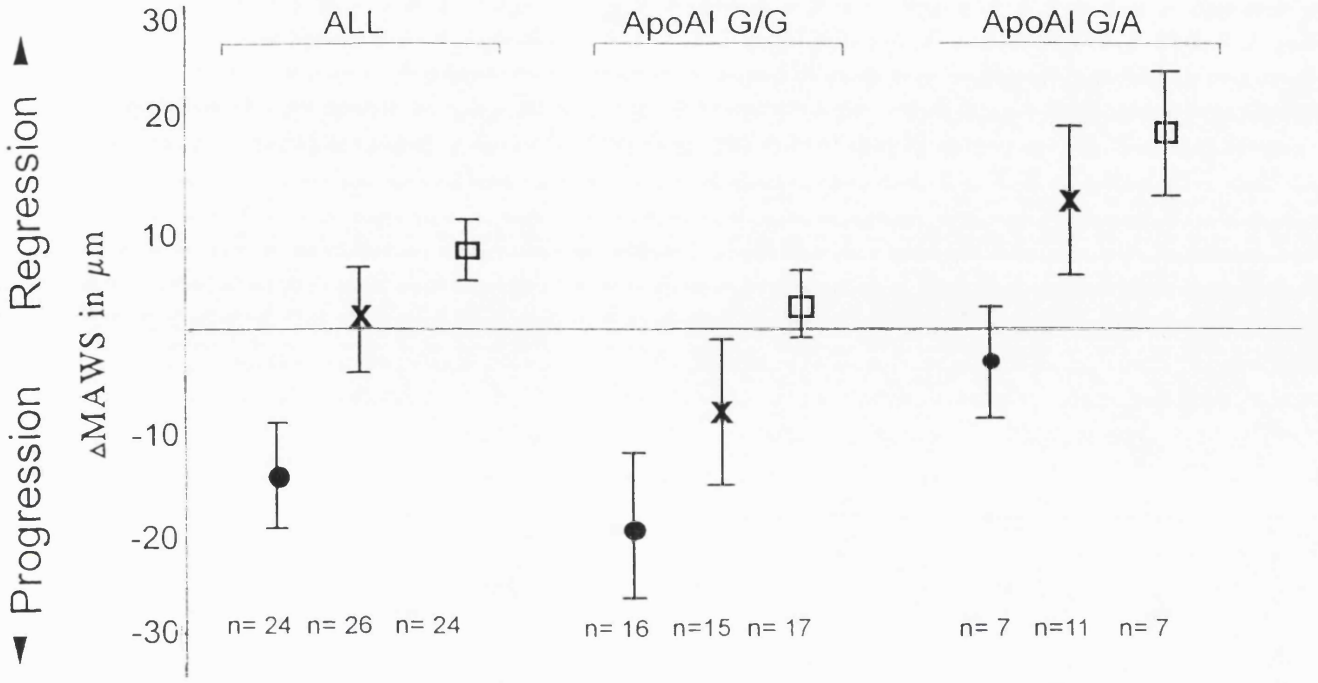
b) Change in atherosclerosis score between angiographies versus genotypes of the apoAI G_{-75} to A polymorphism.

Change (Δ) in Trait (μm) †	Genotypes (number of patients analyzed)			Genotypes (number of segments analyzed)		
	GG (48)	**GA (25)**		**GG (310)**	**GA (172)**	
ΔMAWS	-10.0 ± 5.8	10.0 ± 5.3	*	-7.8 ± 3.2	10.2 ± 3.6	***
ΔMinAWS	-8.2 ± 6.0	7.7 ± 5.8		-6.2 ± 3.2	8.0 ± 3.7	**

correlated ($p<0.001$ for extent of linkage disequilibrium). No significant associations of MAWS and MinAWS with genotypes at the other gene loci (apoE, apoAI-CIII-AIV or LPL) were found.

Associations were examined between genotypes at the four gene loci and the change in atherosclerosis severity during the three year period of the study. Only for the G_{-75} to A substitution in the apoAI gene promoter were significant associations observed, and Table 4.5.2 shows the results for ΔMAWS and ΔMinAWS by these genotypes in the whole patient group using per-patient and segmental analyses. For both per-patient and segmental analyses, the presence of the A_{-75} allele was associated with overall mean regression of atherosclerosis (positive value for ΔMAWS and ΔMinAWS) and genotype GG with overall mean progression (negative value for ΔMAWS and ΔMinAWS). For ΔMAWS this reached statistical significance whether the data was analyzed on a per-patient or segmental basis, and for ΔMinAWS this reached statistical significance in segmental analysis. This association was then examined separately in the three different treatment groups and the data is shown in Figure 4.5. As reported previously (Watts et al. 1992), regardless of genotype, the diet plus cholestyramine group showed, on average, regression of disease over the study period and the usual care group showed, on average, progression of disease; with the diet group being intermediate. For each genotype the same relationship was observed between ΔMAWS or ΔMinAWS and treatment regimen as in the whole sample ie. there was no interaction between treatment regimen and genotype. After adjusting ΔMAWS or ΔMinAWS for the effect of treatment group or in-trial plasma LDL-cholesterol, the associations between change in severity of atherosclerosis and the G_{-75} to A substitution were still significant (ΔMAWS $= 26.6 + 17.4(G_{-75}/A) - 8.5$ (LDL-cholesterol), $R^2x100 = 4.7\%$, $p<0.001$; ΔMinAWS $= 40.7 + 13.4(G_{-75}/A) - 11.6$ (LDL-cholesterol), $R^2x100 = 5.6\%$, $p<0.001$). Despite the strong linkage disequilibrium between the G_{-75} to A substitution and the C_{1100} to T polymorphism in the apoCIII gene [the A_{-75} allele being nearly always found on the same chromosome as the T_{1100} allele (Xu et al. 1994, Chapter 3 of this thesis)], no significant association was observed between coronary atherosclerosis and the C_{1100} to T polymorphism, though, as expected, those who were homozygous for the T_{1100} allele showed, on average, mean regression in segmental analysis (see Table 4.5.3).

Figure 4.5



Change in severity of coronary atherosclerosis over the three year study period in each of the three treatment groups versus genotypes of the G_{-75} to A polymorphism in the apoAI gene promoter. Mean changes in MAWS and MinAWS are shown above and below, respectively. Means are represented by symbols and magnitude of standard errors of these means are represented by vertical lines through the symbols. Number of patients analysed is shown below representations of means and their standard errors obtained by segmental analysis. The p-values for ΔMAWs and ΔMinAWS versus genotypes of the G_{-75} to A polymorphism are 0.0002 and 0.004, respectively. The discrepancy between the number of patients in the entire usual care group and in each of the two genotype groups within the usual care group is due to non-availability of genotype data for one individual.

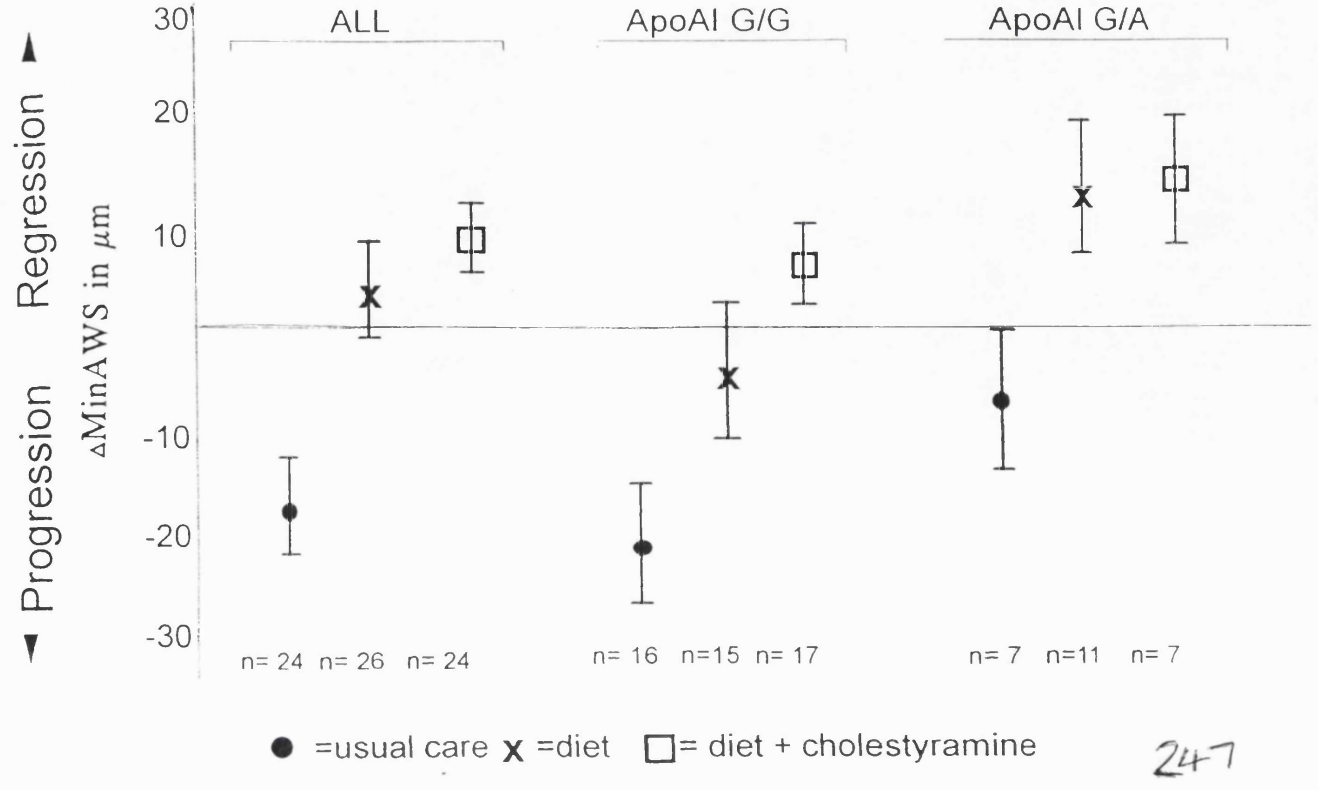


Table 4.5.3 Results with the apoCIII C_{1100} to T polymorphism

(data shown as means ± S.E.s)

	Genotypes of the apoCIII C_{1100} to T polymorphism (number of segments)†		
	CC (259)	CT (180)	TT (50)
Δ MAWS	-2.3 ± 3.5	-2.8 ± 3.8	9.1 ± 6.9
Δ MinAWS	-0.6 ± 3.4	-3.6 ± 4.0	5.3 ± 7.1

† per-patient data was not available for progression of disease versus genotypes of the C_{1100} to T polymorphism.

Unlike the Swedish patients (Chapter 3 of this thesis), the associations observed between severity of prevalent atherosclerosis and genotypes of the apoB signal peptide (SP) polymorphism or haplotypes of the LPL gene were not significant. However the same trend was observed in both groups of patients, with presence of the SP-27 allele or both H- and Stop447 alleles being associated with worst prevalent disease (Table 4.5.4). Presence of H- but not Stop447 was associated with least prevalent disease in both patient groups (Chapter 3 of this thesis and Table 4.5.5). Similar results were obtained for genotypes of the Serine447 to Stop polymorphism. Like STARS, presence of the Ag(a1) epitope or X- allele was associated with worse prevalent atherosclerosis in the Swedish patient sample. Similarly, with the apoCIII C to T polymorphism, homozygosity for the C allele was associated with worse prevalent disease in both Swedish and STARS patient samples.

Table 4.5.6 compares trends observed in the two patient samples for change in severity of atherosclerosis. While genotype GA of the apoAI G_{-75} to A polymorphism was associated with regression in STARS, this same genotype was linked with worse progression in the Swedish patient sample. Within STARS, despite the strong linkage disequilibrium between the apoAI-A_{-75} and apoCIII C_{1100} alleles (see section 4.4) the A_{-75} allele was associated with greater regression of atherosclerosis than was the T_{1100} allele. This difference is most likely to be due to the three STARS patients who had the unpredicted haplotype A_{-75} C_{1100} and those individuals with the T_{1100} allele but not the A_{-75} allele.

Table 4.5.4
Trends observed in Swedish and STARS patient samples for prevalent atherosclerosis

Genotypes	Swedish patient sample (medians of score for global severity of atherosclerosis at baseline. **High median = worse disease**			STARS patient sample (Mean Absolute width of segment in per-patient analysis) **Low MAWS = worse disease**		
	SP27/27 (32)	**SP27/24** (26)	**SP24/24** (12)	**SP27/27** (34)	**SP27/24** (33)	**SP24/24** (6)
apoB signal peptide	2.00	0.80	0.90	270	265	282
	a1/a1 (13)	**a1/d** (35)	**d/d** (22)	**a1/a1** (15)	**a1/d** (43)	**d/d** (15)
apoB Ag(a1/d)	1.30	1.10	0.85	266	262	292
	X-X- (19)	**X+X-** (31)	**X+X+** (20)	**X-X-** (16)	**X+X-** (42)	**X+X+** (15)
apoB XbaI polymorphism	1.90	1.20	0.80	269	265	281
	CC (32)	**CT** (12)	**TT** (5)	**CC** (39) †	**CT** (29) †	**TT** (6) †
apoCIII C_{1100}/T	1.60	0.70	0.90	265	276	264
	Absence of H- and Stop (40)	**Presence of H- but not Stop** (22)	**Presence of H- and Stop** (9)	**Absence of H- and Stop** (35)	**Presence of H- but not Stop** (24)	**Presence of H- and Stop** (15)
Haplotypes of LPL gene	1.00	1.00	2.80	270	282	257
	Ser/Ser (61)	**Ser/Stop** (8)	**Stop/Stop** (0)	**Ser/Ser** (59)	**Ser/Stop** (14)	**Stop/Stop** (1)
Ser447 to Stop in LPL gene	1.00	2.40	-	275	257	254

† results from segmental analysis as per-patient data was not available for genotypes of the apoCIII C_{1100} to T polymorphism.
numbers in brackets = no. of individuals

Table 4.5.5
Per-patient results for prevalent and progression of atherosclerosis for haplotypes of the LPL gene (means ± S.Es shown).

Prevalent or change in measured disease in μm	LPL haplotypes (no. of individuals)			p-value for comparsion of genotype means
	Absence of both H- and Stop447 (35)	Presence of H- but not Stop447 (24)	Presence of both H- and Stop447 (15)	
Prevalent MAWS	270.2 ± 8.8	282.0 ± 12.5	256.5 ± 10.2	0.4 (oneway)
Prevalent MinAWS	223.5 ± 8.5	233.0 ± 11.5	208.5 ± 9.4	0.4 (oneway)
ΔMAWS	-6.2 ± 5.4	-4.1 ± 10.1	6.0 ± 5.4	0.4 (K-W)
ΔMinAWS	-8.0 ± 6.1	-2.3 ± 9.8	9.3 ± 5.0	0.2 (K-W)

oneway = oneway analysis of variance. K-W = non-parametic Kruskal-Wallis test.

Table 4.5.6 Trends observed in Swedish and STARS patient samples for change in severity of atherosclerosis

Genotypes	Swedish patient sample (medians of score for progression of global severity of atherosclerosis **High median = worse progression**			STARS patient sample (ΔMAWS in per-patient analysis) **negative ΔMAWS = progression positive ΔMAWS = regression**		
	GG (53)	**GA** (12)	**AA** (2)	**GG** (48)	**GA** (25)	**AA** (0)
apoAI G_{-75} / A polymorphism	0.70	0.85	1.30	-10.0	10.0	-
	CC (32)	**CT** (30)	**TT** (5)	**CC** (39) †	**CT** (29) †	**TT** (6) †
apoCIII C_{1100} /T polymorphism	0.75	0.80	0.60	-2.3 ± 3.5	-2.8 ± 3.8	9.1 ± 6.9

† results from segmental analysis as per-patient data was not available.
numbers in brackets = no. of individuals.

Section 4.6 Associations between gene polymorphisms and fasting serum lipid and lipoprotein traits.

Associations were examined between fasting lipid and lipoprotein traits and genotypes where the latter showed a significant association with prevalent or change in atherosclerosis in segmental analysis. This was to avoid the problem of making too many comparsions and obtaining associations of spurious significance. The null hypothesis to be tested was that associations between genotypes and atherosclerosis are explained by fasting lipids or lipoproteins. Table 4.6.1 shows means and standard errors of baseline or in-trial lipids for those genotypes showing an effect on severity or progression of coronary atherosclerosis. Since means were similar (data not shown) for genotypes a1/d and d/d, they were combined and compared with genotype a1/a1.

The change in MAWS was previously found to be independently and significantly correlated with both the ratios of cholesterol to HDL-cholesterol and of LDL-cholesterol to HDL-cholesterol (Watts et al. 1992). Therefore associations were examined between genotypes and these ratios in addition to single lipid levels. Homozygosity for the Ag(a1) epitope was associated with higher means of lipid traits with the exception of LDL-cholesterol, though none reached statistical significance. For the G_{-75} to A substitution, Table 4.6.2 shows data for in-trial lipid traits by genotypes. The largest effect of genotype seen was on triglycerides, with those with the GA genotype having mean levels 20.8% greater than for the GG group. However there were no significant differences between traits and genotypes. There were no significant differences in body mass index, prevalance of smokers or age amongst genotypes of any of the polymorphisms studied (data not shown). Haplotypes defined by presence of the Ag(a1) epitope (Val591) and the X+ allele were reported to be associated with elevated apoB and LDL-cholesterol levels in both the Swedish healthy individuals and patients, the effect on apoB levels being significant in the patients (Dunning et al. 1993). However baseline apoB levels were not measured in STARS so the previously reported elevating effects could not be confirmed. In STARS, the presence of the Ag(a1) X+ haplotype (in 9/74 individuals) was associated with a small and insignificant decrease in total, LDL and HDL cholesterol and triglycerides (Table 4.6.1). However, the extremely strong linkage disequilibrium between these two apoB polymorphisms precluded attempts to seek associations between these haplotypes and lipids.

Table 4.6.1

Baseline lipid levels (mean ± standard errors) for genotypes of polymorphisms which showed a significant association with baseline atherosclerosis score.

* = median (interquartile range)

Trait (mM)	Genotypes (number of individuals) of apoB-Ag(a1/d) epitope pair		p-value by unpaired t-test
	a1/a1 (15)	a1/d & d/d (59)	
Cholesterol	7.30 ± 0.23	7.19 ± 0.10	0.9
Triglycerides *	2.14 (1.43-2.77)	2.01 (1.53-2.76)	0.6
HDL-cholesterol	1.27 ± 0.11	1.15 ± 0.03	0.3
LDL-cholesterol	4.98 ± 0.21	5.11 ± 0.12	0.9
Cholesterol/HDL-cholesterol	6.15 ± 0.38	6.60 ± 0.20	0.6
LDL-cholesterol/ HDL-cholesterol	4.28 ± 0.28	4.72 ± 0.18	0.5

Trait (mM)	Genotypes (number of individuals) of Apo B-XbaI polymorphism		p-value by unpaired t-test
	X-X- (16)	X+X- & X+X+ (58)	
Cholesterol	7.53 ± 0.22	7.13 ± 0.10	0.2
Triglycerides *	2.25 (1.41-2.79)	2.01 (1.49-2.72)	0.7
HDL-cholesterol	1.24 ± 0.10	1.15 ± 0.03	0.5
LDL-cholesterol	5.29 ± 0.24	5.02 ± 0.11	0.5
Cholesterol/HDL-cholesterol	6.56 ± 0.42	6.49 ± 0.19	0.8
LDL-cholesterol/ HDL-cholesterol	4.67 ± 0.35	4.63 ± 0.18	0.6

* shown as medians (inter-quartile ranges)

Table 4.6.2

In-trial lipid levels (mean ± standard errors) for genotypes of polymorphisms which showed a significant association with change in extent of atherosclerosis between angiographies.

Trait (mM)	Genotypes (number of individuals) ApoAI-G/A_{-75} polymorphism		p-value by unpaired t-test
	GG (48)	GA (25)	
Cholesterol	6.12 ± 0.14	6.28 ± 0.16	0.502
Triglycerides *	1.78 (0.81 to 3.90)	2.15 (0.83 to 5.60)	0.100
HDL-cholesterol	1.19 ± 0.03	1.17 ± 0.04	0.770
LDL-cholesterol	4.06± 0.14	4.01 ± 0.18	0.825
ApoAI	1.30 ± 0.03	1.32 ± 0.04	0.707
ApoB	1.23 ± 0.03	1.29 ± 0.05	0.352

* shown as geometric mean (95% reference range)

Section 4.7 Possible Mechanisms underlying association of apoB gene polymorphisms with prevalent atherosclerosis

The similar results for MAWS and MinAWS at first angiography by genotypes of the Ag(a1/d) epitope pair and XbaI polymorphism in the apoB gene is a reflection of the very strong linkage disequilibrium between these two sites on the apoB gene. The Ag(a1) epitope was nearly always found on the same chromosome as the X- allele and likewise for the d and X+ alleles. This strong allelic association was also found in a sample of Swedish origin (Dunning et al. 1993) and because of the small sample size, this precluded any further 'haplotype' analysis in the STARS sample. The XbaI polymorphism cannot, by itself underlie these associations with atherosclerosis since it does not result in an amino acid change in the apoB protein molecule (Carlsson et al. 1986). However, it is possible that the Val591 to Ala amino acid change alone, or the Val to Ala change plus other variation in linkage disequilibrium with the X- allele of the XbaI polymorphism is explaining the association with worse coronary atherosclerosis.

The Val591 to Ala amino acid change in the N-terminal trypsin accessible region of apoB replaces one hydrophobic amino acid with another hydrophobic amino acid (Wang et al. 1990) and is outside both the primary LDL-receptor binding domains of apoB (Knott et al. 1986, Innerarity et al. 1987, Soria et al. 1989, Yang et al. 1989, Dunning et al. 1991). The direct involvement of the Val591 to Ala amino acid change in influencing the LDL-receptor affinity of apoB is thus not very likely. This amino acid substitution could have an indirect effect through linkage disequilibrium with another variant in apoB that does affect receptor binding. All individuals heterozygous for the rare apoB mutation that changes Arg3500 to Glu and diminishes the normal binding of apoB to the LDL-receptor (Soria et al. 1989), causing familial defective apoB100 (Vega & Grundy 1986, Innerarity et al. 1987) and who have been haplotyped have the same haplotype (Ludwig & McCarthy 1990, Myant et al. 1991, Rauh et al. 1991, Loux et al. 1993). This haplotype has the Ag(a1) epitope in combination with the X- allele. However the worse prevalent atherosclerosis observed with homozygosity of the a1 epitope is not likely to be explained by the presence of the Arg3500 to Glu mutation, which causes elevated LDL-cholesterol levels (Innerarity et al. 1990), and which is more frequent in patients with CAD compared to healthy controls (Tybjaerg-Hansen et al. 1990). This is because of the low frequency of this mutation (1-6% in samples with

moderate or familial hypercholesterolaemia or Type IIa hyperlipidaemia from Europe and North America, reviewed in Tybjaerg-Hansen and Humphries 1992) and absence of severe hypercholesterolaemia in the STARS patients.

Alternatively, the Val591 to Ala substitution may be associated with, as yet, unidentified missense mutation in the apoB gene that abolishes or reduces binding of apoB to the LDL-receptor, or variation in the apoB promoter or enhancer that alters the rate of synthesis of apoB. Over-production of apoB seems to be the primary metabolic defect in combined hyperlipidaemia (Chait et al. 1980). Combined hyperlipidaemia (Type IIb) is characterized by elevated levels of VLDL or LDL or both (Goldstein et al. 1973) and its prevalence in myocardial infarction survivors is high (Goldstein et al. 1973). Members of families with Type IIb hyperlipidaemia have 10 times the risk of developing CHD than do normal people (Hazzard et al. 1976). Finally the Val591 to Ala substitution could directly or indirectly affect affinity of apoB for arterial proteoglycans or susceptibility of apoB to oxidation. Binding of apoB to proteoglycans in the arterial wall causes accumulation and non-enzymatic modification of the apoB (Steinbrecher et al. 1987). This damage to apoB leads to the modification of LDL, so it can no longer be recognized by LDL-receptors, but rather by specific scavenger receptors on macrophages, leading to foam cell formation and thence to atherogenesis (Sparrow et al. 1989, Keidar et al. 1992, Aviram et al. 1991). Alternatively, variation in apoB might influence suspectibility of LDL to enzymatic modifications in the arterial wall. Various enzymatic modifications of LDL occur (reviewed by Aviram 1993) eg. the action of cholesterol oxidase on LDL increases suspectibility of LDL to oxidation. Modified LDL is associated with increased atherogenicity (reviewed by Aviram 1993).

The association between the X- allele and worse prevalent disease confirms data from other studies; the X- allele has been found to occur more frequently in patients with CAD relative to healthy controls (Hegele et al. 1986, Monsalve et al. 1988, Myant et al. 1989, Paulweber et al. 1990, Renges et al. 1991) and the Ag(a1) epitope (Val591) is associated with a trend towards worse baseline coronary atherosclerosis in a sample of Swedish patients (Chapter 3 of this thesis).

The association between the Ag(a1) epitope or X- allele and more severe coronary atherosclerosis, as evidenced by lower mean values for both MAWS and MinAWS in individuals homozygous for the Ag(a1) epitope or X- allele, cannot be explained solely by effects on meaured fasting lipid or lipoprotein traits. Those individuals with the Ag(a1) epitope or X- alleles had higher mean levels of total cholesterol and triglyceride levels which may be a partial explanation for the worse coronary atherosclerosis, but associations between genotypes and baseline lipids were not significant. This may be because a reduced variability in baseline lipids (could be due to selection for cholesterol levels within a narrower range or environmental factors such as body mass index or presence of atherosclerosis) may have attenuated associations between lipids and genotypes. It is more likely that these associations between genetic variation at the apoB locus and baseline atherosclerosis are explained by an unmeasured lipid parameter such as levels of apoB or LDL subfractions. Case-control studies have reported elevated apoB levels in young myocardial infarction survivors (Hamsten et al. 1986) and in patients with CAD or CHD (Sniderman et al. 1980, Myant et al. 1989, Paulweber et al. 1990, Tong et al. 1992) compared to healthy individuals. Prospective studies have reported elevated apoB levels as an independent risk factor for ischaemic heart disease (IHD) (Salonen et al. 1985, Stampfer et al. 1991, Sigurdsson et al. 1992, Wald et al. 1994). A decrease in apoB of 10% was associated with a 22% decrease in IHD risk, but measurement of apoB detected only 17% of all IHD deaths at a cost of a 5% false-positive rate, with considerable overlap in distributions of apoB in men who died of IHD and in men who did not, but the relationship between apoB and IHD risk (Wald et al. 1994) becomes weaker with increasing age. ApoB is highly correlated with LDL cholesterol, $r=0.91$ (Maciejko et al. 1987), so it follows that both apoB and LDL will exhibit similar associations with the risk of IHD, agreeing with an association observed between LDL-cholesterol and IHD risk (Law et al. 1994). Another unmeasured lipid parameter is postprandial lipaemia which is discussed in Chapter 5 of this thesis.

Section 4.8 Possible mechanisms underlying association of apoAI G_{-75} to A polymorphism with progression of atherosclerosis

Change in severity of coronary atherosclerosis as measured by ΔMAWS and ΔMinAWS was strongly associated with genotypes of the G_{-75} to A substitution in the apoAI

gene promoter in these patients. A striking feature of these associations was the absence of interaction of genotypes with treatment regimen; the pattern of association between ΔMAWS or ΔMinAWS in each of the three treatment regimens was the same for each of the two genotypes, GG and GA (none of the patients were homozygous for the 'A' allele) as for the entire sample. Thus, treatment regimen appears to be modulating ΔMAWS and ΔMinAWS in a manner that is independent of genotypes of the G_{-75} to A polymorphism and of baseline or in-trial lipids. Associations between the G_{-75} to A polymorphism and on-trial lipids were much weaker than those with change in severity of atherosclerosis. Confounding factors at baseline that could have influenced prognosis, such as advanced age, frank hypercholesterolaemia and lipid lowering medication, can be ruled out due to the selection criteria employed at the start of the study. Other lifestyle factors such as smoking status, alcohol intake and type A personality showed a similar distribution amongst the three groups of patients (Watts et al. 1992).

The A_{-75} allele of the G_{-75} to A polymorphism in the apoAI promoter was significantly associated with mean regression of atherosclerosis (means for both ΔMAWS and ΔMinAWS being positive). This polymorphism could itself have a direct effect on plasma levels of apoAI via altered production rate of apoAI (Smith et al. 1992). ApoAI levels have been shown in several epidemiological studies to be inversely related to incidence of CAD (eg. Stampfer et al. 1991) and to severity of CAD (eg. Miller et al. 1981). The A_{-75} allele has been consistently associated with higher apoAI levels in healthy non-smoking men (Jeenah et al. 1990, Pagani et al.1990, Paul-Hayase et al. 1992, Sigurdsson et al. 1992). About 26% of the patients who completed the study were current smokers; previously smoking was shown to abolish the effect of the G_{-75} to A polymorphism on HDL-cholesterol and apoAI levels in men; an example of interaction between the G_{-75} to A polymorphism and smoking (Sigurdsson et al. 1992). This is the most probable explanation for the lack of association between the G_{-75} to A polymorphism and either HDL-cholesterol or apoAI levels in STARS. Jeenah et al. (1990) showed that the strongest effect of the apoAI G_{-75} to A polymorphism was on the HDL_2 subfraction rather than HDL_3. HDL_2 has been suggested to be protective against development of atherosclerosis (Miller et al. 1981), and has been implicated in the well-known inverse relation between HDL and atherosclerosis in a number of case-control studies (eg. Johansson et al. 1991b).

Alternatively, the G_{-75} to A polymorphism could be acting as a marker for other sequence changes within the apoAI-CIII-AIV gene cluster that themselves would be responsible for the change in MAWS or MinAWS seen in association with the G_{-75} to A polymorphism. The most likely candidate is variation within the apoCIII gene, for example in the region affecting transcription of the gene, which might directly affect levels of apoCIII in plasma. Variation in the apoAI-CIII-AIV gene cluster has been reported to be associated with atherosclerosis (Ferns et al. 1985, Deeb et al. 1986, Price et al. 1989), with familial combined hyperlipidaemia (Wojciechowski et al. 1991) and with differences in plasma levels of apoCIII (Shoulders et al. 1991, Paul-Hayase et al. 1992) but apoCIII levels were not measured in the STARS patients and this cannot be tested directly. ApoCIII could be partly responsible for triglyceride accumulation in plasma (Chivot et al. 1990), due in part to its inhibitory effect on lipoprotein lipase (Havel et al. 1970, Ginsberg et al. 1986). Recently Sehayek et al. (1991) demonstrated that apoC molecules, apoCIII among them, inhibit predominantly the apoE-dependent interaction of triglyceride-rich lipoproteins with the LDL receptor in cultured fibroblasts, and another study demonstrated a much reduced uptake of apoE-containing remnant particles via lipoprotein receptors in transgenic mice overexpressing apoCIII (Aalto-Setala et al. 1992). Levels of apoCIII in apoB-containing lipoproteins strongly discriminated between patients with coronary atherosclerosis and healthy controls (Chivot et al. 1990). Results from several laboratories indicate that apoCIII in VLDL is associated with denser, smaller VLDL subclasses that are believed to be particularly atherogenic (Carlson et al. 1976, Kuusi et al. 1980, Le et al. 1988). However, in STARS none of the VLDL classes predicted CAD nor were triglyceride levels correlated with CAD (Watts et al. 1993). ApoCIII has an inhibiting effect on LPL activity (Havel et al. 1970, Ginsberg et al. 1986) and LPL activity has recently been suggested to be one of the major determinants of LDL heterogeneity with low LPL activity promoting a redistribution of apoB in favour of dense LDL particles which are more atherogenic (Karpe et al. 1993c). This accords well with the positive correlation between LDL_3 (dense LDL) and progression of CAD (LDL_3-cholesterol and change in MAWS were negatively correlated) in STARS (Watts et al. 1993). Another unmeasured lipid parameter is postprandial lipaemia which is discussed in Chapter 5 of this thesis.

Section 4.9 Possible reasons for different associations between genetic variation and extent of atherosclerosis.

In the Swedish patient sample (Chapter 3 of this thesis) prevalent atherosclerosis score was associated with genotypes at the apoB signal peptide polymorphism or the apoCIII C_{1100} to T polymorphism or with haplotypes of the HindIII and Serine447 to Stop polymorphisms in the LPL gene, with no significant associations observed between genotypes and progression of atherosclerosis. However, in the STARS patient sample, prevalent atherosclerosis was associated with the apoB Ag(a1/d) or XbaI polymorphisms and change in disease associated with the apoAI G_{-75} to A polymorphism. These different patterns of associations observed in the two patient samples is likely due to differences in the selection criteria employed in the selection of each patient sample, or the methodology used in measuring prevalent and change in severity of atherosclerosis. The Swedish patients, selected on the criteria of surviving a myocardial infarction, were younger (mean $\pm$ SE = 40.4 $\pm$ 0.38 years) than the STARS patients, selected on the criteria of having angiographically defined CAD (mean $\pm$ SE = 50.8 $\pm$ 0.90 years). Only 35 out of the 74 STARS patients had a previous myocardial infarction (Watts et al. 1992). Genetic factors are likely to play a more important role in the presence of disease in the Swedish patient sample than in the STARS sample, due to their younger age and higher frequency of myocardial infarction. The differences in allele frequencies, and therefore of genotype distributions between the two patient samples are too small to be a likely explanation for these differences. In the Swedish patient sample, fifteen proximal coronary arterial segments per individual were examined and points assigned on an arbitrary scale for extent of atherosclerosis and plaque size, while in the STARS patient sample, a maximum of ten coronary artery segments per individual were examined, and both mean and minimum diameter of these segments measured. Thus the system used in STARS may be more accurate and less subject to errors inherent in an arbitrary scale of measurement. The trends observed between genotypes and prevalent atherosclerosis were similar in both groups of patients, with the same alleles/haplotypes associated with worse prevalent disease (section 4.5). However trends observed between genotypes and change in severity of atherosclerosis were less similar between the two patient groups. In the Swedish sample, genotype GA of the apoAI G_{-75} to A polymorphism was associated with worse prognosis while in STARS this

same genotype was associated with better prognosis, with the same trend being evident in both samples with genotypes of the apoCIII C_{1100} to T polymorphism. This may be due to the influence of treatment regimen on prognosis (in STARS) and/or the unpredicted haplotype A_{-75} C_{1100}. None of these differences are likely to be due to varying influence of obesity; both patient samples had a similar body mass distribution (Swedish = 26.4 $\pm$ 0.35 and STARS = 26.2 $\pm$ 0.86, means $\pm$ SE) in Kg/m^2.

It will be of interest to do a much fuller scale study demonstrating more clearly these differences on a per-patient basis. Next, it would be of interest to identify functional mutations causing these effects; these mutations could then be used to identify individuals at risk or who would require additional intervention to reduce their risk of developing CAD.

Chapter 5 - The Swedish Study; effect of common genetic variation at apoB, apoCIII, apoE and LPL gene loci in modulating postprandial lipaemia.

Section 5.1 Summary

A pilot study was carried out to examine the influence on postprandial lipid and lipoprotein metabolism of common genetic variation in the genes coding for apolipoproteins (apo) B, CIII and E and for lipoprotein lipase (LPL), in a group of 30 myocardial infarction survivors and 11 age-matched healthy individuals drawn from the original Swedish sample described in Chapter 2 of this thesis. Postprandial lipid or lipoprotein levels, adjusted for fasting triglycerides, were examined by genotypes. For the signal peptide (SP) polymorphism in the apoB gene, individuals with one or more SP-24 alleles of the apoB signal peptide length polymorphism had a 28% smaller mean area under curve (AUC) ($p=0.06$) for postprandial large chylomicron remnants (intestinal origin) and a 23% smaller mean AUC ($p=0.01$) for large VLDLs (hepatic origin) compared to individuals homozygous for the wild type SP-27 allele. This was due to a slower increase of these lipoproteins in the early postprandial period in carriers of the SP-24 allele (6.5 versus 15mg/l per hour for homozygosity for the SP-27 allele for intestinal lipoproteins), suggesting that length variation in the apoB signal peptide influences the rate of production of postprandial lipoproteins of both intestinal and hepatic origin. For the apoB-XbaI polymorphism, individuals homozygous for the X- allele had a greater area under curve (AUC) for total and Sf 60-400 postprandial triglycerides ($p=0.01$), due mainly to differences in the catabolism of remnants of chylomicron and VLDLs, with a 79% faster decline in the late postprandial period compared to carriers of the X+ allele supporting the hypothesis that variation in linkage disequilibrium with the XbaI polymorphism is influencing catabolism of postprandial lipoproteins.

For the apoCIII-C_{1100} to T polymorphism, the T allele was associated with higher fasting triglycerides and was associated with a 12% larger mean AUC for triglycerides ($p=0.01$ for all lipoprotein fractions) indicating greater postprandial lipaemia. Individuals with the apoE2 allele had a 18% larger mean area under curve (AUC), $p=0.09$ for large very low density lipoproteins (VLDLs) compared to those without the apoE2 allele, supporting the hypothesis of slower catabolism of postprandial lipoproteins in carriers of the apoE2 allele. The H+ allele of the LPL-HindIII polymorphism was associated with a 22%

larger mean AUC for LPL, a measure of total LPL activity over the entire postprandial period (p=0.04).

This study demonstrated that common variation in genes with known roles in lipid metabolism is having an important effect on the magnitude of postprandial lipaemia via production (apoB-signal peptide polymorphism), lipolysis (apoCIII and LPL) and catabolism (apoB-XbaI polymorphism and common apoE alleles) of TG-rich lipoproteins and their remnants.

Section 5.2 Rationale for Study

Common genetic variation, apart from the common apoE variants, has not previously been investigated in relation to the magnitude of postprandial lipaemia. Dr Fredrik Karpe and Dr Anders Hamsten (collaborators in Stockholm, Sweden) recently collected postprandial measurements including TGs and apoB (total and in each lipoprotein fraction defined by Svedberg flotation rate (Sf) range) on a sub-group of the original Swedish sample. Therefore it was an opportunity to test the hypothesis that variation in candidate genes influences magnitude of postprandial lipaemia and explains the associations between genetic variation and fasting lipids or lipoproteins or extent of coronary atherosclerosis. In this subgroup of patients and healthy individuals, selected measurements of postprandial lipaemia were analyzed with regard to genotypes of selected polymorphisms, chosen on the basis of having a high rare allele frequency and because of previous reports of being asssociated with fasting lipid or lipoprotein levels or extent of atherosclerosis (Davignon et al. 1988, Chamberlain et al. 1989, Thorn et al. 1990, Xu et al. 1990a, Heizmann et al. 1991, Hixson et al. 1992, Humphries et al. 1994, Xu et al. 1994).

Section 5.3 Swedish Individuals, their characteristics, gene polymorphisms and allele frequencies.

The 33 myocardial infarction survivors and 11 healthy individuals, who were investigated by Dr Fredrik Karpe and Dr Anders Hamsten with respect to postprandial lipaemia, were a sub-group of the original sample (see Chapter 2); more than 5 years had elapsed between the MI and the postprandial study. They had an oral fat tolerance test and blood was drawn just before the load (fasting sample) and hourly for the first nine hours after

the load. The last sample was taken 12 hours after ingestion of the emulsion. The oral fat tolerance test, blood sampling, fractionation of major plasma lipoproteins, subfractionation of Sf12-400 lipoproteins and cholesterol, TG and apolipoprotein and free fatty acid analyses are described in detail elsewhere (Karpe et al. 1992, 1993a, 1993b, 1993c, 1994).

Two of the patients who were heterozygous for the Asp9 to Asn substitution in exon 2 of the LPL gene (Mailly et al. 1994), were excluded from the subsequent analysis to eliminate any effects that would be due to this rare mutation rather than common genetic variation. No DNA was available for another HTG patient. One healthy individual who was hypertriglyceridaemic was excluded from the analysis previously done (Karpe et al 1992, 1993a, 1993b, 1993c) but is included here since the data was adjusted for fasting TGs prior to analysis. Table 5.3.1 shows the characteristics of each of the three groups of individuals with regard to age, body mass index and mean levels of postprandial lipid traits analyzed. Amongst the three groups, levels of TG-rich postprandial lipoproteins were highly significantly different (with the exception of small chylomicron remnants and small VLDLs). This was mainly due to the large differences between the hypertriglyceridaemic (HTG) patients and the other two groups. Total postprandial LPL activity was significantly lower in the healthy individuals ($p=0.02$) than in either patient group. However fasting plasma levels of free fatty acids were not significantly different amongst the three groups.

For all polymorphisms studied, there was no significant difference in genotype distributions or allele frequency amongst the three groups (healthy individuals, normotriglyceridaemic (NTG) patients and HTG patients) or between this sample and the distributions observed in the larger Swedish sample (Chapter 3 of this thesis).

Table 5.3.2 Allele frequencies in the postprandial sample.

Polymorphism rare allele	apoB-signal peptide SP-24	apoB-XbaI X+	apoCIII-C to T T_{1100}	LPL-HindIII H-	apoE2	apoE4
rare allele frequency	0.27	0.39	0.26	0.26	0.10	0.13

Table 5.3.1 - characteristics of the three study groups (unadjusted levels for postprandial lipid traits). Means ± standard errors are shown unless otherwise stated.

Trait - mean levels	Units	Controls (n=11)	NTG patients (n=16)	HTG patients (n=14)	P-values;Kruskal-Wallis test †
Age	years	49.2±1.0	48.9±0.9	48.9±0.8	N/S
Body mass index	Kg/m^2	24.5±0.8	25.8±1.0	27.4±0.8	N/S
AUC for TGs, all lipoproteins *	mM hours	21.9 (16.3 to 29.3)	25.1 (20.4 to 30.9)	58.9 (47.0 to 73.8)	⟨0.0001
AUC for TGs, chylomicrons *	mM hours	3.7 (2.7 to 5.1)	4.8 (3.6 to 6.4)	15.1 (11.8 to 19.4)	⟨0.0001
AUC for TGs, large chylomicron remnants and large VLDLs *	mM hours	6.9 (4.8 to 9.9)	8.5 (5.9 to 12.3)	27.5 (21.4 to 35.4)	⟨0.0001
AUC for TGs, small chylomicron remnants and small VLDLs *	mM hours	2.8 (2.0 to 4.0)	3.2 (2.5 to 3.9)	7.1 (5.4 to 9.4)	⟨0.0001
AUC for apoB-48 large chylomicron remnants	mg/l hours	97.4±17.7	154.2±25.8	315.5±36.6	0.0003
AUC for apoB-48 small chylomicron remnants *	mg/l hours	37.2 (21.6 to 63.8)	41.7 (29.9 to 58.2)	56.2 (39.5 to 80.1)	N/S
AUC for apoB-100 large VLDLs	mg/l hours	614±133	708±121	1737±161	0.0005
AUC for apoB-100 small VLDLs	mg/l hours	498±112	464±56	747±111	N/S
Total AUC for lipoprotein lipase	milliunits hours	12.3±0.9	18.5±1.9	18.2±1.5	0.02
Fasting levels of free fatty acids	μM/l	581±60	656±68	586±34	N/S
apoCIII at 0hrs	mg/l	157.4±11.8	113.1±10.9	181.3±16.4	0.005
apoCIII at 3hrs	mg/l	145.7±9.5	101.8±10.7	164.2±14.6	0.003
apoCIII at 6hrs	mg/l	126.9±9.5	103.9±11.2	174.7±10.9	0.0007
apoCIII at 12hrs	mg/l	109.5±6.4	83.3±8.0	152.2±10.7	0.0001

For lipoprotein lipase activity, milliunits = nmol of fatty acd released per minute per ml plasma.
N/S = not significant AUC = area under curve
* antilogs of log-transformed means and 95% confidence intervals † comparing unadjusted levels across all three groups

As this study was an exploratory one, generating hypotheses to test in future studies, the arbitrary cut-off value (α) for statistical significance was set at 0.1 instead of the more conventional value of 0.05, to increase the probability of finding at least one 'statistically significant' association, which is $1-(1-\alpha)^n$, from $1-(0.95)^n$ to $1-(0.90)^n$ where n = number of individual null hypotheses being tested (Rothman 1990). This reduction in the probability of mistakenly accepting an incorrect null hypothesis (false negative statement or Type II error) is at the cost of increasing the probability of mistakenly rejecting a correct null hypothesis (false positive statement or Type I error). The result is an increased probability of finding associations that could be worth exploring further, which would have been ignored had the conventional level of statistical significance been ultilized.

Section 5.4 Influence of Fasting TGs on postprandial lipaemia

As previously reported (Karpe et al 1992, 1993a, 1993b, 1993c, 1994), except for AUCs-LPL and levels of fasting free fatty acids (FFA), fasting TGs accounted for much of the variability seen between individuals and between subgroups. Over 60% of sample variance in postprandial lipaemia (as measured by AUCs for TGs) in all lipoprotein fractions was attributable to fasting TGs. The regression equation for $\log_{10}$ of AUC for TGs in all lipoprotein fractions versus $\log_{10}$ fasting TGs, for the whole group of 42 individuals (R^2 = 0.92) was; $Y = 0.99X + 1.29$, where 0.99 = the slope and 1.29 = the intercept on the Y-axis. The regresson equation was similar for each of the three subgroups (data not shown), with the slope ranging between 0.92 for the NTG patients and 1.03 for the HTG patients. 32 to 73% of sample variance in cumulative levels of large and small chylomicron remnants and VLDLs (as measured by AUCs) was explained by levels of fasting TGs.

The strong postive relationship between fasting TGs and magnitude of postprandial lipaemia observed in this study confirms previous findings (Nestel et al. 1964, Pelkonen et al. 1965, Patsch et al. 1983, 1987, 1992, Cohn et al. 1988, Simpson et al. 1990, Groot et al. 1991, O'Meara et al. 1992) making fasting TG levels one of the important determinants of postprandial lipaemia. Because of this, all data was adjusted for fasting TGs prior to examining associations between postprandial lipaemia and common genetic variation.

Table 5.4.

Percentage of total variance in postprandial lipid and lipoprotein traits attributable to fasting TGs

Trait AUC (area under curve)	%age of total sample variance attributable to fasting TGs	F value; its significance
AUC for TGs, all lipoproteins	92.0	462.6; <0.0001
AUC for TGs, chylomicrons	64.0	65.7; <0.0001
AUC for TGs, large chylomicron remnants and large VLDLs	83.3	184.7; <0.0001
AUC for TGs, small chylomicron remants and small VLDLs	73.6	103.1; <0.0001
AUC for apoB-48 large chylomicron remnants	43.8	28.0; <0.0001
AUC for apoB-48 small chylomicron remnants	31.6	14.3; <0.0001
AUC for apoB-100 large VLDLs	72.7	93.0; <0.0001
AUC for apoB-100 small VLDLs	32.1	15.6; <0.0001
Total AUC for LPL	3.7	1.5; N/S
Fasting levels of FFAs	9.3	4.1; 0.05
apoCIII at 0hrs	31.6	17.1; 0.0002
apoCIII at 3hrs	25.1	12.4; 0.001
apoCIII at 6hrs	45.3	30.7; <0.0001
apoCIII at 12hrs	42.6	27.4; <0.0001

Section 5.5 Association of genotypes at the apoB signal peptide polymorphism with postprandial lipaemia and possible underlying mechanisms.

For the signal peptide (SP) length polymorphism, differences were seen between AUCs for apoB-48-containing lipoproteins (small and large chylomicron remnants) and for apoB-100-containing lipoproteins (small and large VLDLs), (Table 5.5) with the presence of the SP-24 allele being associated with a 22.7% smaller AUC (p=0.01) for large VLDLs and a 27.8% smaller AUC (p=0.06) for large chylomicron remnants. Associations between

Table 5.5.

Means ± standard errors (SEs) of Areas Under Curves (AUCs) in patients and healthy individuals combined for absence (homozygosity for the SP-27 allele) and presence of the SP-24 allele of the apoB-signal peptide length polymorphism.

AUC (units = mg/l hours) - adjusted for log fasting TG	Absence of SP-24 allele (n=22)	Presence of SP-24 allele (n=16)	p value from ANOVA
apoB-48 Sf 60-400 large chylomicron remnants	209 ± 21.8	151 ± 20.7	0.06
apoB-48 Sf 20-60 * small chylomicron remnants	47 (37 to 60)	40 (29 to 54)	N/S
apoB-100 Sf 60-400 large VLDLs	1055 ± 75.2	815 ± 78.4	0.01
apoB-100 Sf 20-60 small VLDLs	627 ± 68.8	461 ± 44.1	0.12

numbers in brackets = number of individuals
* antilogs of $\log_{10}$ transformed mean and 95% confidence limits
N/S = not significant

presence/absence of the SP-24 allele and Sf20-60 lipoproteins were weaker, the SP-24 allele still being associated with a 14.9% smaller AUC for small chylomicron remnants and a 26.5% smaller AUC for small VLDLs. The TG composition of Sf 60-400 lipoproteins does not seem to be influenced by genotypes of the signal peptide polymorphism; while the total AUC for TGs in Sf 60-400 lipoproteins is also higher (approximately 15%) for homozygosity for the SP-27 allele. AUC's for cholesterol were not available, so the hypothesis that signal peptide genotypes influence the lipid composition of Sf 60-400 lipoproteins cannot be tested fully here. The association of the SP-24 allele with smaller AUCs for apoB-containing lipoproteins Sf 60-400 on its own cannot distinguish between diminished production or enhanced catabolism of these lipoproteins. Therefore the individual time points were plotted to attempt to distinguish between these two possibilities.

The plasma levels over time for apoB-48 in large chylomicron remnants (intestinal origin) and apoB-100 in large VLDLs (hepatic origin) are shown in Figures 5.5.1 and 5.5.2, respectively, for individuals grouped for presence or absence of the SP-24 allele. For large chylomicron remnants, the initial lines are not parallel, with gradients from 0 to 2.5 hours after the oral fat load being 15mg/l for absence and 6.5mg/l for the presence of the SP-24 allele. This showed that for large apoB-containing lipoproteins of intestinal origin in the early postprandial period, the rate of increase over time in those with only the SP-27 allele was 2.3 times greater than was seen in those with the SP-24 allele. In addition, the peak values at about 5 hours after the oral fat load were approximately 40% lower for those with the SP-24 allele. For large VLDLs, these effects were less marked, but in those with the SP-24 allele, the peak was lower and occured earlier. This effect was consistent in each of the three groups of subjects separately, without adjustment for fasting TGs, with those carrying the SP-24 allele having smaller AUCs for both large chylomicron remnants and large VLDLs (data not shown).

Several association studies have shown associations between genotypes of the apoB signal peptide length polymorphism and fasting lipid or lipoprotein levels (Xu et al 1990a, Boerwinkle et al. 1991, Renges et al 1991, Visvikis et al 1993), extent of atherosclerosis (Hixson et al 1992) and responsiveness of plasma TGs to dietary intervention (Xu et al. 1992). Recently Sturley et al. (1993) demonstrated, using apoB signal peptide alleles fused

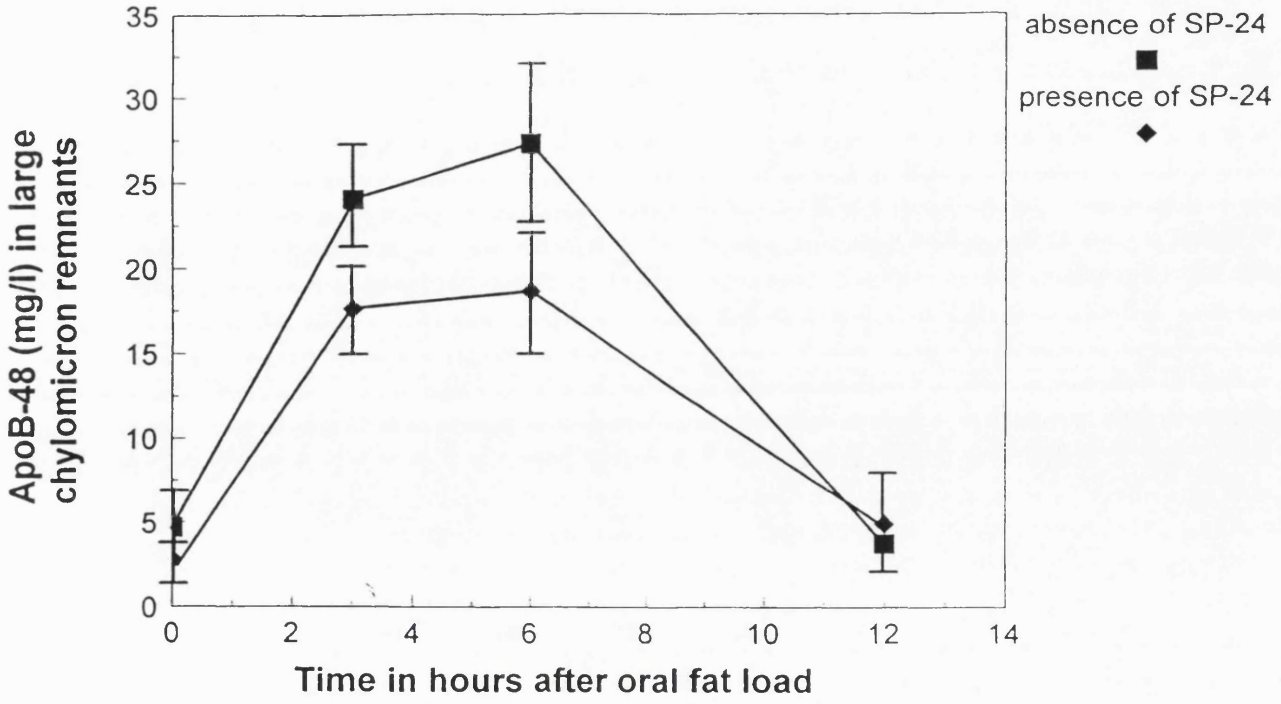


Figure 5.5.1

Means ± S.E.s of apoB-48 (mg/l) in Sf 60-400 lipoproteins (large chylomicron remnants) versus time (in hours) since ingestion of the oral fat load, with the group divided into those homozygous for the SP-27 allele and those carrying at least one copy of the SP-24 allele of the apoB signal peptide polymorphism. Data is shown for the combined group of healthy individuals and patients and is adjusted for fasting TGs.

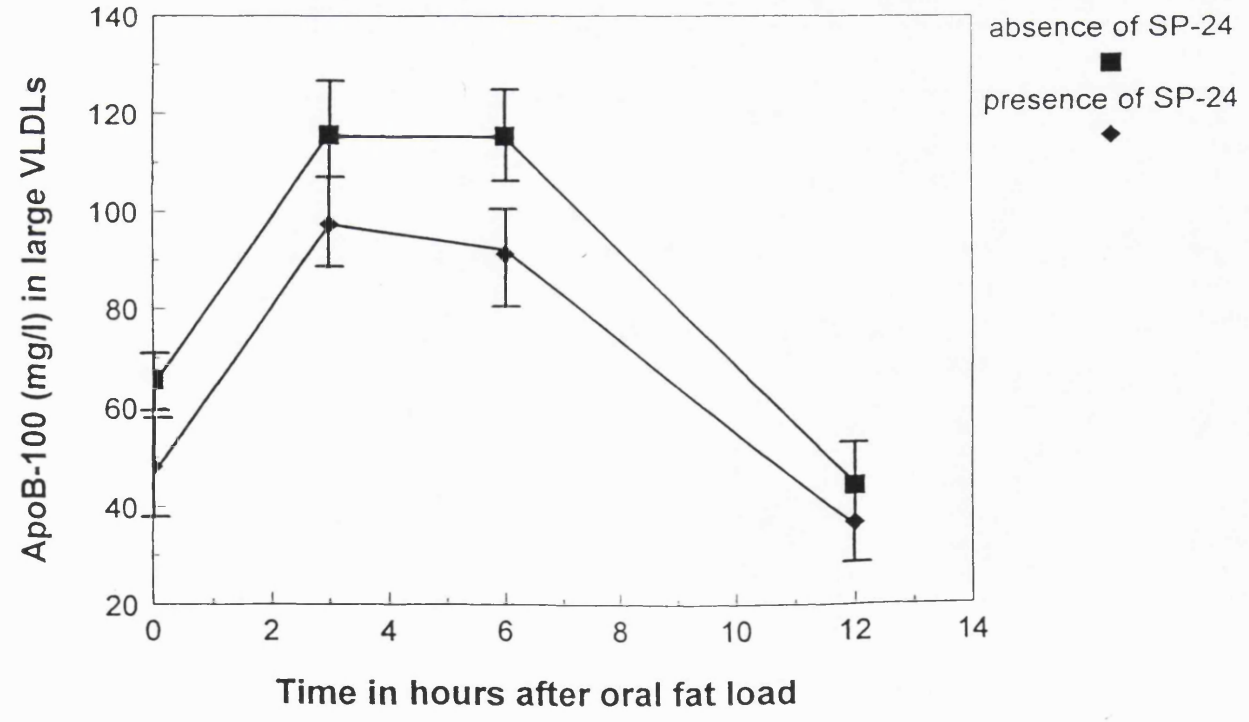


Figure 5.5.2

Means ± standard errors of apoB-100 (mg/l) in Sf 60-400 lipoproteins (large VLDLs) versus time (in hours) since ingestion of the oral fat load, with the group divided into those homozygous for the SP-27 allele and those carrying at least one copy of the SP-24 allele of the apoB signal peptide polymorphism. Data is shown for the combined group of healthy individuals and patients and is adjusted for fasting TGs.

to a secretory protein, invertase and expressed in yeast cells, that the different alleles of the signal peptide length polymorphism may themselves affect the translocation of the apoB polypeptide into the endoplasmic reticulum (ER). Secretions of active invertase effected by the SP-24 and SP-29 [rare variant so far only identified in Mexican-Americans (Boerwinkle et al. 1991)] alleles were 25% and 33%, respectively, relative to the common SP-27 allele. Both these variants mediated inefficient translocation and reduced efficiency of secretion due to alterations in the SP hydrophobicity (Sturley et al. 1994).

A molecular modelling strategy (Brasseur et al. 1988) was applied to predict the degree and angle of insertion of the three apoB SP variants into a hydrophobic environment such as a lipid membrane (Sturley et al. 1994). They predict an optimal angle of insertion (54°) for the apoB SP-27 allele and a sub-optimal one (23°) for the SP-29 allele. The SP-24 allele is predicted to have an angle of insertion (45°) within the optimal range, but due to the deletion of 3 hydrophobic amino acids, Leu-Ala-Leu, the SP-24 allele is predicted to lack sufficient total hydrophobicity to penetrate the lipid-water interface effectively. Sturley et al. (1994) have listed four parameters that are important for determining maximal secretion of the yeast invertase fused to the apoB signal peptide, and hence maximum penetration by the signal peptide through the lipid-water interface. These are 1) angle of insertion to the lipid-water interface of 30-60° ie. oblique orientation is essential to proper function of the SP. 2) Large difference between hydrophobic and hydrophilic energies to induce lipid destabilization at the interface and allow penetration deep into the lipid matrix. 3) The peptide helical structure at the interface must be sufficently stable, as reflected by the need for a maximal difference between the hydrophilic and hydrophobic centres of the molecule. 4) The peptide must have a stable helical structure - important for maximal destabilization of the lipid matrix.

Sturley et al. (1994) suggest that alteration to the orientation of signal peptides, by introducting mutations, disrupts the functional properties of the signal peptide (directing translocation of the protein across the ER and/or the local disruption of the lipid bilayer) by altering hydrophilic/phobic content, and/or the secondary structure of the SP. Studies with lipid vesicles support the hypothesis that translocation is due to direct interaction with the lipid membrane (Geller et al. 1985). The decreased translocation efficiency in-vitro of the

SP-24 protein relative to the SP-27 protein accords well with the decreased responsiveness of plasma triglycerides to change of diet in individuals with one or more SP-24 alleles compared to those without this allele in the North Karelia dietary intervention study (Xu et al 1990a). The similar results for intestinal and hepatic Sf 60-400 lipoproteins suggests that the effect of the reduced length and hydrophobicity variation in the signal peptide caused by the deletion of the Leu-Ala-Leu sequence exerts its influence on both intestinal and hepatic secretion of apoB-containing lipoproteins postprandially.

Section 5.6 Association of genotypes of the apoB-XbaI polymorphism and the Ag (x/y) epitope pair with postprandial lipaemia and possible underlying mechanisms

For the apoB-XbaI polymorphism, differences were seen between AUCs of total TGs in all, Sf 60-400 and Sf 20-60 lipoproteins for genotypes stratified by presence and absence of the X+ allele. Means and 95% confidence limits are shown in Table 5.6.1 for these AUC's for those individuals with and without the X+ allele and in Table 5.6.2 for those with and without the Ag(x) epitope. For all lipoprotein fractions, AUCs were 13% larger ($p=0.01$) in individuals homozygous for the X- allele compared to those carrying at least one X+ allele. For the Sf 60-400 lipoproteins, AUC's were 20% larger ($p=0.01$) in individuals homozygous for the X- allele. Smaller differences, but in the same direction throughout, were observed for presence/absence of the Ag(x) epitope, the AUC for total TGs being 10.3% larger in carriers of the Ag(x) epitope. Figure 5.6 shows mean levels of total postprandial TGs (adjusted for fasting TGs) in the combined group versus time elapsed since ingestion of the oral fat load, in individuals grouped for presence and absence of the X+ allele, respectively. The lines are approximately parallel until 4 hours, when the rate of decline is faster for the X-X- group. Between 9 and 12 hours postprandium, the gradient for the X-X- group was 0.25mM/hour and that for the X+ group was 0.14mM/hour.

In terms of postprandial lipid metabolism, genotypes of the XbaI polymorphism exerted their strongest effect in the late postprandial period, when plasma levels of TGs are decreasing because the catabolism of the lipoproteins is exceeding their synthesis; during this period (9-12 hours) the downwards gradient for total postprandial TG levels in X-X- individuals was nearly twice that for individuals carrying the X+ allele. This is not the first finding of an influence of genotypes of the XbaI polymorphism upon levels of TGs; such

Table 5.6.1.

Means and 95% confidence limits of Areas Under Curves (AUCs) for triglycerides (TGs) in patients and healthy individuals combined for absence (homozygosity for the X- allele) and presence of the X+ allele of the apoB-XbaI polymorphism in exon 26.

AUC (units = mM hours) - adjusted for log fasting TG	Absence of X+ allele X-X- (n=14)	Presence of X+ allele X+X- and X+X+ (n=27)	p value from ANOVA
AUC TGs; all lipoproteins *	36.3 (31.8 to 41.5)	31.6 (30.2 to 33.1)	0.01
AUC TGs; Sf 60-400 large chylomicron remnants and large VLDLs *	13.5 (11.3 to 16.2)	11.2 (9.8 to 12.9)	0.01
AUC TGs; Sf 20-60 small chylomicron remnants and small VLDLs *	3.7 (2.9 to 4.7)	4.1 (3.7 to 4.6)	0.3
AUC apoB-48 Sf 20-60*†	45.6 (36.0 to 57.8)	42.6 (32.5 to 55.7)	0.6
AUC apoB-100 Sf 20-60†	475 ± 53.1	598 ±62.1	0.4
AUC apoB-100 in IDL †	458 ± 50.6	524 ± 46.1	0.4

† units are mg/l hours
numbers in brackets = number of individuals
* antilogs of $\log_{10}$-transformed mean and 95% confidence limits.
IDL = intermediate density lipoproteins

Table 5.6.2

Means and 95% confidence limits of Areas Under Curves (AUCs) for triglycerides (TGs) in patients and healthy individuals combined for absence (homozygosity for the Ag(y) epitope) and presence of the Ag(x) epitope of the apoB Ag(x/y) epitope in exon 29.

AUC (units = mM hours) - adjusted for log fasting TG	presence of Ag(x) epitope xy and xx (n=17)	Absence of Ag(x) epitope yy (n=24)	p value from ANOVA
AUC TGs; all lipoproteins *	35.2 (32.0 to 38.8)	31.9 (29.9 to 34.1)	0.08
AUC TGs; Sf 60-400 large chylomicron remnants and large VLDLs *	12.7 (10.7 to 15.2)	11.5 (10.0 to 13.3)	0.4
AUC TGs; Sf 20-60 small chylomicron remnants and small VLDLs *	3.6 (3.0 to 4.3)	4.3 (3.9 to 4.8)	0.2
AUC apoB-48 Sf 20-60* †	49.5 (40.2 to 61.0)	39.8 (29.8 to 53.1)	0.3
AUC apoB-100 Sf 20-60 †	460 ± 51.0	619 ± 64.6	0.14
AUC apoB-100 in IDL †	442 ± 48.5	544 ± 46.7	0.15

† units are mg/l hours
numbers in brackets = number of individuals
* antilogs of $\log_{10}$-transformed mean and 95% confidence limits.
IDL = intermediate density lipoproteins

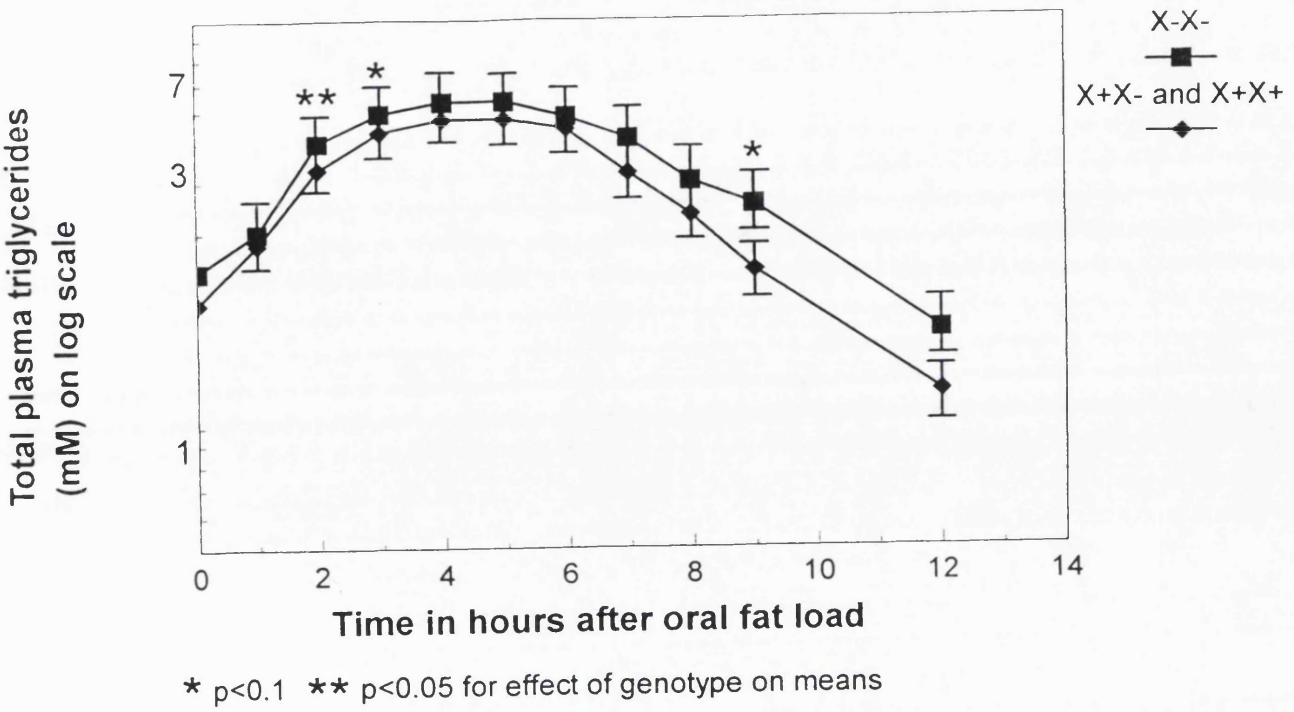


Figure 5.6
Means ± standard errors of total plasma TGs (log_{10} scale) in mM, adjusted for fasting TGs versus time (in hours) since ingestion of the oral fat load, for healthy individuals and patients combined, with the group divided into those homozygous for the X- allele (no cutting site) and those carrying at least one copy of the X+ allele of the apoB-XbaI polymorphism.

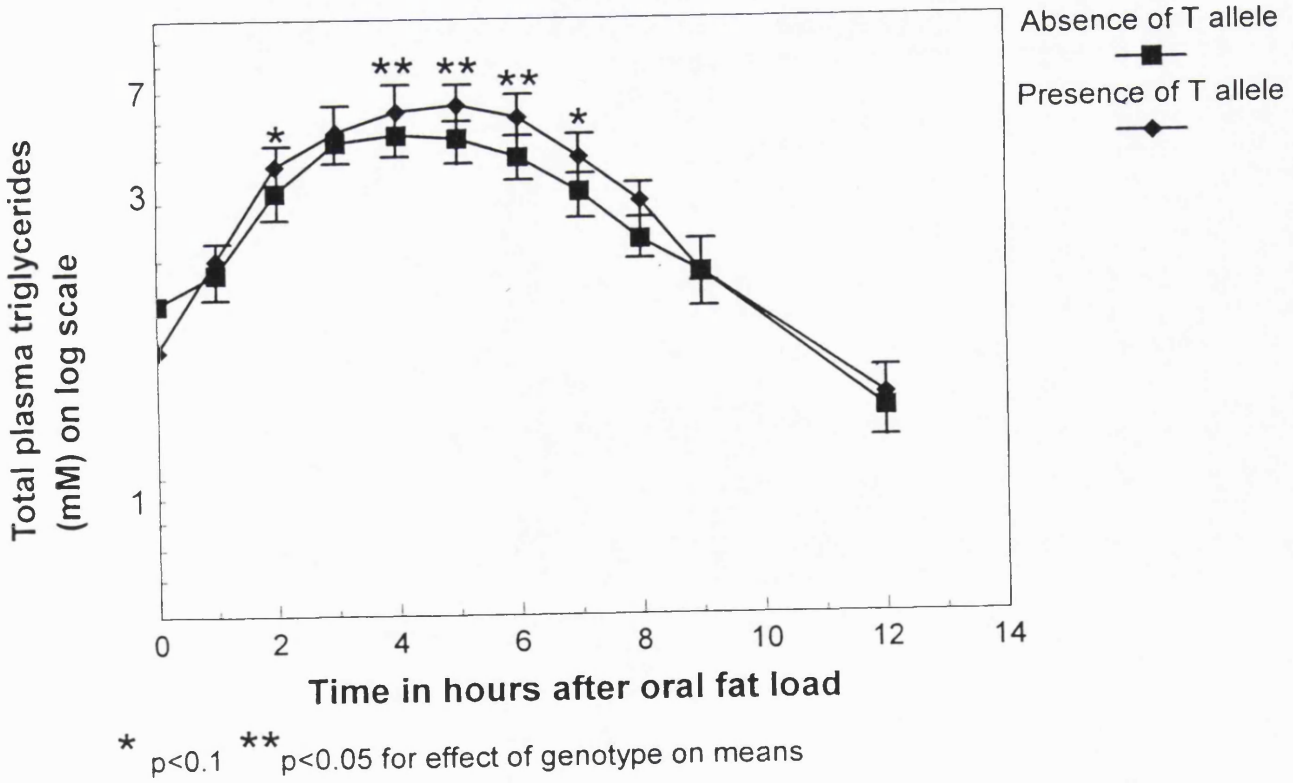


Figure 5.7
Means ± standard errors of total plasma TGs (log_{10} scale) in mM, adjusted for fasting TGs versus time (in hours) since ingestion of the oral fat load, with the group divided into those homozygous for the C_{1100} allele and those carrying at least one copy of the T_{1100} allele of the C_{1100} to T polymorphism in the apoCIII gene. Data is shown for the combined group of healthy individuals and patients.

associations have previously been reported for fasting TGs, with the X+ allele associated with raised levels (Law et al. 1986, Talmud et al. 1987, Jenner et al. 1988) but it gives a possible explanation for occasional findings of associations between the XbaI polymorphism and HDL levels (eg. Myant et al. 1989) due to the strong inverse relationship between HDL and postprandial lipaemia (Patsch et al. 1983, 1987). The XbaI polymorphism itself does not alter the apoB amino acid sequence (Carlsson et al 1986) so it must be in linkage disequilibrium with other functional variation in the apoB gene that could itself modulate the receptor-mediated uptake of remnant lipoproteins.

The LDL receptor binds both LDL which contains only apoB-100, and apoE-containing remnants of chylomicrons and VLDLs produced by LPL-mediated lipolysis of TG-rich lipoproteins (Brown & Goldstein 1986). Only about 50% (depending on apoE phenotype) of VLDL particles synthesized by the liver ever enter the IDL and LDL density range, the rest being catabolized in the VLDL density range (Demant et al. 1991). In addition, IDL particles can be cleared directly from the circulation via apoE binding to the LDL-receptor (Packard et al. 1985). However, the LDL-receptor related protein (LRP), rather than the LDL-receptor, may play a major role in the uptake and catabolism of postprandial remnant proteins (Herz et al. 1988). Evidence for this comes from the normal clearance of chylomicron remnants in individuals with homozygous familial hypercholesterolaemia (FH) (Havel et al. 1982) and from Watanable heritable hyperlipidaemic (WHHL) rabbits which also rapidly clear an appreciable fraction of large VLDL particles from the circulation (Yamada et al. 1989). Direct removal of large VLDL ($VLDL_1$) from the circulation has been demonstrated in both normal and homozygous FH subjects (James et al. 1989), raising the possibility that the LDL-receptor may not be the agent responsible for this catabolic route even in non-FH individuals.

The receptor binding domain of apoB-100 only becomes available to the LDL-receptor once the VLDL has been metabolized to LDL (Chappell et al. 1991). The lack of appreciable apoB-100-mediated binding of VLDLs to LDL-receptors may also be due to interactions of apoE or apoB-100 or both with other components of VLDLs such as apoCs (Krul et al. 1985). The conformation of apoB-100 in VLDL particles may also be masking the receptor binding domain (Krul et al. 1985). Recently, Yang et al. (1993) suggested that

removal of apoCs during lipolysis of VLDLs affects the accessibility and conformation of apoB-100 in the individual VLDL subfractions which, in turn, may regulate the association of apoB-100-containing lipoproteins with cell surface receptors. The only way in which sequence variation in apoB-100 could directly affect binding of remnant lipoproteins to the LDL-receptor would be if it shifted the equilibrium between VLDL and LDL in favour of LDL due to greater affinity of binding of LDL to the receptor. Such an effect was demonstrated for homozygosity of apoE4 (Demant et al. 1991), this has been discussed in more detail in Section 1.4.6). Alternatively, the XbaI polymorphism could be in linkage disequilibrium with variation in apoB that affects its conformation in VLDLs and thus alters interactions of other apolipoproteins (apoE and apoC) with these VLDLs. This would, in turn, affect the number of apoEs and/or apoCs, thus altering the ratio of apoEs to apoCs. There are four to six apoE molecules, but only one apoB-100 molecule per VLDL particle (Russell et al. 1989). Alterations in this ratio have been demonstrated in-vivo to directly affect receptor-mediated uptake of apoE-containing remnant lipoproteins (Aalto-Selta et al. 1992).

Taken together, these findings support the hypothesis that the X+ allele may be in linkage disequilibrium with variation in the apoB gene that would affect receptor-mediated clearance of remnants of triglyceride-rich lipoproteins of hepatic origin, either by changing the affinity of apoB-100 for the LDL-receptor or by changing the conformation of apoB-100 in VLDLs and hence the number and orientation of apoEs on the VLDL surface. Candidates for such variation are the Ag(x/y) epitope pair (Breguet et al. 1990) which appears to be determined by the apoB protein having Pro2712 and Asn 4311 [Ag(y)] or Leu2712 and Ser4311 [Ag(x)] (Dunning et al. 1992) and the Ag(a1/d) epitope pair created by the Val591 to Ala polymorphism (Wang et al. 1990). The percentage of sample variance in fasting apoB levels attributable to the apoB XbaI polymorphism was approximately equal to the sum of percentages of sample variance attributable to the Ag(x) epitope plus the haplotype defined by the Ag(a1) epitope in combination with the X+ allele (Dunning 1994, reviewed in Humphries 1993), suggesting that the XbaI effect may be mediated by the combination of these three amino acid changes in the apoB protein. Protein modelling has demonstrated that the N4311S substitution would reduce the number of possible hydrogen bonds participating in tertiary structure formation by one, and that the P2712L substitution appears to be in a

short helical region, which would be disturbed by the presence of Pro, and thus affect the secondary structure of apoB (Dunning et al. 1992). It is thus plausible that the Ag(x/y) epitope would affect the conformation of apoB in VLDLs and hence interactions between apoB and other apolipoproteins. From the data in Tables 5.6.1 and 5.6.2, it appears that apoB with the Ag(x) epitope (most of which comes from apoB encoded by the X- allele) favours a lipoprotein distribution towards the Sf 60-400 range and away from the Sf 20-60 and IDL ranges. One possibility is that the apoB protein bearing the Ag(x) epitope may adopt a conformation that increases the number of apoEs on the VLDL, and therefore favour apoE- rather than apoB-mediated uptake of lipoproteins. The similar results obtained with the Ag(x/y) epitopes or the XbaI polymorphism are not surprising, given the strong linkage disequilibrium between alleles at these two polymorphic sites, with all Ag(x) epitopes being encoded by X- bearing chromosomes (Dunning et al. 1992). Because of this strong allelic association, such an analysis has no power to discriminate whether the Ag(x) or X- alleles are having the largest effect on plasma lipid levels.

Section 5.7 Association of genotypes of the apoCIII C_{1100} to T polymorphism with postprandial lipaemia and possible underlying mechanisms

Compared to those homozygous for the C_{1100} allele, those carrying the T_{1100} allele had postprandial lipaemia that was 12% higher ($p=0.03$) and 34% and 29% greater AUCs for Sf>400 and Sf 60-400 lipoprotein fractions respectively. As shown in Figure 5.7. and Table 5.7.1, those with the T_{1100} allele had greater postprandial lipaemia in all, Sf>400 and Sf60-400 lipoproteins. The same trend was also evident in the three groups separately, when adjusted for fasting TGs (data not shown). This greater lipaemia was most pronounced in the mid-postprandial period (4-6 hours) as shown in Figure 5.7 (Page 274). Between 0 and 3 hours, time courses in the two groups were approximately parallel, however between 3 and 5 hours, levels were still rising in those with the T_{1100} allele while in those without this allele, total TGs were levelling out. From 5 hours onwards levels fell in both groups, with the excess lipaemia in the carriers of the T_{1100} allele disappearing by 9 hours postprandially. Carriers of the T_{1100} allele had 20% greater AUC's for LPL, a measure of freely circulating LPL activity (Table 5.7.1). This trend was also observed in the three groups separately (data not shown).

Table 5.7.1
Antilogs of log_{10} transformed means and 95% confidence limits of Areas Under Curves (AUCs) for triglycerides (TGs) in patients and healthy individuals, combined for absence (homozygosity for the C allele) and presence of the T allele of the apoCIII C_{1100} to T polymorphism.

AUC (units = mM hours) - adjusted for log fasting TG	Absence of T allele CC (n=21)	Presence of T allele CT and TT (n=19)	p value from ANOVA
AUC TGs; all lipoproteins	31.6 (29.4 to 34.0)	35.5 (32.8 to 38.3)	0.03
AUC TGs; Sf >400 chylomicrons	5.8 (4.7 to 7.0)	7.8 (6.2 to 9.7)	0.03
AUC TGs; Sf 60-400 large chylomicron remnants and large VLDLs	10.7 (9.2 to 12.5)	13.8 (11.8 to 16.1)	0.02
AUC for LPL (= freely circulating LPL activity)	15.2 ± 1.3	18.2 ± 1.4	0.10

Table 5.7.2
Means ± standard errors of postprandial apoCIII levels (adjusted for log fasting TGs) in patients and healthy individuals combined for genotypes of the apoCIII C_{1100} to T polymorphism (absence and presence of the T allele)

Adjusted apoCIII levels; time after oral fat load (mg/dl)	Absence of T allele CC (21)	Presence of T allele CT and TT (17)	p value from ANOVA
0 hours	157 ± 11.1	138 ± 9.2	0.15
3 hours	144 ± 9.6	125 ± 10.1	0.13
6 hours	142 ± 7.5	126 ± 8.9	0.14
12 hours	121 ± 7.2	105 ± 7.7	0.14

numbers in brackets = maximum number of individuals

Total apoCIII levels (adjusted for fasting TGs) decreased during the 12 hour postprandial period (Table 5.7.2). The mean apoCIII level at 12 hours was significantly lower ($p<0.001$) than the mean level at 0 hours, in the whole group, as well as in groups stratified for presence/absence of the T_{1100} allele ($p<0.001$), using the paired T-test. Adjusted mean apoCIII levels dropped by 29.8% and 31.4% for absence and presence of the T_{1100} allele, respectively. This downward trend in apoCIII levels was also observed in the healthy individuals, NTG patients and HTG patients separately and for both absence and presence of the T_{1100} allele (data not shown). This was statistically significant ($p<0.05$) except for the HTG patients, when divided into presence and absence of the T_{1100} allele. This suggests that whatever the mechanism that is mediating the decrease in total apoCIII levels, it is not associated with variation in linkage disequilibrium with the apoCIII gene. ApoCIII is known to shift towards newly synthesized TG-rich lipoproteins from HDL during postprandial lipaemia (Havel et al. 1973, discussed in Section 1.5.2) and during conversion of these lipoproteins to remnants, apoCIII transfers back to HDL from the remnants of TG-rich lipoproteins (Eisenberg et al. 1979). However the reduction in apoCIII levels can only be explained by the speculation that apoC's, including apoCIII, leave the plasma compartment together with the remnants formed during lipolysis (Nestel et al. 1982, Barr et al. 1985). Data was not available on the relative distribution of apoCIII between TG-rich lipoproteins and HDL, so the hypothesis that variation at the apoCIII locus influences this apoCIII distribution between different lipoproteins cannot be tested here. A possible underlying mechanism would be that variation in apoCIII affects the exclusion pressure of this apolipoprotein and hence the distribution of apoCIII between lipoproteins of differing surface pressures. The concept of exclusion and surface pressures has been discussed in relation to apoAIV in Section 1.5.3. Such a hypothesis appears feasible since a lower mean total apoCIII level in the T_{1100} carriers was associated with greater postprandial lipaemia and greater free circulating LPL activity (Table 5.7.1). The correlation coefficient for the interrelationship between the AUC for TGs (all lipoproteins) and the AUC for LPL was 0.28 for the whole postprandial sample (not significant) and similar for presence and absence of the T_{1100} allele (data not shown). As discussed in Sections 1.5.2 and 1.6.3, high plasma apoCIII levels are associated with HTG and with inhibition of lipolysis. At all four time points, total apoCIII levels were lower for T_{1100} carriers in the whole sample (Table 5.7.2), the healthy individuals and HTG patients (data not shown) compared to those without this

allele. However the trend was reversed in the NTG patients (data not shown), whether data was adjusted for fasting TGs or not. Confirmatory studies on independent samples are needed to ascertain the effect of genotypes of the C_{1100} to T polymorphism on total fasting and postprandial apoCIII levels, before any inferences can be drawn.

Many studies have shown associations between variation at the apoAI-CIII-AIV gene cluster and hyperlipidaemia (Rees et al. 1985, Hayden et al. 1987, Henderson et al. 1987, Aalto-Setala et al. 1992 and reviewed in Humphries et al. 1990, 1992). Of the known polymorphisms in this gene cluster, the recently discovered silent C_{1100} to T polymorphism, in the wobble position of amino acid 14 in exon 3 of the apoCIII gene was chosen (Xu et al. 1994), because of the relatively high rare allele frequency and because in two studies of healthy individuals and patients the T allele was associated with significantly higher TG levels (Xu et al. 1994, Chapter 3 of this thesis). The rare allele of the 3'-apoCIII-SstI polymorphism (S+) which has been associated with hypertriglyceridaemia (Rees et al. 1985, Henderson et al. 1987, Aalto-Setala et al. 1992) is always found on the same chromosome as the T allele of the apoCIII C_{1100} to T polymorphism (Xu et al. 1994). Haplotypes specified by polymorphisms in the apoCIII promoter region and the 3'apoCIII-SstI polymorphism confer either protection against or suspectibility to severe hypertriglyceridaemia (Dammerman et al. 1993). The significantly higher levels of postprandial triglycerides in both chylomicrons and the Sf 60-400 fraction observed in carriers of the T_{1100} allele is thus in accord with previous results on fasting samples. It suggests that the association of the T allele with higher postabsorptive triglyceride levels may be an indicator of worse postprandial lipaemia, which could be due to genetic variation, in linkage disequilibrium with the C_{1100} to T polymorphism, altering the level of expression of one of the apolipoproteins, with apoCIII being the most likely.

Elevated levels of plasma apoCIII could be partly responsible for the TG accumulation in plasma (Chivot et al. 1990), due in part to its inhibitory effect on LPL (Havel et al. 1970, Ginsberg et al. 1986), but recently Aalto-Selta et al (1992) demonstrated, in transgenic mice overexpressing apoCIII, a much reduced uptake of apoE-containing remnant particles via lipoprotein receptors, compared to control mice. This suggests that apoCIII could have a major effect postprandially via both reduced lipolysis and reduced clearance of TG-rich

lipoproteins, by interfering with functioning of LPL and apoE, respectively. The individuals with the T_{1100} allele had 20% higher AUCs for LPL than those without this allele ($p = 0.1$, Table 5.7.1). This might be explained, in part, by the greater postprandial lipaemia in these individuals, which would mean more circulating TG-rich lipoproteins for LPL to attach to. The non-parallel associations between the T_{1100} allele and lower apoCIII levels and greater postprandial lipaemia, after adjustment for fasting TGs, suggest that it is not the total postprandial apoCIII levels that are of primary importance. Data from other studies supports this hypothesis; in a recent study, the distribution of apoCIII between high density lipoproteins (HDLs) and non-HDLs was a stronger predictor than plasma apoCIII levels, of coronary atherosclerosis (Blankenhorn et al. 1990). The opposite trend of less pronounced lipaemia in carriers of the T allele in the whole group (Figure 5.7) when unadjusted TG levels are plotted against time elapsed since ingestion of the oral fat load suggests that variation (as yet unknown) associated with the C_{1100} to T polymorphism is modulating postprandial lipaemia in a fashion dependent on fasting TGs. This variation could be in the apoCIII promoter region affecting apoCIII production, or in the coding region affecting efficiency of apoCIII in inhibiting LPL, or stability of binding of apoCIII to lipoproteins affecting receptor mediated uptake of these lipoproteins via apoE.

Section 5.8 Association of common apoE alleles with postprandial lipaemia and possible underlying mechanisms

Because of small numbers, the combined sample was divided into those carrying and lacking the apoE2 allele (7 and 31 individuals respectively), and those carrying and lacking the apoE4 allele (9 and 30 individuals respectively) and examined in relation to AUCs for apoB-48- and apoB-100-containing lipoproteins. Table 5.8.1 shows means $\pm$ SEs of AUCs for apoB100-containing lipoproteins in presence and absence of the apoE2 allele. The mean AUC for large chylomicron remnants was 33% larger in apoE2 carriers but this difference did not reach statistical significance. Mean AUCs for small and large VLDLs, respectively, were 21% and 18% larger in apoE2 carriers. No statistically significant differences were observed with respect to presence or absence of the apoE4 allele (Table 5.8.2). In Figure 5.8.1, for apoB-100 levels in large VLDLs (an approximation of the number of these lipoprotein particles), data is plotted versus time since the oral fat load, in the individuals with or without the apoE2 allele; the AUC seen in those with the apoE2 allele is roughly 1.2

Table 5.8.1

Means ± standard errors of AUCs for apoB-containing lipoproteins in patients and healthy individuals combined versus presence and absence of the apoE2 allele.

AUCs; units = mg/l hours adjusted for log fasting TG	Absence of apoE2 (n=31) E3/3, E4/3 and E4/4	Presence of apoE2 (n=7) E2/3 and E2/4	p value from ANOVA
apoB-48 Sf 60-400 large chylomicron remnants	174 ± 16.8	231 ± 41.3	0.25
apoB-48 Sf 20-60 * small chylomicron remnants	44 (36.4 to 52.3)	43 (20.4 to 89.1)	0.18
apoB-100 Sf 60-400 large VLDLs	931 ± 64.4	1096 ± 120.4	0.09
apoB-100 Sf 20-60 small VLDLs	537 ± 45.5	648 ± 152.5	0.03

Table 5.8.2

Means ± standard errors of AUCs for apoB-containing lipoproteins in patients and healthy individuals combined versus presence and absence of the apoE4 allele.

AUCs; units = mg/l hours adjusted for log fasting TG	Absence of apoE4 (n=27) E2/3 and E3/3	Presence of apoE4 (n=9) E4/3 and E4/4	p value from ANOVA
apoB-48 Sf 60-400 large chylomicron remnants	176 ± 18.4	183 ± 21.3	0.94
apoB-48 Sf 20-60 * small chylomicron remnants	41 (31.9 to 51.5)	47 (34.5 to 65.4)	0.39
apoB-100 Sf 60-400 large VLDLs	915 ± 64.3	1022 ± 135.4	0.78
apoB-100 Sf 20-60 small VLDLs	537 ± 53.2	566 ± 104.9	0.71

* antilogs of $\log_{10}$ transformed mean and 95% confidence limits numbers in brackets = number of individuals

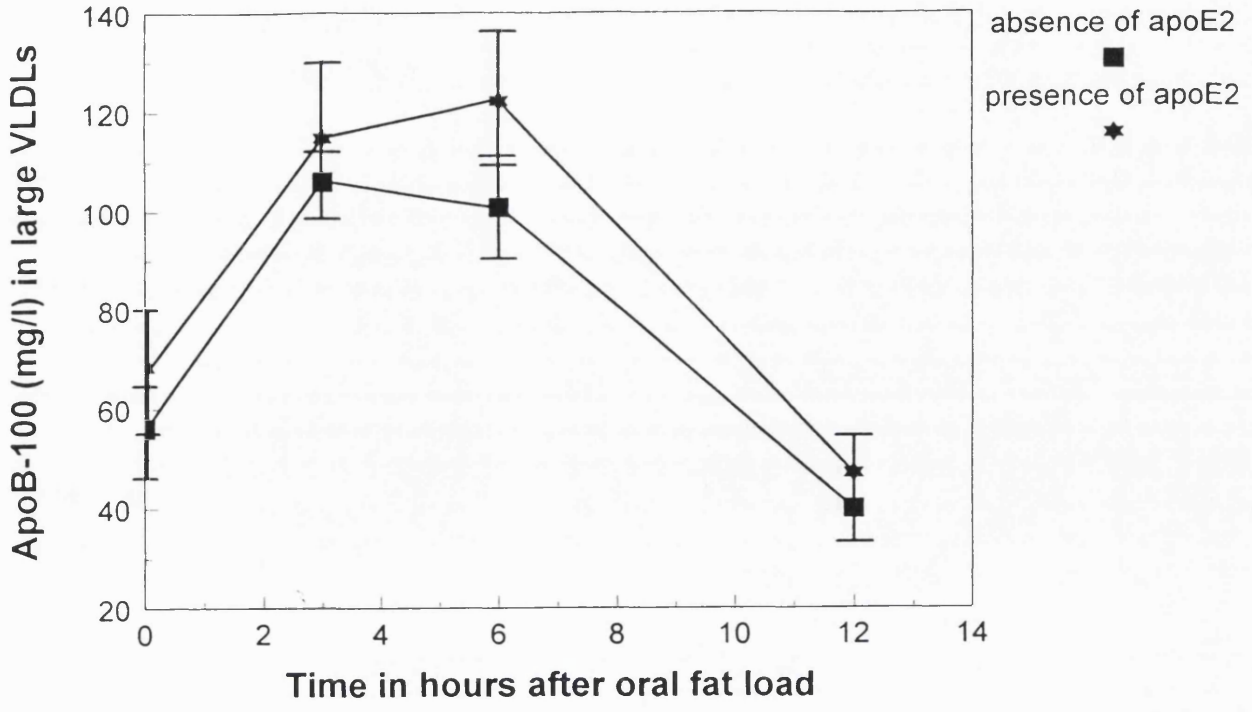


Figure 5.8.1

Means ± S.E.s of apoB-100 (mg/l) in Sf 60-400 lipoproteins (large VLDLs) versus time (in hours) since ingestion of the oral fat load, with the group divided into those carrying the apoE2 allele and those without the apoE2 allele. Data is shown for the combined group of healthy individuals and patients and is adjusted for fasting TGs.

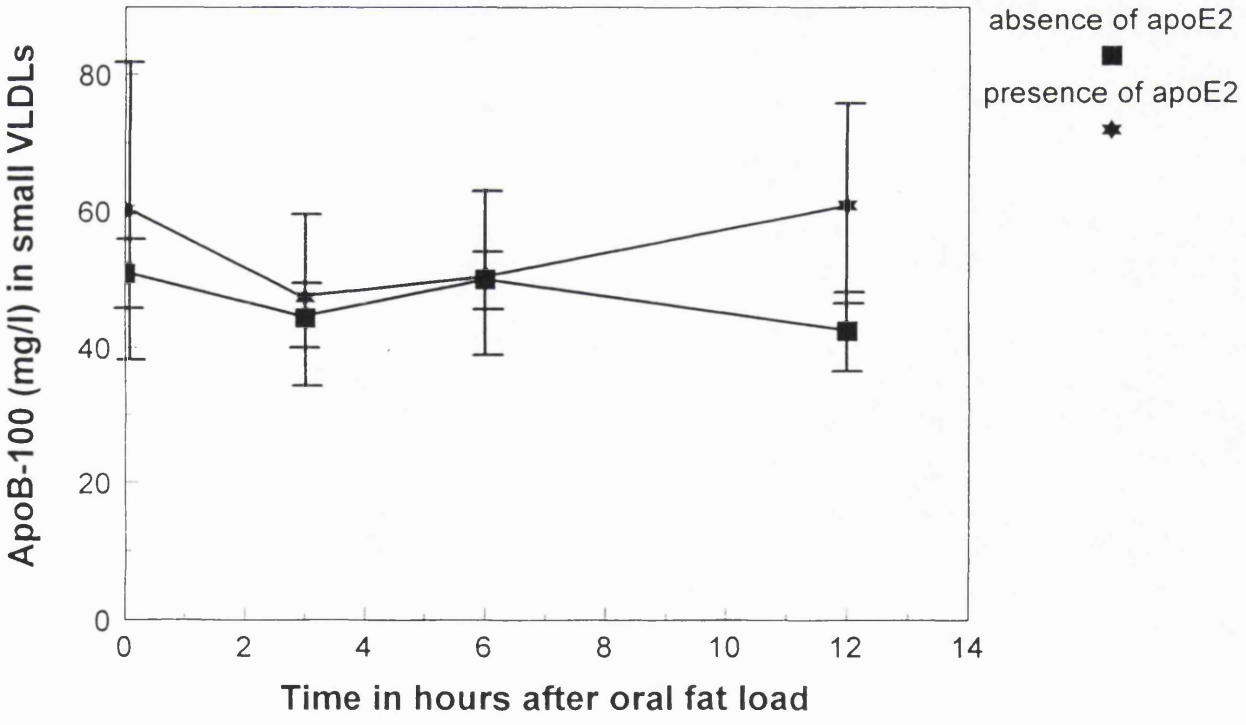


Figure 5.8.2

Means ± S.E.s of apoB-100 (mg/l) in Sf 20-60 lipoproteins (small VLDLs) versus time (in hours) since ingestion of the oral fat load, with the group divided into those carrying the apoE2 allele and those without the apoE2 allele. Data is shown for the combined group of healthy individuals and patients and is adjusted for fasting TGs.

times greater than that observed in those with all other apoE genotypes. In the early postprandial period as shown in Figure 5.8.1, the time course of large VLDLs was approximately parallel in both groups; however between 3 and 6 hours, in apoE2 carriers levels continued to rise while in individuals without this allele, levels were already falling. In the late postprandial period, levels dropped more quickly in apoE2 carriers, indicating that differences between the two groups were most pronounced in the mid-postprandial period.

The time course of apoB-100 levels in small VLDLs did not relate to apoE genotypes when stratified by presence or absence of the apoE2 allele (Table 5.8.3) despite the significant difference in AUC's ($p=0.03$) when individuals were stratified by apoE genotypes. Levels of apoB in small VLDLs fell in the first 3 hours, followed by a slight rise between 3 and 6 hours for both presence and absence of the apoE2 allele; however for carriers of the apoE2 allele, levels continued to rise between 6 and 12 hours while they fell in individuals lacking this allele. At all time points postprandially, except for 12 hours, apoB-48 levels in both small and large chylomicron remnants were higher in apoE2 carriers than in individuals without this allele (Table 5.8.3); this reached statistical significance ($p=0.03$) for large chylomicron remnants at 3 hours.

The trend towards larger AUCs for apoB-100-containing lipoproteins in carriers of the apoE2 alleles is in agreement with the suggestion of slower uptake of remnant lipoproteins; this was particularly striking for the Sf20-60 fraction, where from 6 to 12 hours postprandially, levels rise in apoE2 carriers but fall in non-carriers (Figure 5.8.2 and Table 5.8.3). Due to small sample size, it is not possible to demonstrate unequivocally at which stage of the postprandial period, the apoE2 allele is exerting its greatest effect on levels of either apoB-48- or apoB-100- containing lipoproteins. ApoE has been shown to be involved in uptake and catabolism of remnants of TG-rich lipoproteins, with apoE4 being catabolized fastest in-vivo (Gregg et al. 1986), despite in-vitro studies showing a similar binding of apoE3 and apoE4 to receptors (Weisgraber 1982); the most plausible explanation being the differential distribution of apoE3 and apoE4 amongst lipoprotein particles, with lipoproteins of lower density relatively enriched in apoE4 (Steinmetz et al 1989).

The grossly-diminshed receptor-binding activity of apoE2 relative to apoE3 and apoE4

Table 5.8.3
Means ± standard errors of time points for apoB-containing lipoproteins in patients and healthy individuals combined versus presence and absence of the apoE2 allele.

apoB in mg/l adjusted for log fasting TG	Time since oral fat load (in hours)	Absence of apoE2 (n=31) E3/3, E4/3 and E4/4	Presence of apoE2 (n=7) E2/3 and E2/4	p value from ANOVA
apoB-48 Sf 60-400	0	3.6 ± 0.8	5.0 ± 1.7	0.43
large chylomicron remnants	3	19.5 ± 1.5	29.2 ± 5.1	0.02
	6	22.2 ± 2.9	29.1 ± 5.7	0.28
	12	4.5 ± 1.2	3.2 ± 1.8	0.64
apoB-48 Sf 20-60	0	4.4 ± 0.6	5.8 ± 1.2	0.30
small chylomicron remnants	3	7.3 ± 0.7	8.4 ± 2.4	0.52
	6	5.1 ± 0.8	5.8 ± 1.2	0.69
	12	2.7 ± 0.7	2.6 ± 0.6	0.97
apoB-100 Sf 20-60	0	50.8 ± 5.2	60.2 ± 20.2	0.53
small VLDLs	3	44.3 ± 4.3	47.3 ± 11.9	0.78
	6	50.0 ± 4.5	50.7 ± 11.0	0.95
	12	42.4 ± 4.9	60.9 ± 15.0	0.15

numbers in brackets = number of individuals

is well documented (Schneider 1981, Weisgraber 1982). Boerwinkle et al. (1994) measured multiple parameters of postprandial response in 397 individuals participating in the Atherosclerosis Risk in Communities Study. For each of the common apoE genotypes, E2/3, E3/3 and E4/3, postprandial TGs and retinyl palmitate (which gives a measure of intestinally dervived chylomicrons and their remnants) levels were measured. The profile of postprandial TG levels, adjusted for fasting TGs, was not different among apoE genotypes. However the profile of adjusted retinyl palmitate for genotype E2/3 from 4 to 8 hours postprandially was significantly different from the other apoE genotypes. During this time, for E3/3 and E4/3 there was a small rise but for E2/3, there was a more abrupt rise from 823μg/dl to 1463μg/dl; about 33% higher than for other apoE genotypes at 8 hours postprandially. This data from Boerwinkle et al. (1994) indicates that the final step of the postprandial remnant pathway (their removal from the circulation) is delayed in E2/3 individuals, most probably due to the diminshed affinity of apoE2 for receptors (Schneider 1981, Weisgraber 1982).

Section 5.9 Association of genotypes of the LPL-HindIII polymorphism with postprandial lipaemia and possible underlying mechanisms

AUCs for postprandial (freely circulating) LPL activity and levels of fasting free fatty acids (FFAs) were significantly higher in those with the genotype H+H+ of the HindIII polymorphism compared to carriers of the rare allele (H-), as shown in Table 5.9. Individuals homozygous for the H+ allele had 21.7% higher AUCs for LPL activity and 23.6% higher levels of fasting FFAs. When the three groups; healthy individuals, NTG patients and HTG patients were analyzed separately, the genotype H+H+ was associated with 11.4% lower, 32% higher and 19.9% higher mean AUCs for LPL activity in the healthy individuals, NTG patients and HTG patients, respectively (not significant). The healthy individuals and NTG patients with this genotype had higher mean free fatty acid levels (data not shown), with the converse applying to the HTG patients ($p=0.02$ for the healthy individuals). The H+H+ genotype was associated with greater total postprandial lipaemia in the NTG patients but not in the other two groups; this was not significant (data not shown). These conflicting results amongst the three groups is likely to be due to the existence of genetic variation that is in linkage disequilibrium with the H+ allele; stratification of individuals into groups lacking and carrying the H- allele will not necessarily stratify them into groups carrying and lacking the variant. In Chapter 6 of this thesis, a

Table 5.9.

Means ± standard errors for selected traits in patients and healthy individuals combined, by genotypes of the HindIII polymorphism in the lipoprotein lipase gene.

Trait adjusted for log fasting TG	absence of H- allele H+H+ (n=21)	Presence of H- allele H+H- (n=19)	p value from ANOVA
AUC for LPL (milliunits hours)	18.5 ± 1.7	15.2 ± 0.9	0.04
levels of fasting free fatty acids	680 ± 53.3	550 ± 32.5	0.05
AUC TGs; all lipoproteins *	32.8 (30.0 - 35.8)	33.7 (31.3 - 36.3)	0.64

* antilogs of $\log_{10}$ transformed mean and 95% confidence limits
numbers in brackets = number of individuals

potential causative genetic variation, the N291S substitution is found associated with the H+ allele, but six of the seven Ser291 carriers in the postprandial sample (six are patients) also carry the H- allele.

Figure 5.9.1 shows the time course of freely circulating LPL activity in milliunits (μU/ml) during the postprandial period in individuals with or without the H- allele. Data for time points was not adjusted for fasting TGs because such adjustment had no effect on means. In carriers of the H- allele, plasma LPL activity is lower throughout the entire 12 hour period. The time course is similar during the first 3 hours but rises more slowly in H- carriers between 3 and 6 hours, reaching a lower peak (17% lower, N/S). In the latter half of the postprandial period, plasma LPL activity falls along a similar course in both groups, with the difference in means reaching statistical significance ($p=0.03$) at 9 and 12 hours. Total postprandial TG levels, adjusted for fasting TGs, were almost identical in those with and without the H- allele at all time points throughout the postprandial period (Figure 5.9.2).

The binding of LPL to proteoglycans such as heparin is thought to be weakened by local accumulation of FFAs arising from the LPL-mediated hydrolysis of TGs (Saxena et al. 1989, Olivecrona et al. 1990, Peterson et al. 1990); hence excess FFAs released from the LPL-mediated metabolism of TG-rich lipoproteins exert feed-back control of lipolysis. The high degree of parallelism and correlation between total postprandial LPL activity (=AUCs for LPL) and AUCs for 18:2 FFAs ($p<0.05$) in this postprandial sample (Karpe et al. 1992) supports the hypothesis of the proposed fatty acid feed-back control system of LPL during physiological conditions in humans. Since levels of FFAs did not change much throughout the entire postprandial period in this sample [<5% in the healthy individuals and patients combined (Karpe et al. 1992)], levels of fasting FFAs are a good predictor of what happens to FFA levels during the postprandial period. The significantly higher total postprandial LPL activity (measure of activity not associated with the endothelium i.e. in the capillary lumen) and higher fasting FFAs over the entire postprandial period, in those with the H+H+ genotype in this study suggests that the LPL enzyme coded for by the H+ allele of the HindIII polymorphism is more suspectible to dissociation from the endothelium brought about by accumulation of FFAs. Recent studies have reported that LPL increases uptake of

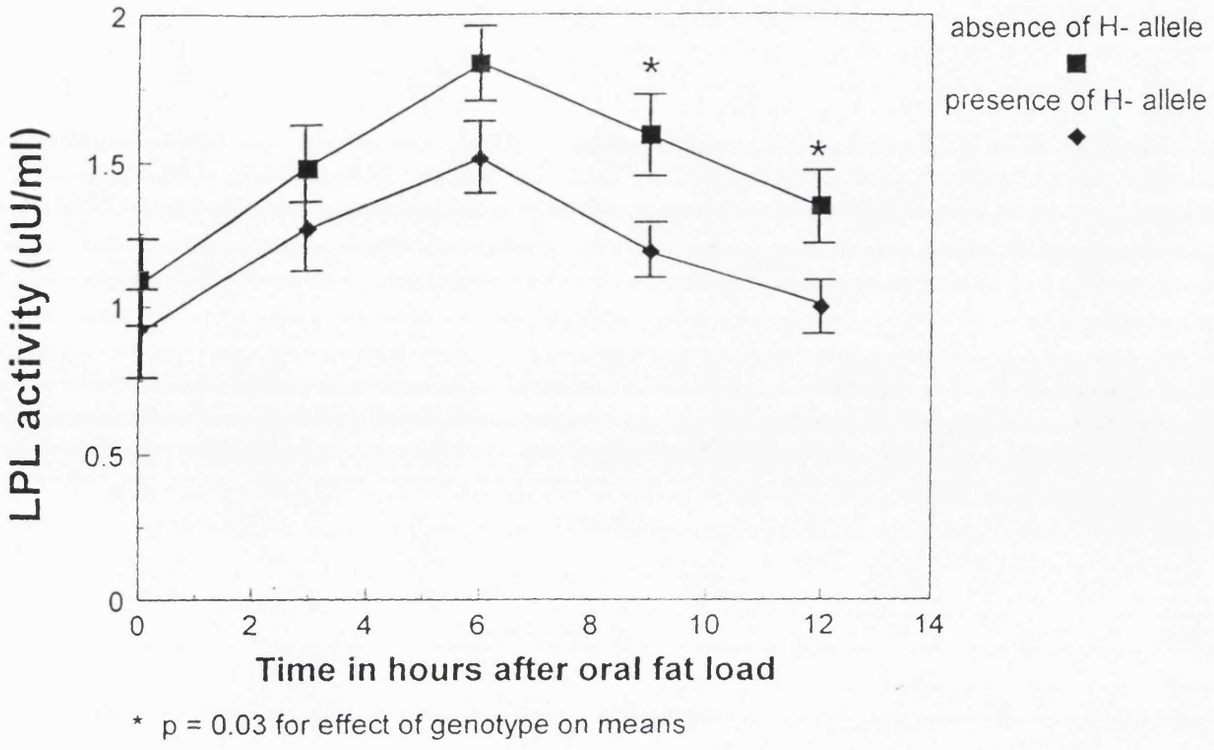


Figure 5.9.1

Means ± standard errors of postprandial LPL activity in microunits per ml versus time (in hours) since ingestion of the oral fat load, with the group divided into those homozygous for the H+ allele (absence of H- allele) and those carrying at least one copy of the H- allele of the HindIII polymorphism in the LPL gene. Data is not adjusted for fasting TGs becasuse such adjustment had no effect on levels, and is shown for the combined group of healthy individuals and patients.

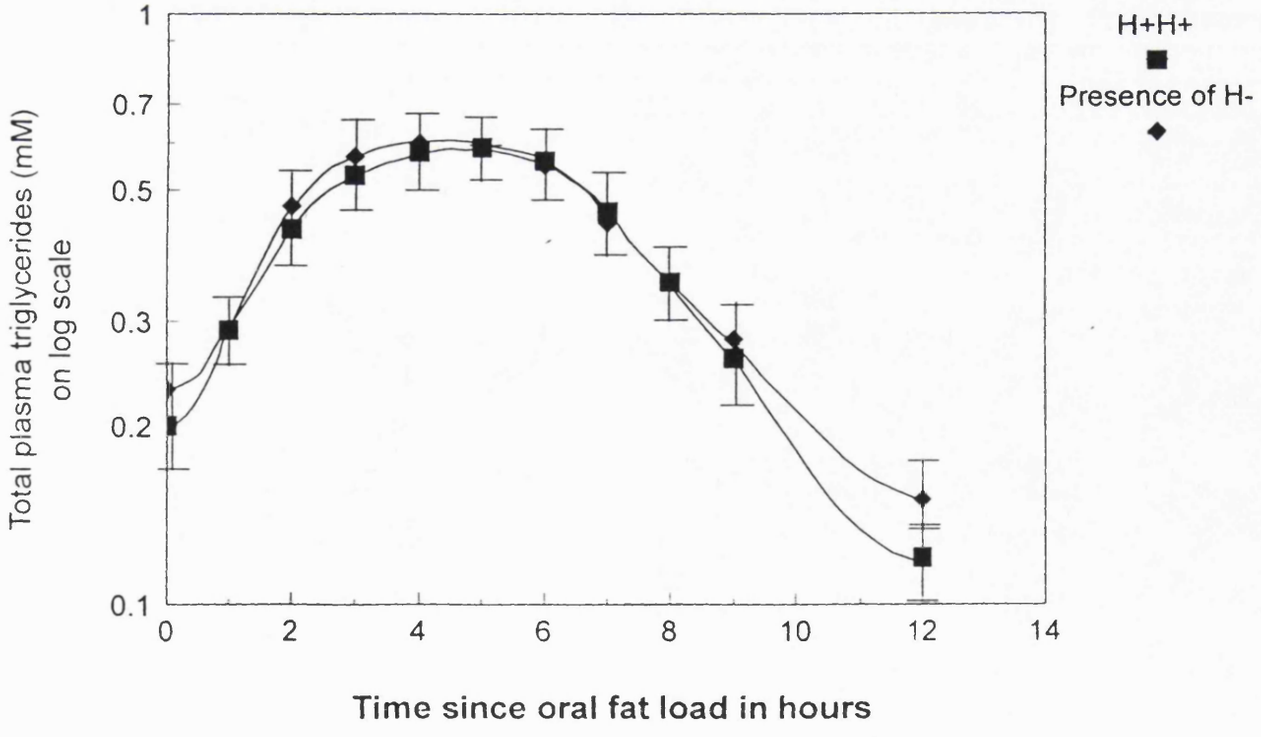


Figure 5.9.2

Means ± standard errors of total plasma TGs (log_{10} scale) in mM, adjusted for fasting TGs versus time (in hours) since ingestion of the oral fat load, with the group divided into those homozygous for the H+ allele and those carrying at least one copy of the H- allele of the HindIII polymorphism in the LPL gene. Data is shown for the combined group of healthy individuals and patients.

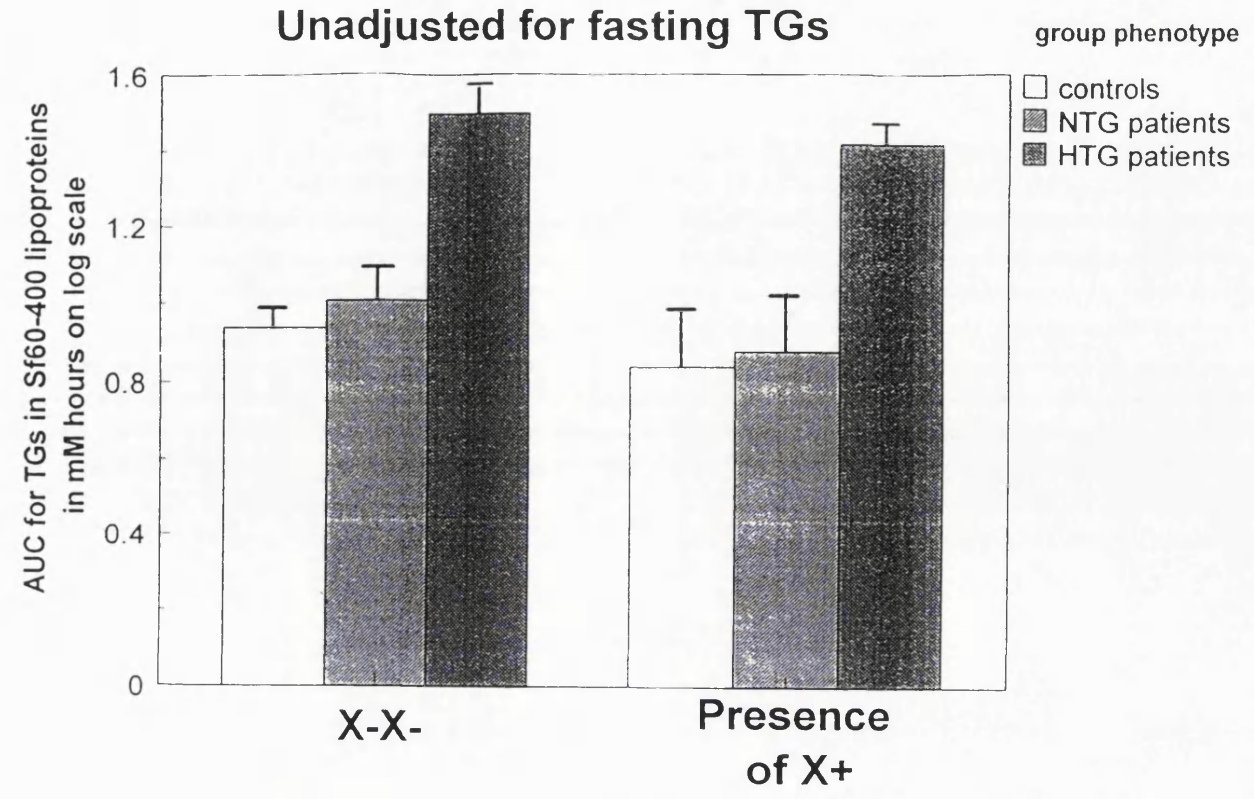
remnant lipoproteins by hepatic LRP (LDL-receptor related protein), which is thought to be independent of TG hydrolysis by LPL (Beisiegel et al. 1991) and that, in HepG2 cells, such uptake leads to reduced secretion of TG-rich particles (Williams et al. 1991). LPL with a higher affinity for lipoproteins or for heparin-like proteoglycans (coded for by the H- allele) might cause greater LPL-mediated binding of lipoproteins to LRP and thus increased uptake of LPL-lipoprotein complexes by LRP. LPL has been suggested to be a ligand for LRP (Beisiegel et al. 1991, Saxena et al. 1991 and discussed in Section 1.8.1). This might explain the lower freely circulating LPL activity postprandially in carriers of the H- allele, as compared to those without this allele. Under this hypothesis, this changed affinity for LRP would be due to sequence variation in linkage disequilibrium with the polymorphic HindIII site that affects the binding of LPL to lipoproteins and the susceptibility of this binding to local accumulation of FFAs postprandially. Screening, sequencing and impact of such variation on fasting and postprandial lipids is described in Chapter 6 of this thesis.

Section 5.10 Interactions between genotypes and group phenotype in modulating postprandial lipaemia

Because of the small sample size, the forty-one individuals (16 NTG patients, 14 HTG patients and 11 healthy individuals) were pooled and group phenotype (healthy individuals, NTG patients and HTG patients) was included as the covariate in analysis of variance (ANOVA) to test the hypothesis that there is interaction between genotypes and group phenotype in modulating magnitude of postprandial response, after removing variation attributed to level of fasting TGs. This ANOVA was only carried out for lipid traits where genotype had a significant impact. The covariate was chosen because these three groups were previously demonstrated to differ greatly with respect to magnitude of postprandial lipaemia (Karpe et al. 1992, 1993a, 1993b).

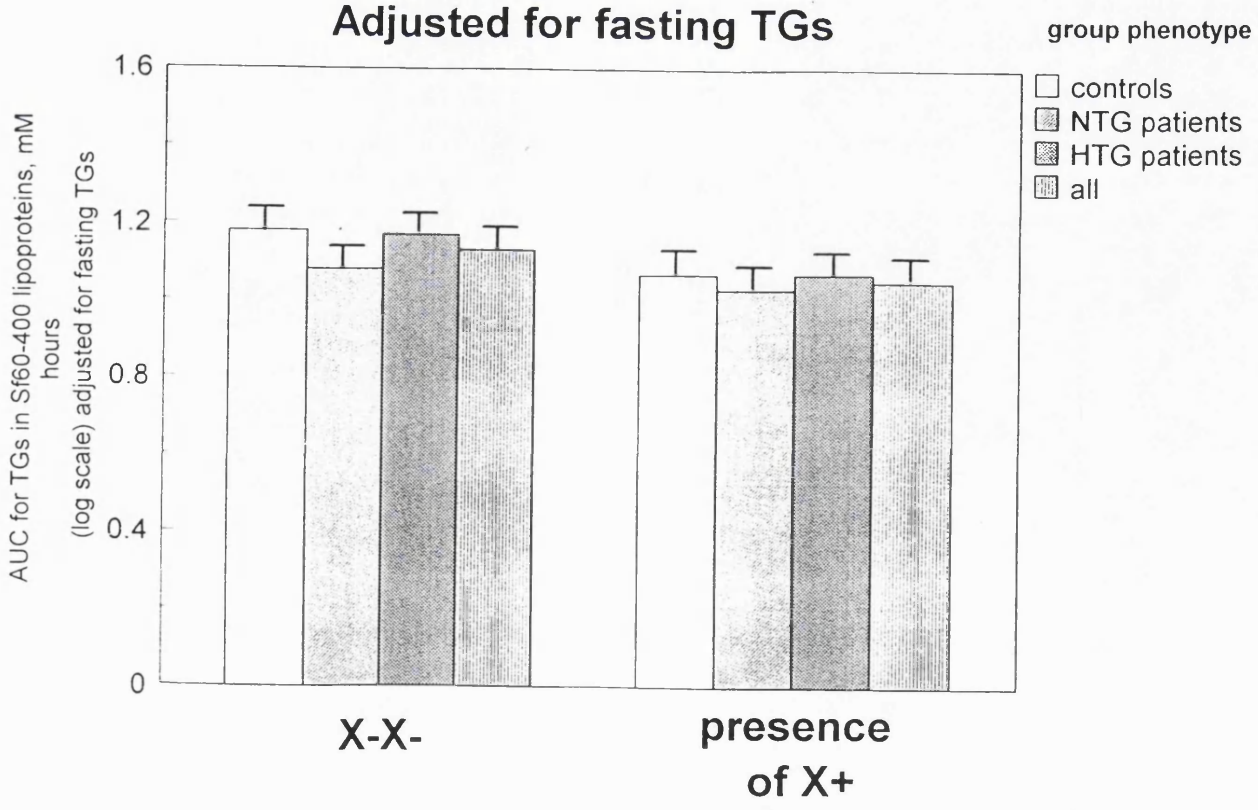
For some of the postprandial lipid traits there was evidence for interaction between genotype and group phenotype, because either the magnitude or the direction of the effect associated with genotypes was different amongst the three groups of individuals. Adjusting the data for fasting TGs removed much of this interaction and Figure 5.10.1 shows an example of this; the top part shows unadjusted AUCs for TGs in large chylomicron remnants and VLDLs by presence/absence of the X+ allele of the apoB-XbaI polymorphism for each

Figure 5.10.1.



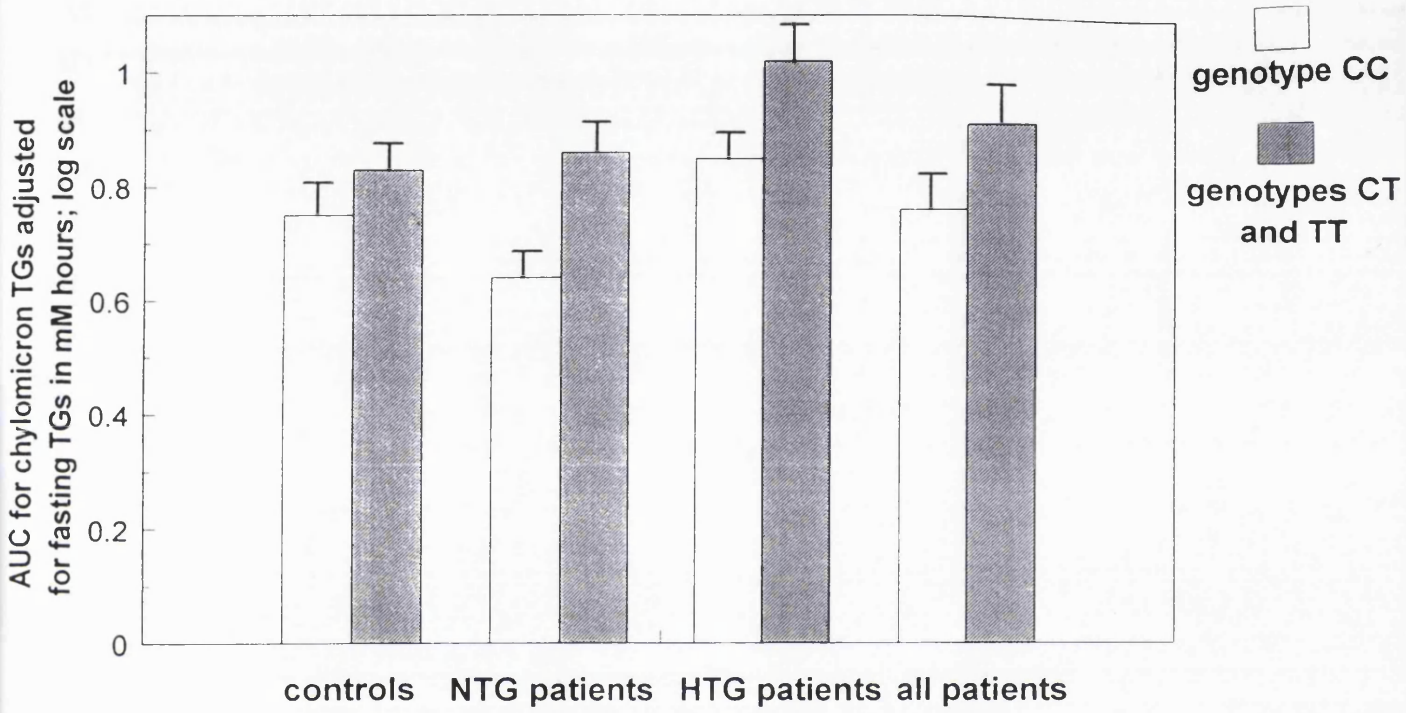
Interaction between group phenotype and genotypes; p<0.0001

Means ± standard errors of AUCs for TGs in large chylomicron remnants and large VLDLs (above) and adjusted for fasting TGs (below), with groups divided into those homozygous for the X- allele and those carrying at least one copy of the X+ allele of the apoB-XbaI polymorphism, for each of the three group phenotypes; healthy individuals, NTG patients and HTG patients. Interaction between group phenotype and genotypes is highly significant for the unadjusted data ($p < 0.0001$) but disappears when data is adjusted for fasting TGs.



Interaction between group phenotype and genotypes; not significant

Figure 5.10.2



Interaction between group phenotype and genotypes
(i) controls, NTG patients and HTG patients; p = 0.03
(ii) controls, all patients; not significant

Means ± standard errors of AUCs for chylomicron triglycerides adjusted for fasting TGs, for each of the four group phenotypes; healthy individuals, NTG patients, HTG patients and all patients. For each group, means ± standard errors are shown for those homozygous for the C allele and those carrying at least one T allele of the apoCIII-C_{1100} to T polymorphism. Interaction between group phenotype and genotypes is significant when NTG and HTG patients are considered as separate groups, but disappears when all patients are combined.

of the three groups, where interaction between genotypes and groups is highly significant ($p<0.0001$). When the data is adjusted for fasting triglycerides (bottom part of Figure 5.10.1), interaction between genotypes and groups is no longer significant. However there were four instances where there was statistically significant evidence ($p<0.05$) for interaction between genotypes and group phenotype even after adjustment for fasting TGs. These were between the apoB signal peptide polymorphism and the AUC for large VLDLs, the apoB-X+ allele and the AUC for total TGs, the apoCIII-T_{1100} allele and the AUC for chylomicron TGs and the apoE2 allele and the AUC for small VLDLs. For the apoB-signal peptide polymorphism, this was due to a negligible effect of genotypes on the trait in the NTG patients and a large effect in the HTG patients compared to the healthy individuals. For the apoB-XbaI polymorphism, this was due to a larger effect of the trait associated with genotypes in the healthy individuals than in either of the two patient groups. For apoCIII, this was due to a larger effect on the trait associated with genotypes in the two patient groups compared to the healthy individuals. For apoCIII, when the NTG and HTG patients were combined into a single group and compared to the healthy indivduals, group phenotype was no longer associated with genotype differences on postprandial lipaemia ($p>0.05$ for interaction). This is demonstrated in Figure 5.10.2 for AUCs-chylomicron TGs (adjusted for fasting TGs) and presence/absence of the apoCIII-T_{1100} allele. For apoE, this was due to the opposing direction of effect associated with the apoE2 allele in healthy individuals and HTG patients (there were no apoE2 carriers in the NTG patient group).

Section 5.11 Overall Discussion of impact of common genetic variation on postprandial lipaemia.

In this pilot study, associations between common genetic variation and postprandial lipaemia in a group of 41 patients and healthy individuals were examined. Because the statistical power of this study was limited, only large effects of genotypes on lipid traits were detectable. Variation in the apoB, apoCIII, apoE and LPL genes is demonstrated to have a moderate impact on magnitude of postprandial lipaemia, after removing variability in postprandial lipaemia attributable to fasting TGs. This study has demonstrated the feasibility of using small numbers of well characterized subjects to investigate effects of genetic variation of moderate size on different aspects of postprandial metabolism. Variation at the apoB signal peptide region appears to influence production of TG-rich lipoproteins. Lipolysis

of these lipoproteins seems to be modulated by genetic variation in the apoCIII and LPL genes. Catabolism of remnants of TG-rich lipoproteins might be influenced by genetic variation in linkage disequilibrium with the apoB-XbaI polymorphism and by the common apoE alleles. This confirms and extends mechanism of associations with genotypes using fasting lipids and lipoproteins. The two questions that need to be answered are whether the impact of common genetic variation on postprandial lipaemia would be confirmed in larger samples (n=50 or more) of patients with atherosclerosis or diabetes and healthy individuals, separately and whether effects of common genetic variation on postprandial lipaemia are modulated by genetic or environmental factors. Examples of genetic factors include mutations in the LDL-receptor causing familial hypercholesterolaemia, or mutations in LPL or apoCII genes causing Type I hyperlipoproteinaemia. Examples of environmental factors include diet (eg. changing composition of the administered oral load), gender, obesity and exercise. The eventual aim is to identify causative mutations, in linkage disequilibrium with the apoB-XbaI, apoCIII C_{1100} to T and LPL-HindIII polymorphisms, none of which alter the amino acid sequence of the gene products, that themselves underlie aberrant metabolism of postprandial TG-rich lipoproteins and their remnants.

Previously in this postprandial sample, small chylomicron remnants were found to relate significantly and postively to progression of coronary atherosclerosis suggesting that these lipoproteins are implicated in progression of atherosclerosis (Karpe et al. 1994). The gene polymorphism which had the largest effect on levels of small chylomicron remnants was the apoB-XbaI polymorphism, however this effect did not reach statistical significance (Table 5.6.1). It is possible that in a larger group of patients a significant association might be found between this or other polymorphisms and levels of these remnant lipoproteins. Larger patient samples will be needed to identify the common genetic variation that determines plasma levels of these remnants and therefore progression of atherosclerosis. When such causative mutations are identified, these may be useful, in conjunction with elevated fasting lipids, in identifying those individuals, at risk of atherosclerosis due to aberrant postprandial lipid metabolism. Other candidate genes could also be investigated in relation to postprandial lipid metabolism; examples are the genes encoding CETP (cholesteryl ester transfer protein), LCAT (lecithin:cholesterol acyltransferase), hepatic lipase and MTTP (microsomal TG transfer protein). The genes for all four proteins have already been cloned

(McLean et al. 1986, Stahnke et al. 1987, Agellon et al. 1990, Sharp et al. 1993) and mutations found in all four genes (e.g. Hegele et al. 1993, Ikewaki et al. 1993, Klein et al. 1993, Shoulders et al. 1993). As discussed in Sections 1.7.2 and 1.7.4, CETP, LCAT and hepatic lipase all have a modulating effect on the HDL fraction, and hence it is conviceable that mutations in any of these proteins will have an effect on postprandial lipoprotein metabolism. MTTP deficiency, as discussed in Section 1.8.3, underlies some cases of abetalipoproteinaemia (Sharp et al. 1993), which is characterized by the absence of detectable circulating apoB-containing lipoproteins. MTTP deficiency has not been investigated in relation to postprandial lipoprotein metabolism, to date but it is conviceable that due to absence of apoB-containing lipoprotiens, differences will be observed postprandium. Larger samples could also be investigated with regard to associations between common genetic polymorphisms at the apoB, apoAI, apoCIII and LPL genes, with a limited number of time points measured (e.g. 3.5 and 8 hours after the oral fat load, as in Boerwinkle et al. 1994) for parameters of postprandial lipaemia. The rationale for selecting these two time points (Boerwinkle et al. 1994) was to obtain basic information about the shape of the postprandial curve for selected, further analyses and to include a late postprandial sample which has been shown to discrimate strongly between patients and healthy individuals (Patsch et al. 1992).

CHAPTER 6 - THE SEARCH FOR CAUSATIVE GENETIC VARIATION IN LINKAGE DISEQUILIBRIUM WITH ALLELES OF THE HINDIII POLYMORPHISM IN THE LIPOPROTEIN LIPASE GENE.

Section 6.1. Summary

A search was made, using PCR and Single Strand Conformation Polymorphism, in exons of the LPL gene to identify sequence variation, in linkage disequilibrium with alleles of the HindIII polymorphism that would explain, at least in part, the associations seen between fasting lipids and lipoproteins (Chapter 3), postprandial traits (Chapter 5), variances of lipid traits and correlations between pairs of metabolically related lipid traits (this chapter), and genotypes of the HindIII polymorphism. An A_{1127} to G substitution in exon 6 was found, which changes Asn291 to Ser. The frequency of Ser291 was 0.035 in the combined Swedish sample, being similar in the healthy individuals and patients, with Ser291 being found on the same chromosome as the H+ allele of the HindIII polymorphism. The effects of N291S on fasting and postprandial lipid and lipoprotein traits were evaluated, but found to be smaller than those associated with genotypes of the HindIII polymorphism. Thus the inference is that N291S is only explaining some of the associations seen with the HindIII polymorphism and that there is other, as yet undiscovered, variation in the LPL gene that explains the rest of these associations.

Section 6.2. Rationale of Study

Since the HindIII polymorphism in the LPL gene is within an intron, it cannot be the direct cause of the alterations in plasma TG levels and both TG and cholesterol in the VLDL fraction, associated with genotypes, in the Swedish healthy individuals (Tables 3.5.8 and 3.5.9). Thus it must be in linkage disequilibrium with other variation in the LPL gene that itself changes the amino acid sequence of LPL and underlies the raised TG and VLDL levels seen in association with the H+ allele. Conversely, the H- allele could be in linkage disequilibrium with a variant that predisposes to lower TG levels. This two-sided hypothesis was used as the starting point for the search, using PCR and Single Strand Conformation Polymorphism (SSCP), of exons for genetic variation associated with either the H+ or H- alleles that changes the amino acid sequence. The aim was to evaluate the effect of such variation on fasting and postprandial lipids and lipoproteins, and to ascertain if such variation

would explain some or all of the effects associated with the HindIII polymorphism.

Section 6.3 Setting the Scene; Statistical comparsions for interrelationships between plasma traits in presence and absence of the H- allele of the HindIII polymorphism.

In the Swedish healthy individuals and patients, the standard deviations were smaller for log_{10}-transformed values of VLDL-cholesterol, TG and VLDL-TG in those carrying at least one H- allele of the HindIII polymorphism than in those homozygous for H+. The F-test was applied to test the null hypothesis that there is no difference between variances obtained with genotype H+H+ and those obtained when the genotypes H+H- and H-H- were combined. Results of the F-test used to compare variances in the presence and absence of the H- allele are shown in Table 6.3.1.

Table 6.3.1 F-test for comparing variances between groups of individuals carrying and lacking the H- allele, respectively.

	Healthy Individuals			
Trait	Variance for H+H+ (n=52)	Variance for presence of H- (n=35)	F-value	Significance of F-value
Log_{10}-VLDL cholesterol	0.096	0.058	1.67	$p < 0.1$
Log_{10}-TG	0.058	0.026	2.25	$p < 0.05$
Log_{10}-VLDL TG	0.109	0.063	1.74	$p < 0.1$
	Patients			
	Variance for H+H+ (n=49)	Variance for presence of H- (n=37)	F-value	Significance of F-value
Log_{10}-VLDL cholesterol	0.090	0.068	1.33	$p < 0.2$
Log_{10}-TG	0.063	0.040	1.56	$p < 0.1$
Log_{10}-VLDL TG	0.096	0.063	1.54	$p < 0.1$

From the results of the F-test and the smaller variances observed in both healthy

individuals and patients carrying at least one H- allele compared to those observed in individuals homozygous for H+, it was hypothesized that genotypes at the LPL gene loci may have an effect on the interrelationship between LPL activity and lipid traits, as well as between some of the other metabolically related plasma lipid traits. The assay system, using a lipid soluble substrate (Nilsson-Ehle et al. 1977) would reveal defects in both LPL catalytic activity and lipid-binding ability. Therefore altered inter-relationships between plasma traits might be due to either altered catalytic activity or altered binding affinity of LPL for TG-rich lipoproteins or alterations in both of these. The enzyme encoded by the H- allele would mediate a tighter interrelationship between traits, due to genetic variation in linkage disequilibrium with this allele causing a change in LPL primary sequence and an increased affinity of LPL for substrate TG-rich lipoproteins. The converse could also apply i.e. variation in linkage disequilibrium with the H+ allele would lead to decreased affinity of LPL for TG-rich lipoproteins. Additional evidence supporting this hypothesis comes from the results in Chapter 5 of this thesis, where the total postprandial LPL activity and fasting free fatty acids (FFAs) over the entire postprandial period was significantly higher in those with the H+H+ genotype than in carriers of the H- allele (Section 5.9). Those results suggested that the LPL enzyme coded for by the H+ allele of the HindIII polymorphism is more suspectible to dissociation brought about by accumulation of FFAs.

Relationships between pairs of variables were therefore investigated, using correlation analysis, in whole groups and haplotype combinations of the HindIII and Serine447 to Stop polymorphisms. The square of the correlation coefficient (R^2, the coefficient of determination) is the measure of the proportion of variation in one trait that is determined by variation in the other trait. The healthy individuals (Table 6.3.2) and patients (Table 6.3.3) were analyzed separately. The R^2 values were similar between the group carrying the H- allele but without the Stop447 allele and the group carrying both H- and Stop447 alleles. Therefore these two groups carrying the H- allele were combined and compared to the group homozygous for H+. The results for R^2x100 (= percentage of variation in one trait determined by variation in the other trait) from the healthy individuals and patients were similar, in that for three out of four pairs of traits, R^2x100 was greater in the presence of the H- allele in both groups. Therefore the two groups were combined and results are shown in Table 6.3.4. The presence of the H- allele, which was associated with smaller variances

Table 6.3.2 Coefficients of Determination (R^2x100) for pairwise comparsions of serum traits for combinations of genotypes of the LPL-HindIII polymorphism, in the healthy individuals.

Traits	Healthy Individuals					
	All	H+H+ Ser/Ser	H+H- and H-H- combined			Significance. of difference between R^2 values for H+H+ and (H+H- & H-H- combined)
			Ser/Ser	Presence of Stop	All H-carriers	
LPL activity and TG §	17.8 **	13.1	23.2	35.8	22.5 *	N/S
LPL activity and HDL-cholesterol	8.0	4.0	15.3	19.9	14.4	N/S
HDL-cholesterol and VLDL-cholesterol §	16.1 **	4.9	13.2	29.3	30.1 **	N/S
HDL-cholesterol and TG §	9.1 *	10.0	26.7 *	50.8	16.9 *	N/S

§ correlations carried out on $\log_{10}$-transformed data.
* $p < 0.01$, ** $p < 0.001$
N/S = not significant

Numbers of individuals with LPL activity data was 64 while the numbers of individuals with data on lipid traits was 90. All correlations were carried out on unadjusted data, to give an estimate of the in-vivo stituation with regard to interactions between traits.

Table 6.3.3 Coefficients of Determination (R^2x100) for pairwise comparsions of serum traits for combinations of genotypes of the LPL-HindIII polymorphism, in the patients.

Traits	Patients					
	All	H+H+ Ser/Ser	H+H- and H-H- combined			Significance of difference between R^2 values for H+H+ and (H+H- & H-H- combined)
			Ser/Ser	Presence of Stop	All H- carriers	
LPL activity and TG §	9.9	1.9	22.7	25.3	18.5	N/S
LPL activity and HDL-cholesterol	16.9	25.5	8.7	10.4	6.2	N/S
HDL-cholesterol and VLDL-cholesterol §	24.5 **	17.9 *	31.6 *	63.1	37.9 *	N/S
HDL-cholesterol and TG §	15.7 **	11.0	21.9	53.0	27.2 **	N/S

§ correlations carried out on $\log_{10}$-transformed data.
* $p < 0.01$, ** $p < 0.001$.

N/S = not significant

Numbers of individuals with LPL activity data was 36 while the numbers of individuals with data on lipid traits was 85. All correlations were carried out on unadjusted data, to give an estimate of the in-vivo stituation with regard to interactions between traits.

Table 6.3.4 Coefficients of Determination (R^2x100) for pairwise comparsions of serum traits for combinations of genotypes of the LPL-HindIII polymorphism, in the combined sample of healthy individuals and patients.

Traits	Healthy individuals and patients combined					
	All	H+H+ Ser/Ser	H+H- and H-H- combined			Significance of difference in R^2 values between H+H+ and (H+H- & H-H- combined)
			Ser/Ser	Presence of Stop	All H- carriers	
LPL activity and TG §	21.9 **	11.7 *	29.6 *	58.2	31.8 **	N/S
LPL activity and HDL-cholesterol	15.6 **	10.4	22.1	33.3	22.5 **	N/S
HDL-cholesterol and VLDL-cholesterol §	31.2 **	19.6 **	51.6 **	60.2 **	51.7 **	$p < 0.01$
HDL-cholesterol and TG §	24.3 **	15.3 **	42.1 **	48.5 **	42.1 **	$p < 0.05$

§ correlations carried out on $\log_{10}$-transformed data.
* $p < 0.01$, ** $p < 0.001$.

N/S = not significant
Numbers of individuals with LPL activity data was 100 while the numbers of individuals with data on lipid traits was 175. All correlations were carried out on unadjusted data, to give an estimate of the in-vivo stituation with regard to interactions between traits. Estimates for the healthy individuals and patients were similar so the data for the combined group are presented here.

in both the healthy individuals and the patients (Table 6.3.1), was associated with larger coefficients of determination, for all pairwise combinations made, with the exception of LPL activity and HDL-cholesterol in the patients, probably attributable both to the small numbers of patients who had data on LPL activity and to factors such as hyperlipidaemia that disrupt the effect of variation associated with alleles of the HindIII polymorphism on interrelationships between LPL activity and HDL-cholesterol. In the combined sample, correlation analysis was also carried out for the HDL2b and HDL3b subclasses instead of HDL-cholesterol for presence and absence of the H- allele. Results are shown in Table 6.3.5; results were similar for presence/absence of the H- allele when either HDL2b or HDL3b was used as one of the two interdependent variables in the correlation analysis. This indicates that whatever the mechanism underlying the stronger interrelationships (larger coefficents of determination) in the presence of the H- allele is, it does not distinguish between the HDL subclasses.

In the combined sample, coefficients of determination were significantly different in groups carrying and lacking the H- allele, for HDL-cholesterol and either VLDL-cholesterol or TG, probably due to the larger number of individuals analyzed. Correlation plots for TG (log_{10} scale) plotted against HDL-cholesterol are shown in Figure 6.3.1 (for absence of H- allele) and Figure 6.3.2 (for presence of H- allele). The slope is steeper and the 95% confidence limits (indicated by the shaded area around the regression line) are smaller in the presence of the H- allele. These results implicate that the H- allele rather than the Stop447 allele is mediating the stronger affinity of LPL for TG-rich lipoproteins than the enzyme encoded by the H+Serine447 allele. This might be caused by sequence variation in the LPL gene segment coding for the interfacial lipid-binding region located to exon 4 (Deeb & Peng 1989), that could be in linkage disequilibrium with alleles of the HindIII polymorphism. This variation could be in linkage disequilibrium with the H+ allele, leading to decreased affinity for TG-rich lipoproteins or be in linkage disequilibrium with the H- allele and cause increased affinity for these lipoproteins.

Therefore the exons in the LPL gene were searched for causative genetic variation, in linkage disequilibrium with the HindIII polymorphism and that could underlie the postulated altered affinity of LPL for substrate TG-rich lipoproteins.

TABLE 6.3.5

COEFFICIENTS OF DETERMINATION (R^2 x 100) FOR PAIRWISE COMPARISONS OF SERUM TRAITS FOR COMBINATIONS OF GENOTYPES OF LPL GENE POLYMORPHISMS

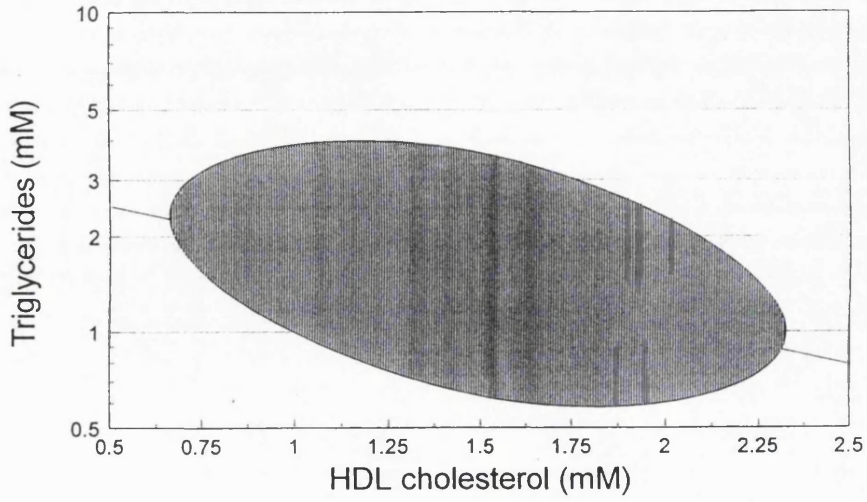
Traits	Healthy individuals and patients combined Genotypes of LPL-HindIII RFLP			
	ALL (99)	H+H+ (55)	H+H- and H-H- combined (43)	Sig. of differences between H+H+ and (H+H- and H-H- combined)
HDL2b and triglycerides[a] ‡	48.0 *	35.2 *	58.1 *	N/S
HDL3b and triglycerides[a] †	40.4 *	25.5 *	60.0 *	$p < 0.05$
HDL2b and VLDL-cholesterol[a]‡	50.4 *	36.4 *	65.3 *	$p < 0.05$
HDL3b and VLDL-cholesterol[a]†	37.2 *	23.6 *	57.8 *	$p < 0.05$
HDL2b and LPL activity †	18.4 *	9.3	27.9 *	N/S
HDL3b and LPL activity ‡	3.4	0.9	21.8 *	$p < 0.01$

[a] Correlations carried out on $\log_{10}$- transformed data * $p < 0.001$
† Positive correlation between these two traits
‡ Negative correlation between these two traits

All correlations were carried out on unadjusted data, to give an estimate of the in-vivo situation with regard to interactions between traits. Estimates for the healthy individuals and patients were similar so the data from the combined group are presented here.

Figure 6.3.1

Plot of Triglyceride levels verus HDL-cholesterol levels in absence of H- allele

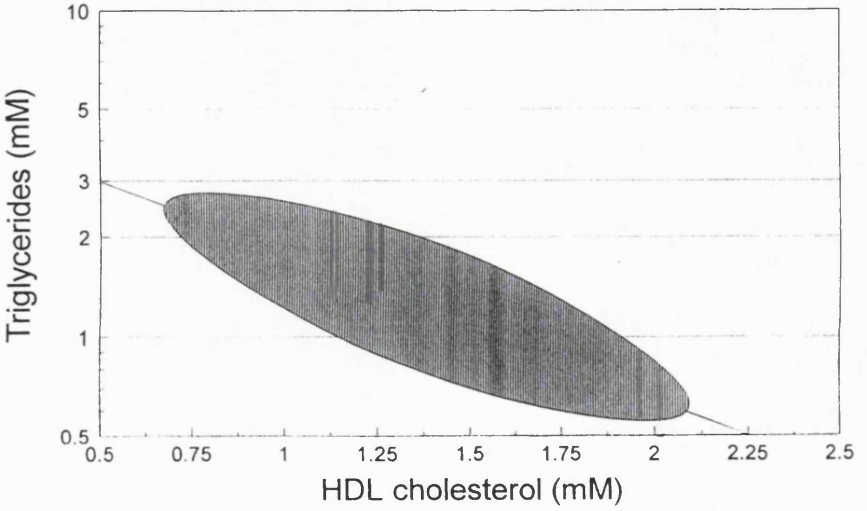


Triglycerides are on a logarithmic scale
Slope (S.E.) = -0.29 (0.07)

Correlation plot of triglycerides (log_{10} scale) versus HDL-cholesterol for absence of the H-allele in the combined sample of healthy individuals and patients.

Figure 6.3.2

Plot of Triglyceride levels verus HDL-cholesterol levels in presence of H- allele



Triglycerides are on a logarithmic scale
Slope (S.E.) = -0.49 (0.07)

Correlation plot of triglycerides (log_{10} scale) versus HDL-cholesterol for presence of the H-allele in the combined sample of healthy individuals and patients.

Section 6.4. The Search for Causative Variation in the LPL gene by SSCP and Sequencing.

Exons 2 to 9, inclusive, of the LPL gene were PCR-amplified and screened using Single Stranded Conformation Analysis (SSCP) as described in Chapter 2. Exon 1 was not included since it only encodes the first three amino acid residues of the mature LPL protein (Deeb & Peng 1989 and see Table 1.6.4). Therefore it is not very likely that genetic variation mediating altered affinity of LPL for TG-rich lipoproteins would be present in exon 1. Twenty-seven individuals from the Swedish sample were selected for the SSCP-based screening of LPL exons. Criteria were either genotype H+H+ and low LPL activity (& low affinity for substrate lipoproteins) or presence of the H- allele and high LPL activity (& high affinity for substrate lipoproteins). This took into account the two sides of the hypothesis outlined in Section 6.3, that variation in linkage disequilibrium with either the H+ or H- alleles of the HindIII polymorphism influences affinity of LPL for lipoproteins, with the enzyme coded for by the H+ or H- alleles having lesser or greater affinity for TG-rich lipoproteins, respectively. Table 6.4 shows the characteristics of the selected individuals (their HindIII genotypes and LPL activities) and a summary of the results from SSCP screening of exons.

Figure 6.4.1 shows the SSCP results from selected samples for exon 2 in the LPL gene. As previously reported (Mailly et al., 1994), the D9N substitution, which is caused by a G_{280} to A substitution, caused a mobility shift in both the single stranded (ss) and double stranded (ds) DNA regions of the SSCP gel. Individuals heterozygous for Asn9 (ID numbers 4094 and 2053) have four bands in the ss region, due to retarded mobility of PCR fragments containing A_{280}, and two bands in the ds region, due to heteroduplex formation in the ds region. SSCP results for exon 4 are displayed in Figure 6.4.2, with two individuals (ID numbers 4015 and 4038) exhibiting a double band in the ss region. Sequencing of one of these individuals (ID number 4035) revealed that this individual is heterozygous for a G to A substitution (Figure 6.4.3). This base substitution occurs in the wobble position of the codon coding for Glu119, thus it does not change the amino acid sequence of the LPL polypeptide. For this reason, this silent G to A substitution was not investigated any further. This substitution appears to be on a H+ chromosome; five out of six individuals with this silent base change who were investigated had the H+H- genotype but the sixth was

Table 6.4 Characteristics of individuals selected for SSCP screening in the LPL gene and results from SSCP

ID number	HindIII genotype	LPL activity (mU)	Exons of the LPL gene screened by SSCP							
			2	3	4	5	6	7	8	9
4015	+-	137	N	N	ss	N	N	N	N	N
4026	+-	158	N	N	N	N	N	N	N	N
4032	++	35	N	N	N	N	N	N	N	N
4033	++	63	N	N	N	N	N	N	N	N
4034	++	58	N	N	N	N	N	N	N	N
4035	+-	120	N	N	ss	X	N	N	X	N
4038	++	61	N	N	ss	N	N	N	N	N
4047	+-	111	N	N	N	N	N	N	N	N
4053	+-	129	N	N	ss	N	N	X	X	X
4056	–	114	N	N	N	N	N	N	N	ss
4060	++	63	N	N	N	N	N	N	N	N
4065	++	48	N	N	N	N	N	N	N	X
4074	++	63	X	X	N	X	N	N	N	N
4088	+-	127	N	N	N	N	N	N	N	N
4089	+-	122	N	N	N	N	ds	N	N	N
4092	+-	158	ss, ds	N	ss	N	N	N	X	N
4094	++	65	N	N	N	N	N	N	N	N
2023	++	50	N	N	N	N	N	N	N	N
2042	++	46	ss, ds	N	N	N	N	N	N	N
2053	++	49	N	N	N	N	N	N	N	N
2056	++	52	N	N	N	N	N	N	N	N
2063	+-	114	N	N	ss	N	N	N	N	N
2064	++	51	N	N	N	N	N	N	N	N
2077	+-	122	N	N	N	N	ds	N	X	N
2084	++	60	N	N	N	N	N	N	N	N
2122	++	59	N	N	N	N	N	N	N	N
2126	++	56	N	N	N	N	N	N	N	N

N = 'normal' appearance on SSCP gel. X = failure of PCR-amplification
ss and ds = mobility shift in single and double stranded DNA regions, respectively, on SSCP gel.

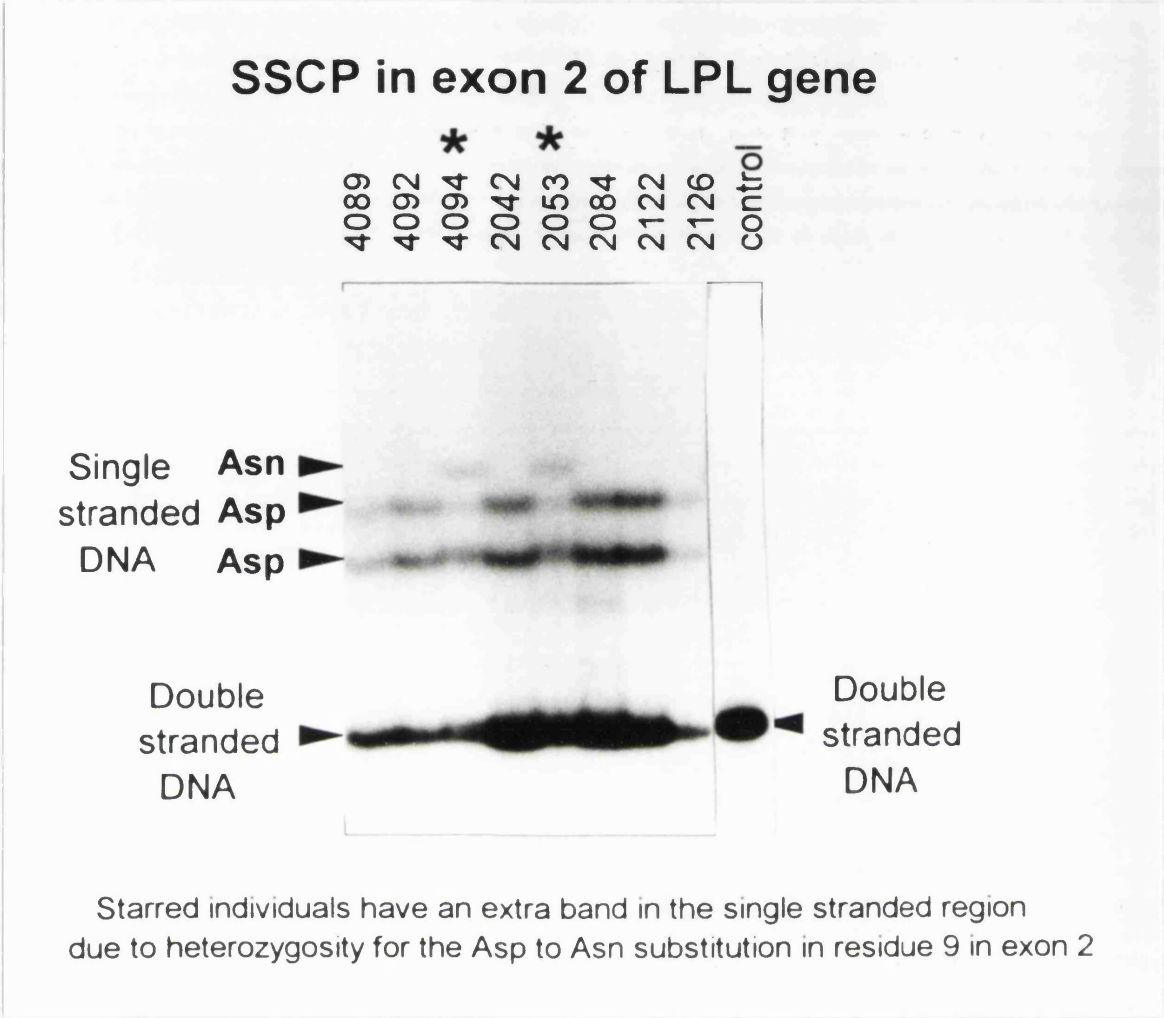


Figure 6.4.1

Single Stranded Conformation Analysis (SSCP) - results for exon 2 of the LPL gene. Samples were run on a 5% polyacrylamide gel (ratio of acrylamide to bis = 49:1) containing 10% glycerol, for 16 hours at a current of 12mA, followed by overnight exposure to Hyperfilm-MP.

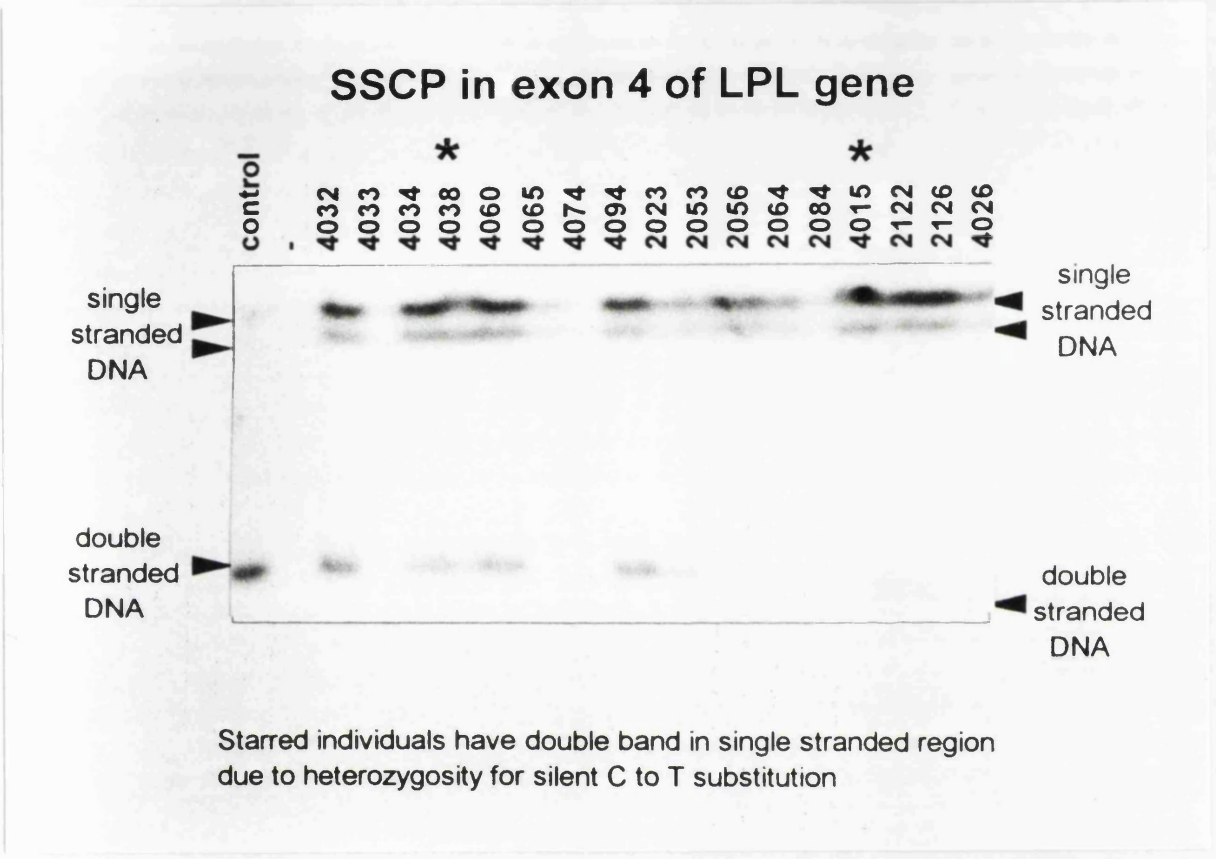
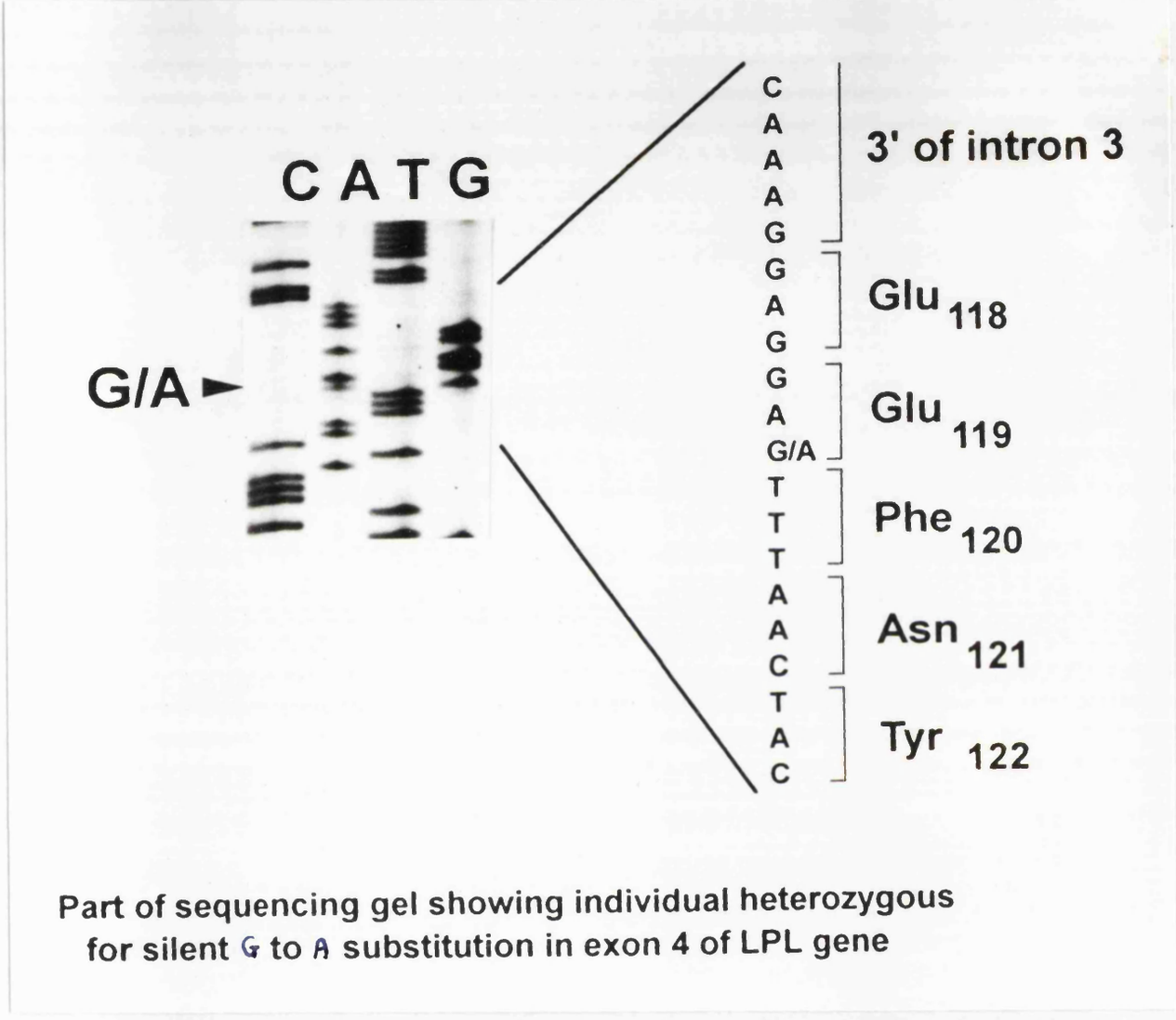


Figure 6.4.2

Single Stranded Conformation Analysis (SSCP) - results for exon 4 of the LPL gene. Samples were run on a 5% polyacrylamide gel (ratio of acrylamide to bis = 49:1) containing 10% glycerol, for 15 hours at a current of 15mA, then 2 hours at 23mA, followed by overnight exposure to Hyperfilm-MP.



Part of sequencing gel showing individual heterozygous for silent G to A substitution in exon 4 of LPL gene

Figure 6.4.3

Samples were run for 1.5 hours at a constant power of 65W. The above results are from DNA amplified from Swedish individual with ID number 4035.

homozygous for H+.

SSCP of exon 6 revealed a double band in the ds region in two individuals, due to heteroduplex formation (Figure 6.4.4); the mismatched heteroduplexes migrating more slowly than those without a mismatch. Sequencing of these two individuals (ID numbers 4089 and 2077) revealed an A_{1127} to G substitution (Figure 6.4.5) which changes Asn to Ser at residue 291 of the mature LPL polypeptide. For exon 9, one of the individuals screened by SSCP exhibited a slower migrating band in the ss region. This individual (ID 4056) is homozygous for Stop447, therefore to check that this mobility shift was due to homozygosity for Stop447 and not to another unidentified mutation in exon 9, individuals with different genotypes of the Serine447 to Stop polymorphism in exon 9 were run on another SSCP gel, and results are shown in Figure 6.4.6. This confirmed that the SSCP mobility shift was due to the C_{1595} to G transversion underlying the Serine447 to Stop polymorphism, and not to another base substitution on exon 9. So exon 9 was not investigated any further. The entire Swedish sample was screened for the N291S substitution using Allele Specific Oligonucleotides (Table 2.6 and Figure 2.7.2.3).

Section 6.5 The N291S Substitution; frequency of Ser291 and association with fasting lipids and lipoproteins

Table 6.5.1 shows the frequencies of the Ser291 variant in the Swedish sample. Six healthy individuals and six MI survivors (patients) were heterozygous for Ser291, so the frequency of Ser291 is similar in both groups. This is in contrast to the previously reported observation by Ma et al. (1993a) of the absence of Ser291 in a sample of 150 healthy Caucasian individuals. It will therefore be of interest to genotype independent samples for the N291S substitution to ascertain whether this is a polymorphism in the general population. In the study by Ma et al. (1993a), 2 out of 15 Type III HLP patients were heterozygous for Ser291, frequency of Ser291 = 0.067, not significantly different from that in the combined Swedish sample (2/30 versus 12/348, $\chi^2 = 0.804$, $p < 0.5$ at 1 degree of freedom). LPL and apoE genotypes are shown for LPL-Ser291 carriers in Table 6.5.2. All Ser291 carriers have at least one V+ and one H+ alleles and all except the individual with ID number 2067 are homozygous for Ser447. The individual with ID number 2067 is heterozygous for Stop447. Therefore it appears that the Ser291 variant is only on the V+H+ Ser447 haplotype, though

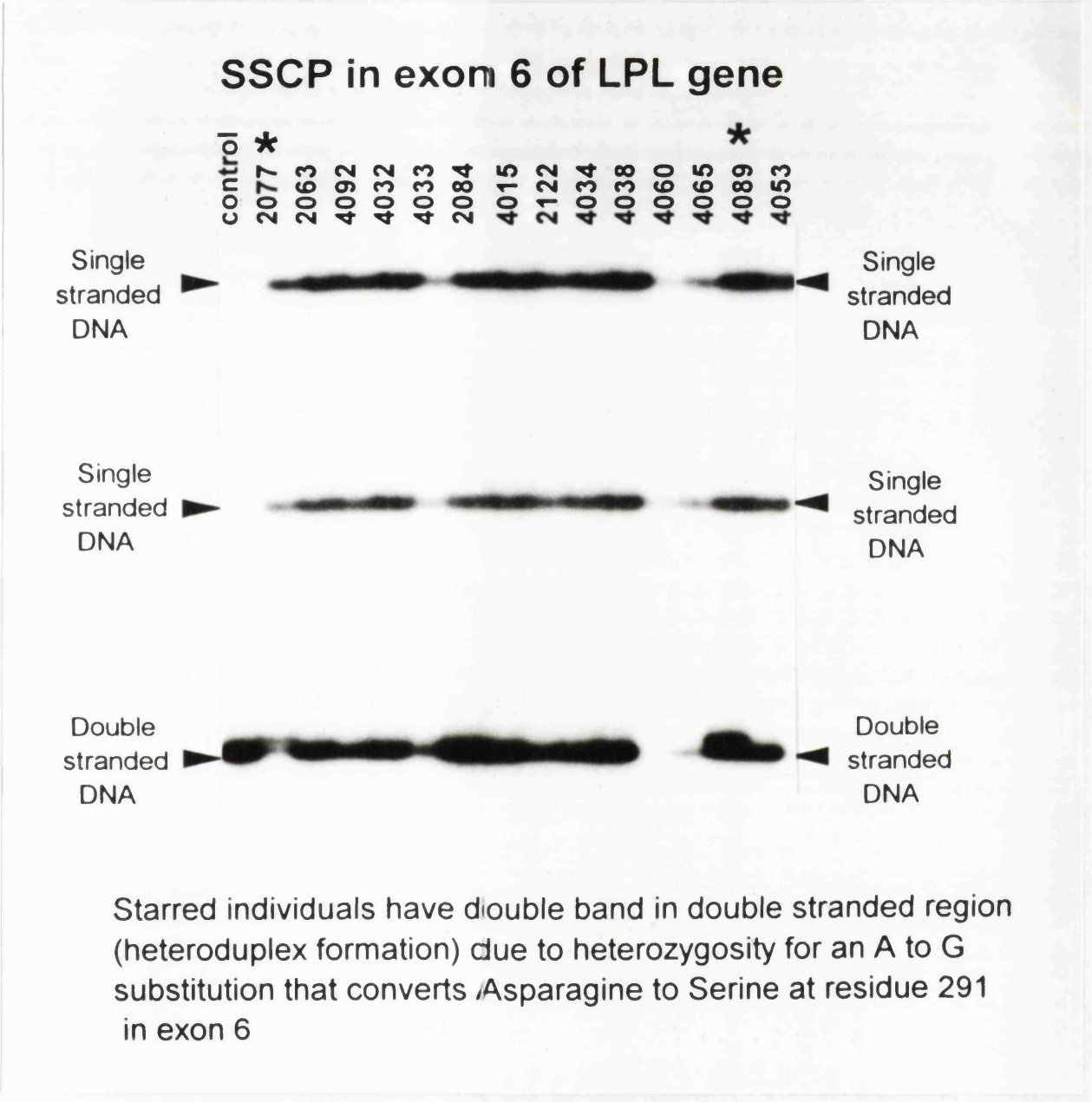
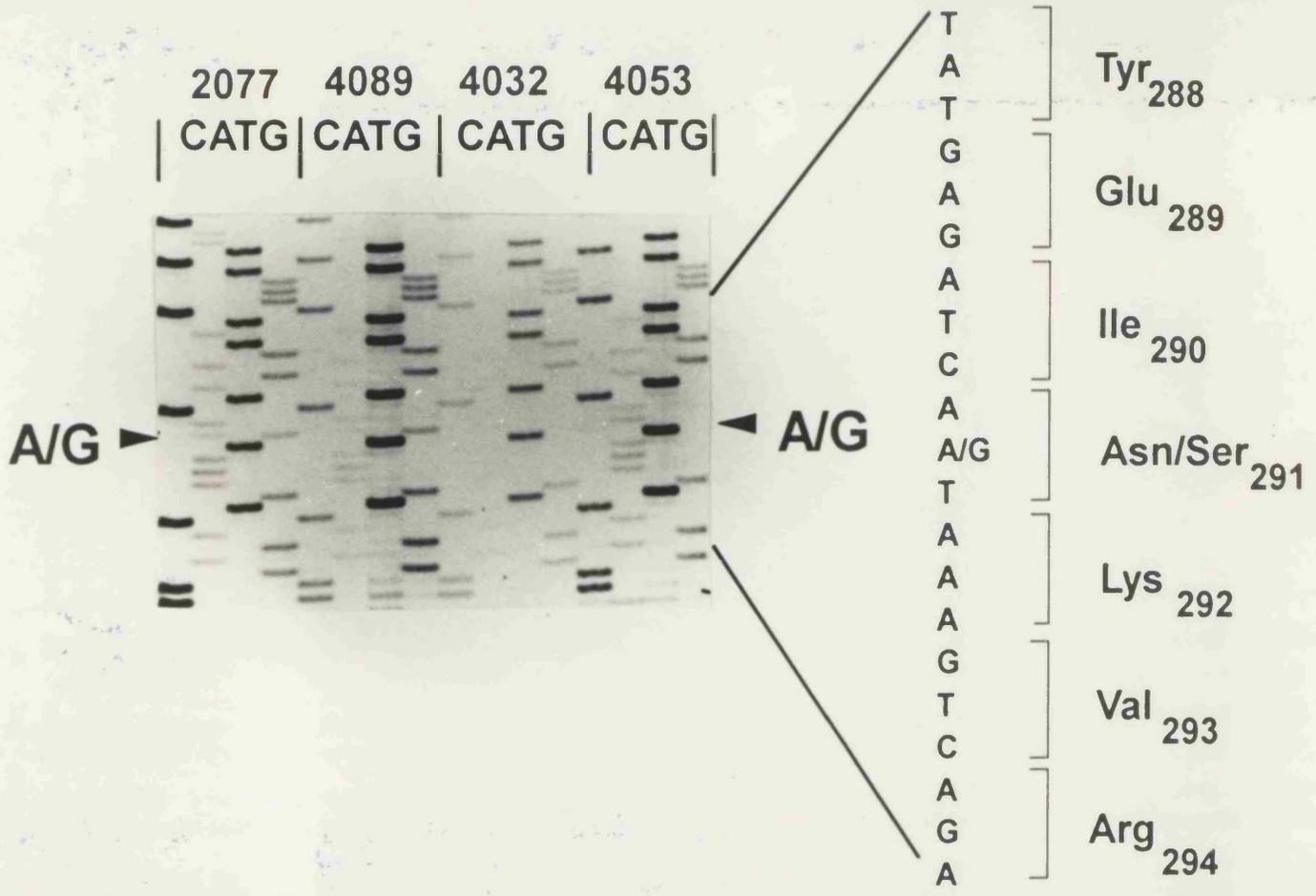


Figure 6.4.4

Single Stranded Conformation Analysis (SSCP) - results for exon 6 of the LPL gene. Samples were run on a 5% polyacrylamide gel (ratio of acrylamide to bis = 49:1) containing 10% glycerol, for 19 hours at a current of 15mA, followed by overnight exposure to Hyperfilm-MP.



Part of sequencing gel of exon 6 in LPL gene. Individuals with ID numbers 2077 and 4089 are heterozygous for an A to G substitution which changes Asp to Ser at residue 291, while the other 2 individuals are homozygous for Asn.

Figure 6.4.5

Samples were run for 3 hours at a constant power of 65W.

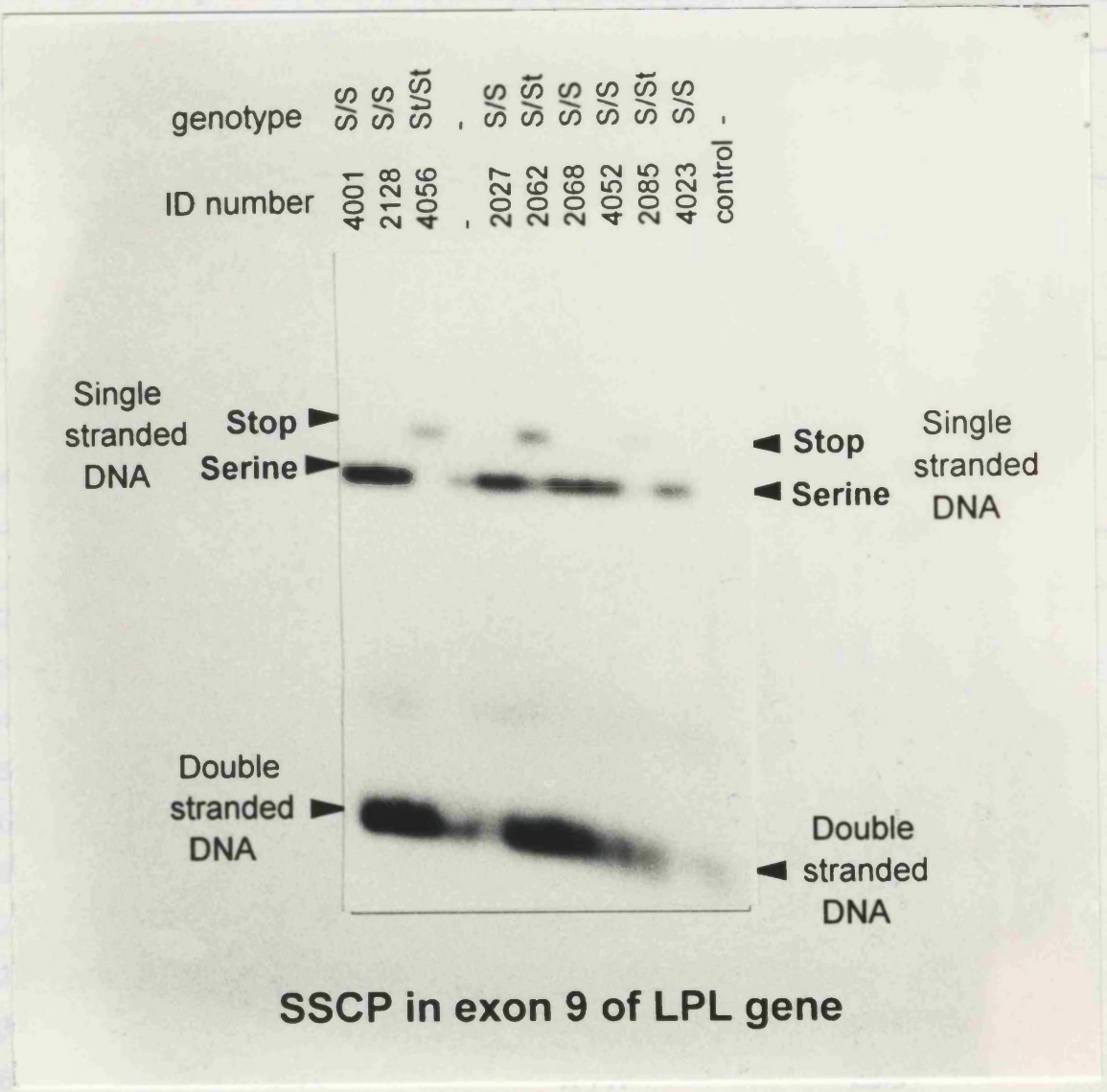


Figure 6.4.6

Single Stranded Conformation Analysis (SSCP) - results for exon 9 of the LPL gene. Samples were run on a 5% polyacrylamide gel (ratio of acrylamide to bis = 49:1) containing 10% glycerol, for 18 hours at a current of 15mA, followed by overnight exposure to Hyperfilm-MP. The C_{1595} to G transinversion underlying the Serine447 to Stop polymorphism underlies the differential mobilities of the PCR fragments produced from the Serine and Stop alleles.

Table 6.5.1 Frequencies of LPL-Ser291 in the Swedish healthy individuals and patients

Sample	No. of individuals homozygous for Asn291	No. of individuals carrying Ser291	Frequency of Ser291
Swedish healthy individuals	83	6	0.034
Swedish MI survivors	79	6	0.035

Table 6.5.2 Genotypes at LPL and apoE gene loci in LPL-Ser291 carriers

ID number	LPL-PvuII genotype	LPL-HindIII genotype	apoE genotype
4020	V+V+	H+H+	E4/3
4022	V+V-	H+H-	E4/3
4046	V+V-	H+H-	E4/3
4051	V+V+	H+H-	E3/3
4069	V+V+	H+H+	E3/3
4089	V+V-	H+H-	E3/3
2022	V+V-	H+H+	E3/3
2025	V+V-	H+H-	E3/3
2039	V+V-	H+H-	E4/3
2067	V+V-	H+H-	E3/3
2068	V+V+	H+H-	E3/3
2077	V+V-	H+H-	E3/3

Footnotes; 4020 to 4089 = Swedish healthy individuals, 2022 to 2077 = Swedish MI survivors. All these individuals, except 2067 were homozygous for LPL-Ser447.

this will need confirmation in independent samples.

The proportion of total sample variance in each fasting plasma trait (adjusted for age and BMI first) explained by genotypes of the Asn291 to Ser substitution for the healthy individuals and patients is given in Table 6.5.3. The largest effects are on HDL-cholesterol and apoAI in the healthy individuals and on total cholesterol in the patients. The effect of genotypes at the N291S substitution on the other traits analysed are negilible in both the healthy individuals and patients. This may be due to the small numbers of Ser291 carriers, therefore the effect of N291S within a larger sample may be more appreciable. Table 6.5.4 shows the means and standard errors for absence and presence of Ser291 in the two groups; for those traits which showed significant or nearly significant associations with genotypes in either group (total cholesterol, HDL-cholesterol and apoAI) or those which are of interest (TGs and LPL activity) because of the potential direct effect of the N291S substitution on LPL activity and hence on TG levels. The presence of Ser291 was associated with lower cholesterol and apoB in both groups; however for the other three traits shown, effects were not consistent. For the healthy individuals, Ser291 was associated with higher HDL-cholesterol levels, but lower in the patients. Mean apoAI levels were 11.4% higher in the presence of Ser291 in the healthy individuals, but in the patients means were only 2% higher in Ser291 carriers. The trend was the opposite for LPL activity i.e. lower in the healthy individuals but higher in the patients. The effect of N291S on TG in the healthy individuals was negilible but associated with a definite lowering effect in the patients.

It is difficult to draw any definite conclusions on the effect of Ser291 on fasting plasma lipid and lipoprotein traits due to the small numbers of Ser291 carriers; however the effect of N291S on fasting lipid traits, with the exception of HDL-cholesterol, is smaller than that associated with the HindIII polymorphism (Table 3.5.8) e.g. for total TGs, VLDL-cholesterol and VLDL-TG, the HindIII polymorphism explained 7.4%, 6.2% and 6.2% of sample variance, respectively while N291S explained only 0.1%-0.2% of sample variance in these traits. This suggests that the N291S substitution is not explaining all of the effect associated with genotypes of the HindIII polymorphism. The characteristics of each of the Ser291 carriers with regard to fasting lipid traits are shown in Table 6.5.5, which also displays the means $\pm$ S.Es for individuals homozygous for Asn291. This demonstrates the

Table 6.5.3 Percentage of total sample variance (R^2x100) in traits adjusted for age and body mass index attributable to genotypes of the N291S polymorphism in the LPL gene, in the Swedish sample

No exclusion of the hyperlipidaemic individuals in the healthy individuals

Fasting Plasma Trait	Healthy Individuals	Patients
Total cholesterol	0.1	6.2 **
VLDL-cholesterol †	0.2	0.7
LDL-cholesterol	0.6	2.7
HDL-cholesterol	5.6 **	0.5
Total TG †	0.1	1.1
VLDL-TG †	0.1	1.4
LDL-TG	1.0	0.6
HDL-TG	0.5	0.6
apoB	1.8	0.2
apoAI	3.9 *	0.1
LPL activity (postheparin)	0.5	0.1

† regression carried out on $\log_{10}$-transformed data.

** $p<0.05$, * $p<0.1$

Table 6.5.4 Means ± S.E.s (data adjusted for age and BMI) for absence and presence of Ser291 in the Swedish sample, where significant or nearly significant results were obtained from ANOVA for either the healthy individuals or patients.

Trait (mmol/l)	Healthy individuals			MI survivors		
	Asn/Asn (n=83)	Asn/Ser (n=6)	F and p values from ANOVAs*	Asn/Asn (n=79)	Asn/Ser (n=6)	F and p values from ANOVAs*
Total-cholesterol	6.12 ± 0.13	5.90 ± 0.35	F=0.2, p=0.7	7.36 ± 0.14	6.21 ± 0.17	F=5.4, p=0.02
HDL-cholesterol	1.41 ± 0.03	1.52 ± 0.19	F=4.5, p=0.04	1.13 ± 0.02	1.06 ± 0.06	F=0.4, p=0.5
Triglycerides †	1.26 (1.14-1.41)	1.27 (0.82-1.96)	F=0.1, p=0.8	2.27 (2.03-2.43)	1.87 (1.29-2.73)	F=0.9, p=0.3
apoAI (mg/dl)	123.4 ± 1.9	137.5 ± 9.9	F=3.5, p=0.06	111.6 ± 2.5 (n=56)	113.8 ± 4.8 (n=5)	F=0.07, p=0.8
apoB (mg/dl)	108.9 ± 2.1	98.8 ± 8.3	F=1.4, p=0.2	127.1 ± 2.4	123.3 ± 4.3	F=0.2, p=0.7
postheparin LPL activity (milliunits)	96.8 ± 4.8 (n=60)	90.9 ± 11.0 (n=5)	F=0.2, p=0.7	73.9 ± 4.2 (n=31)	76.2 ± 13.9 (n=5)	F=0.04, p=0.8

† shown as antilogs of $\log_{10}$-transformed means and 95% confidence limits
* ANOVAs versus absence/presence of Ser291, were carried out on data adjusted for age and BMI.

Triglycerides and post-heparin LPL activity are included in this table, even though associations with genoytpes were insignificant in both healthy individuals and patients, because of the hypothesis that the Asn291 to Ser substitution might affect LPL activity and hence triglyceride levels. Therefore trends observed for presence and absence of the Ser291 allele were of interest.

Table 6.5.5 Characteristics (adjusted for age and BMI) of LPL-Ser291 carriers in the Swedish sample, for fasting plasma traits.

ID	age in years	BMI in Kg/m^2	total-C	VLDL-C	LDL-C	HDL-C	TG	VLDL-TG	LDL-TG	HDL-TG	apoB mg/dl	apoAI mg/dl	LPL activity milliunits
4020	43	22.5	5.54	0.77	3.92	0.81	1.74	1.06	0.44	0.16	ND	110.4	91
4022	35	25.3	5.84	0.26	3.33	1.96	0.80	0.34	0.28	0.17	81	151.0	84
4046	40	23.9	6.04	0.19	3.81	2.00	0.85	0.33	0.34	0.16	112	162.5	69
4051	42	24.7	7.48	0.95	5.48	1.29	2.37	1.80	0.37	0.20	124	130.9	ND
4069	37	17.2	5.56	0.50	3.29	1.29	1.26	0.73	0.24	0.15	95	109.0	ND
4089	41	25.6	4.97	0.36	2.73	1.77	1.18	0.77	0.28	0.13	83	161.0	121
Asn/ Asn	40.5 ±0.44	24.7 ±0.27	6.12 ±0.13	0.43 † (0.37-0.49)	4.00 ±0.10	1.41 ±0.03	1.26 † (1.14-1.41)	0.69 † (0.59-0.81)	0.35 ±0.01	0.15 ±0.01	108.9 ±2.1	123.2 ±2.0	96.8±4.8
2022	44	28.2	5.89	0.35	4.08	1.16	1.42	0.52	0.66	0.17	117	109.9	68
2025	31	30.8	6.76	0.88	4.88	1.20	2.33	1.50	0.34	0.15	110	127.0	87
2039	40	33.5	5.94	0.30	4.44	1.20	1.04	0.60	0.41	0.09	138	122.8	57
2067	36	27.0	6.32	1.26	4.38	0.83	2.17	1.71	0.35	0.15	117	ND	ND
2068	36	26.8	6.61	0.99	4.62	1.04	2.21	1.74	0.36	0.15	133	108.0	44
2077	41	25.4	5.74	1.44	3.35	0.96	2.63	1.88	0.42	0.17	124	101.0	124
Asn/ Asn	40.4 ±0.39	26.3 ±0.37	7.36 ±0.14	0.89 † (0.78-1.02)	4.97 ±0.12	1.13 ±0.02	2.27 † (2.03-2.43)	1.55 † (1.35-1.78)	0.46 ±0.02	0.16 ±0.01	127.1 ±2.4	111.9 ±2.6	73.9±4.2

Means ± S.E.s are shown for Asn/Asn except for † where means are shown as antilogs of log_{10}-transformed means and 95% confidence intervals
With the exception of age and BMI, levels are adjusted for age and BMI.
Unless otherwise stated, units of traits are mmol/l. C = cholesterol, TG = triglycerides

heterogenity of levels in individual Ser291 carriers; levels were both higher and lower in Ser291 carriers compared to the mean level for homozygosity for Asn291. The most consistent effects were on plasma and LDL-cholesterol levels, where all except one Ser291 carrier (I.D. 4051) had levels below the mean for Asn/Asn for healthy individuals and patients, respectively. For LDL-TGs, in the patients, all except one Ser291 carrier (I.D. 2022) had lower levels than the mean for individuals homozygous for Asn291, while in the healthy individuals, levels of LDL-TGs in Ser291 carriers were both higher and lower than the mean for Asn/Asn individuals. Effects of Ser291 on VLDL- and HDL-cholesterol levels, plasma TGs, VLDL-TGs and apoB total and lipoprotein TGs, apoB, apoAI and postheparin LPL activity were less consistent, with some individuals having lower and others having higher levels than the mean level for Asn/Asn, within both the healthy individuals and patients. HDL-TG levels in Ser291 carriers showed little deviation from the means for Asn/Asn individuals except for the two individuals with I.D. numbers 4051 (who had high HDL-TGs) and 2039 (who had low HDL-TGs).

Section 6.6 The effect of the N291S substitution on postprandial lipid and lipoprotein traits.

Measures of postprandial lipaemia and lipoprotein metabolism (adjusted for fasting TGs first; see Section 5.4) were analysed versus genotypes of N291S to evaluate whether this substitution would affect magnitude of postprandial lipaemia or metabolism of potentially atherogenic remnant lipoproteins. Table 6.6.1 shows the means and standard errors or medians and 95% confidence limits for the major parameters of postprandial lipaemia, for absence and presence of Ser291 in the combined group of healthy individuals and patients. There was no interaction between group phenotype (healthy individuals, NTG patients and HTG patients) and genotypes of N291S in modulating postprandial lipaemia or parameters of postprandial lipid metabolism. Presence of Ser291 was associated with 11% and 22% higher levels of TG in all and Sf60-400 lipoproteins but 18% and 17% lower levels of TG in Sf>400 and Sf20-60 lipoproteins. Ser291 carriers had higher levels of apoB-48 and apoB-100 in Sf60-400 lipoproteins, but lower levels of apoB-48 and apoB-100 in Sf20-60 lipoproteins. The mean AUC for LPL (indicating total postprandial LPL activity; a measure of circulating LPL activity in the capillary lumen) was higher for Ser291 carriers. However the mean levels of fasting FFAs were lower in Ser291 carriers. None of these associations

Table 6.6.1 Postprandial lipaemia in the Swedish sub-sample (described in Chapter 6) in relation to genotypes of the Asn291 to Ser substitution in the LPL gene.

Trait (adjusted for fasting TG)	units	Absence of Ser291 (n=34)	Presence of Ser291 (n=7)	p value from ANOVA
AUC TGs; all lipoproteins †	mM hours	32.7 (30.9 to 34.6)	36.3 (29.5 to 44.6)	0.2
AUC TGs; Sf>400 chylomicrons †	"	6.8 (5.8 to 8.0)	5.6 (3.4 to 9.4)	0.2
AUC TGs; Sf 60-400 large chylomicron remnants and large VLDLs †	"	11.6 (10.3 to 13.0)	14.2 (9.8 to 20.4)	0.1
AUC TGs; Sf 20-60 small chylomicron remnants and small VLDLs †	"	4.1 (3.7 to 4.5)	3.4 (2.3 to 5.3)	0.1
apoB-48 Sf 60-400 large chylomicron remnants	mg/l hours	177 ± 17.8	223 ± 30.3	0.6
apoB-48 Sf 20-60 † small chylomicron remnants	"	44 (36 to 54)	41 (26 to 65)	0.8
apoB-100 Sf 60-400 large VLDLs	"	954 ± 64.2	980 ± 140.4	0.5
apoB-100 Sf 20-60 small VLDLs	"	572 ± 51.7	457 ± 53.0	0.6
AUC for LPL	milliunits hours	16.6 ± 1.1	18.7 ± 2.8	0.7
levels of fasting free fatty acids	μmol/l	621 ± 36.7	597 ± 77.9	0.9

† antilogs of $\log_{10}$-transformed means and 95% confidence limits.
AUC = area under curve

reached statistical significance due to the small number of Ser291 carriers.

Figure 6.6.1 shows mean levels of total postprandial TGs (adjusted for fasting TGs) in the combined group versus time elapsed since ingestion of the oral fat load, in individuals grouped for presence and absence of the Ser291 allele, respectively. At all time points, the mean level of total postprandial TGs adjusted for fasting TGs was higher for presence of Ser291; this difference became more pronounced in the late postprandial period. At 8, 9 and 12 hours postprandium, mean levels were 17.4%, 17.6% and 25.8% higher, respectively, than for absence of Ser291. Using antilogs, the rates of decline of TG levels between 9 and 12 hours postprandium were similar, being 0.17mM for absence of Ser291 and 0.16mM for presence of Ser291. The time course for large chylomicron remnants postprandially is presented in Figure 6.2.2. During the first 3 hours, levels of these lipoproteins are similar for both presence and absence of Ser291. However between 3 and 6 hours postprandium, mean levels rose by only 5.3% in absence of Ser291 while levels rose by 31.0% in Ser291 carriers. Between 6 and 12 hours postprandium, the net rate of catabolism of large chylomicron remnants was similar for both groups, being 3.13mg/l for absence of Ser291 and 3.49mg/l for presence of Ser291. The time course of large VLDLs throughout the entire postprandial period was virtually identical for presence and absence of Ser291 (data not shown). Figure 6.6.3 shows the time course of unstimulated LPL activity, where heparin was not administered, in milliunits (μU/ml) during the postprandial period in individuals with or without the Ser291 allele. In carriers of the Ser291 allele, mean LPL activity is higher at all five time points. During the first 3 hours postprandium, mean unstimulated LPL activity in the capillary lumen rose by 44% and 36% in the presence and absence of Ser291, respectively. The time courses between 3 and 6 hours and between 9 and 12 hours are approximately parallel in both groups. Between 6 and 9 hours postprandium, mean unstimulated LPL activity declined by 21% and 16% in the presence and absence of Ser291, respectively. The peak, at 6 hours postprandium, was 20% higher (N/S) in presence of Ser291.

Table 6.6.2 shows the characteristics for postprandial lipaemia adjusted for fasting TGs for each of the Ser291 carriers. With the exception of individuals no. 2067 and 2077, Ser291 carriers had higher AUCs for TGs for all and Sf60-400 lipoproteins. Effects of

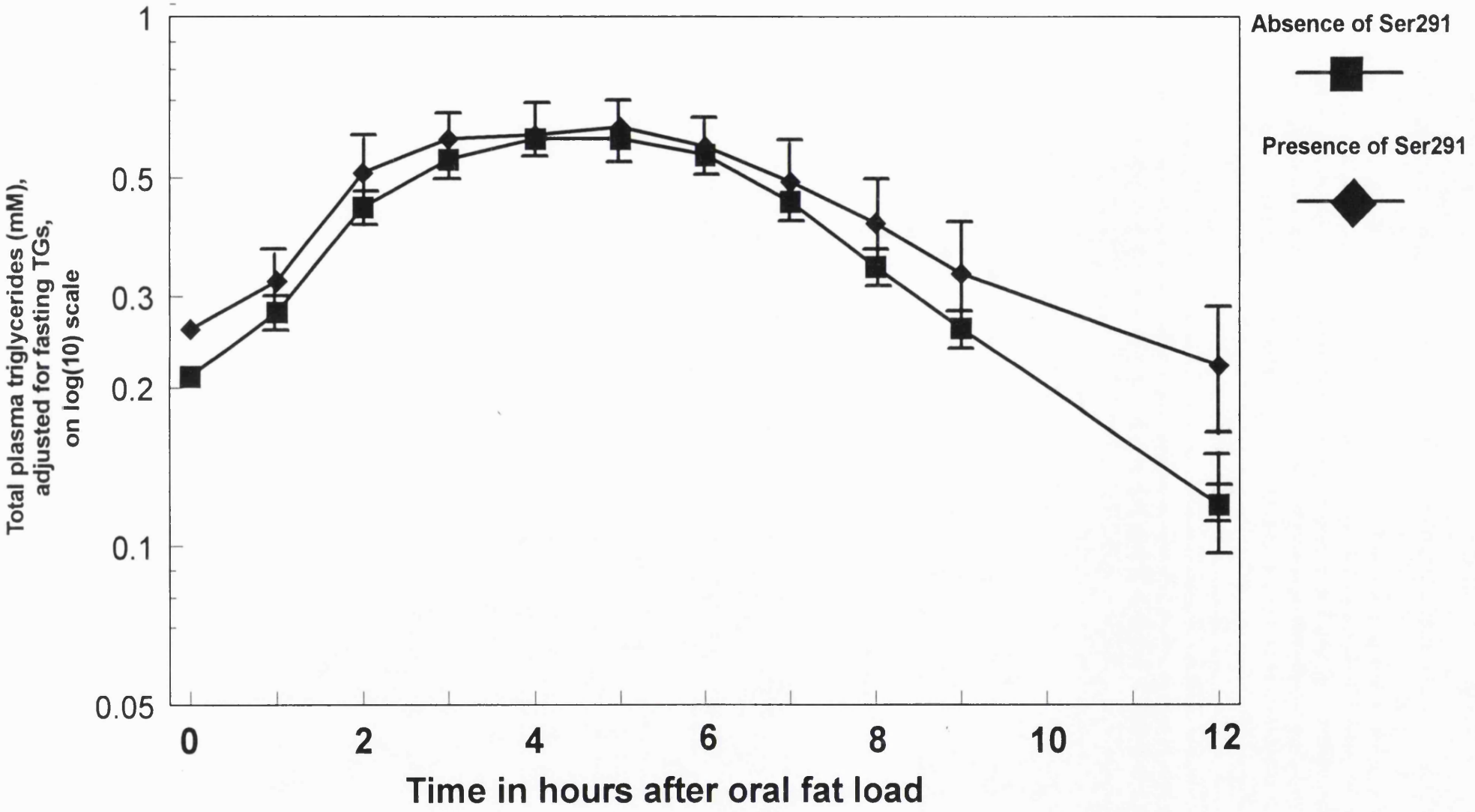
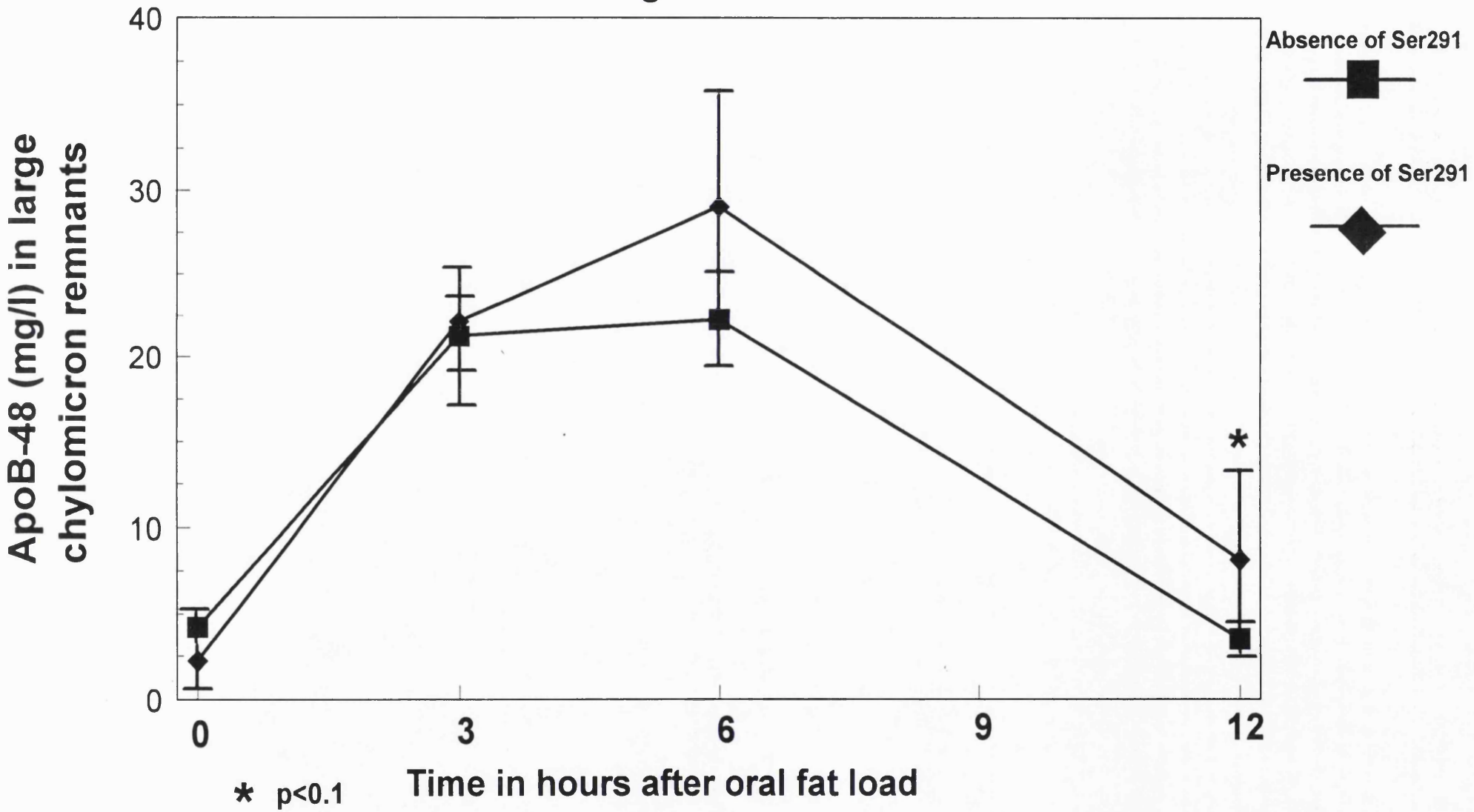


Figure 6.6.1

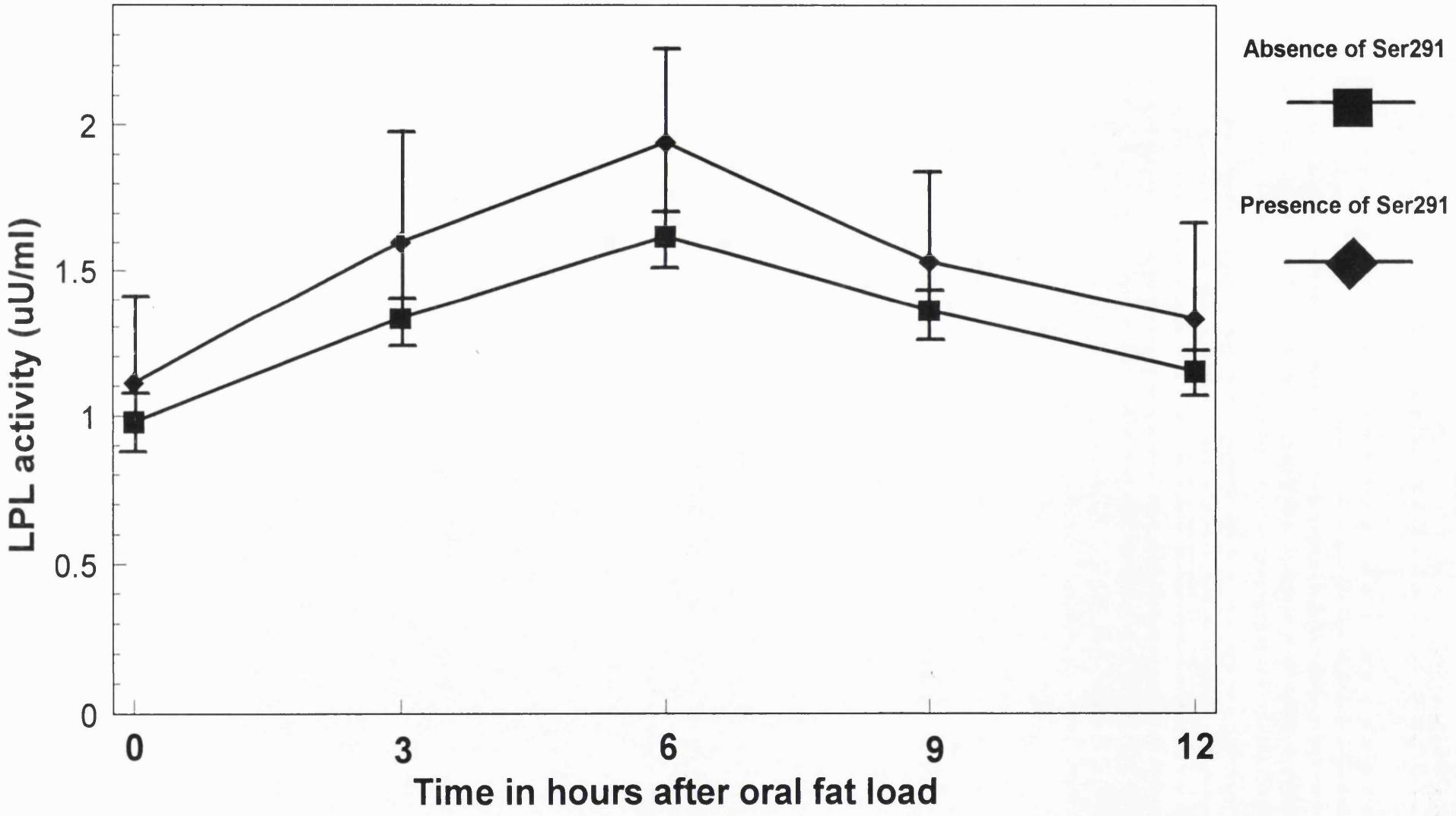
Means and their standard errors of total plasma TGs (log_{10} scale), adjusted for fasting TGs versus time (in hours) since ingestion of the oral fat load, with the group divided into those homozygous for LPL-Asn291 and those heterozygous for LPL-Ser291. Data is shown for the combined group of healthy individuals and patients.

Figure 6.6.2



Means and their standard errors of apoB-48 (mg/l) in Sf 60-400 lipoproteins (large chylomicron remnants), versus time (in hours) since ingestion of the oral fat load, with the group divided into those homozygous for LPL-Asn291 and those heterozygous for LPL-Ser291. Data is shown for the combined group of healthy individuals and patients and is adjusted for fasting TGs.

Figure 6.6.3



Means and their standard errors of postprandial LPL activity in microunits per ml versus time (in hours) since ingestion of the oral fat load, with the group divided into those homozygous for LPL-Asn291 and those heterozygous for LPL-Ser291. Data is not adjusted for fasting TGs and is shown for the combined group of healthy individuals and patients.

Table 6.6.2 Characteristics of postprandial lipid and lipoprotein traits in those Swedish individuals with LPL Ser291 and who had postprandial measurements taken.
Levels are adjusted for fasting TGs.

ID	group - see footnote	age	AUC for TG; (total)	AUC for TG; Sf>400	AUC for TG; Sf 60-400	AUC for TG; Sf 20-60	ApoB-48 Sf 60-400	ApoB-48 Sf 20-60	ApoB-100; Sf 60-400	ApoB-100; Sf 20-60	AUC; LPL	fasting free fatty acids
units	-	years	mM hours	mM hours	mM hours	mM hours	mg/l hours	mg/l hours	mg/l hours	mg/l hours	munits hours	μmol/l
4089	1	49	39.6	8.7	16.6	4.6	73.1	193.5	628.0	1068.8	15.7	404
2022	2	52	44.6	7.2	16.2	1.3	42.3	343.1	352.8	554.8	32.6	865
2039	2	50	39.2	6.2	23.8	3.7	30.2	254.3	340.2	1431.3	20.1	566
2067	2	43	23.6	2.0	8.6	3.8	30.0	122.1	458.9	580.9	11.8	887
2025	3	41	34.8	10.6	16.4	4.4	ND	231.7	ND	1125.2	18.7	558
2068	3	45	44.7	4.3	16.3	4.0	42.5	191.4	506.6	1117.8	10.0	536
2077	3	49	32.4	5.2	7.9	4.2	ND	ND	ND	ND	22.0	362
Absence of Ser291 (n=34)	1,2,3	49.4 ±8.5	32.7 † (30.9-34.6)	6.8 † (5.8-8.0)	11.6 † (10.3-13.0)	4.1 † (3.7-4.5)	177 ±17.8	44 † (36-54)	954 ±64.2	572 ±51.7	16.6 ±1.1	621 ±36.7

† data shown as antilogs of $\log_{10}$-transformed means and 95% confidence limits
In all other cases data is shown as means and standard errors of means.
Group; 1 = healthy individuals, 2 = NTG patients, 3 = HTG patients.

N291S on AUCs for TGs in Sf>400 and Sf20-60 lipoproteins were less consistent, with three individuals having higher levels and four individuals having lower levels than for homozygosity for Asn291. AUC's for apoB-48 in Sf60-400 and Sf20-60 lipoproteins and for apoB-100 in Sf 60-400 lipoproteins were, in general, lower for Ser291 carriers, with the exception of the individual with I.D. no 4089 for Sf 60-400 lipoproteins and the individual with I.D. no. 2067 for small chylomicron remnants (Table 6.6.3). There was less consistency for an effect of N291S on small VLDLs or on circulating LPL activity (measured by AUC for LPL) or on fasting free fatty acid levels (Table 6.6.3).

Section 6.7 Associations between the N291S substitution and atherosclerosis score in the Swedish patients

Associations were sought between genotypes of the N291S substitution and both baseline severity and progression of atherosclerosis in the Swedish patients. Median scores for baseline coronary atherosclerosis (at the first angiography) were almost identical in absence of Ser291 (median score = 0.95) and presence of Ser291 (median score = 0.90). However the median score for global progression of coronary atheroslerosis (on a scale from 0, which means no progression, to 4.0, which means severe progression during the study period) was 86% higher in Ser291 carriers than in those homozygous for Asn291 (1.3 versus 0.7; not significant). Figure 6.7 shows a box plot of progression scores for coronary atherosclerosis for those with and without Ser291. Ser291 carriers had greater extent of global coronary stenosis at baseline than did Asn/Asn individuals (medians being 1.40 and 1.15, respectively, not significant) and worse progression of discrete stenoses between angiographies (medians being 0.90 and 0, respectively, not significant). This suggests that N291S has little effect on established atherosclerosis but has a greater effect on progression of disease.

Table 6.6.3 Characteristics of postprandial lipid and lipoprotein traits in those Swedish individuals with LPL Ser291 and who had postprandial measurements taken.
Levels are <u>not</u> adjusted for fasting TGs, to show the actual levels in each individual

ID	Fasting TG (at 0hr)	AUC for TG; (total)	AUC for TG; Sf>400	AUC for TG; Sf 60-400	AUC for TG; Sf 20-60	ApoB-48 Sf 60-400	ApoB-48 Sf 20-60	ApoB-100 Sf 60-400	ApoB-100 Sf 20-60	AUC; LPL	fasting free fatty acids
units	mM	mM hours	mM hours	mM hours	mM hours	mg/l hours	mg/l hours	mg/l hours	mg/l hours	munits hours	μmol/l
4089	0.99	23.3	5.1	8.7	3.0	54.2	120.5	476.1	600.3	14.6	341
Healthy † individuals without Ser291	1.12 (0.81-1.56)	21.9 (15.7-30.4)	3.5 (2.5-5.1)	6.8 (4.5-10.1)	2.8 (1.9-4.1)	95 ±19.4	36 (20-66)	501 ± 124.2	615 ±147.5	12.1 ±0.9	605 ±60.7
2022	1.46	38.5	6.5	14.3	1.2	40.5	332.9	330.2	501.3	32.3	847
2039	1.49	34.5	5.7	21.6	3.5	29.3	247.4	324.4	1399.5	19.8	550
2067	1.55	21.6	1.9	8.2	3.7	29.9	121.5	456.2	591.3	11.6	875
NTG patients † without Ser291	1.26 (1.02-1.55)	24.0 (18.9-30.4)	4.9 (3.5-6.8)	7.6 (5.0-11.5)	3.4 (2.7-4.2)	136 ±27.0	45 (29-69)	490 ±69.3	679 ±137.8	17.9 ±2.0	633 ±80.5
2025	1.93	39.6	13.0	21.1	5.3	ND	266.6	ND	1369.8	19.0	572
2068	4.15	108.0	12.3	58.4	9.5	81.0	349.9	831.8	2180.1	11.8	638
2077	2.49	47.4	8.4	14.3	6.4	ND	ND	ND	ND	22.8	405
HTG patients † without Ser291	3.02 (2.33-3.92)	58.9 (46.0-75.4)	16.6 (12.5-22.0)	28.2 (22.0-36.1)	7.1 (4.9-10.2)	317 ±44.7	54 (36-80)	738 ±123.7	1727 ±189.5	18.3 ±1.7	599 ±40.0

† unadjusted data is shown

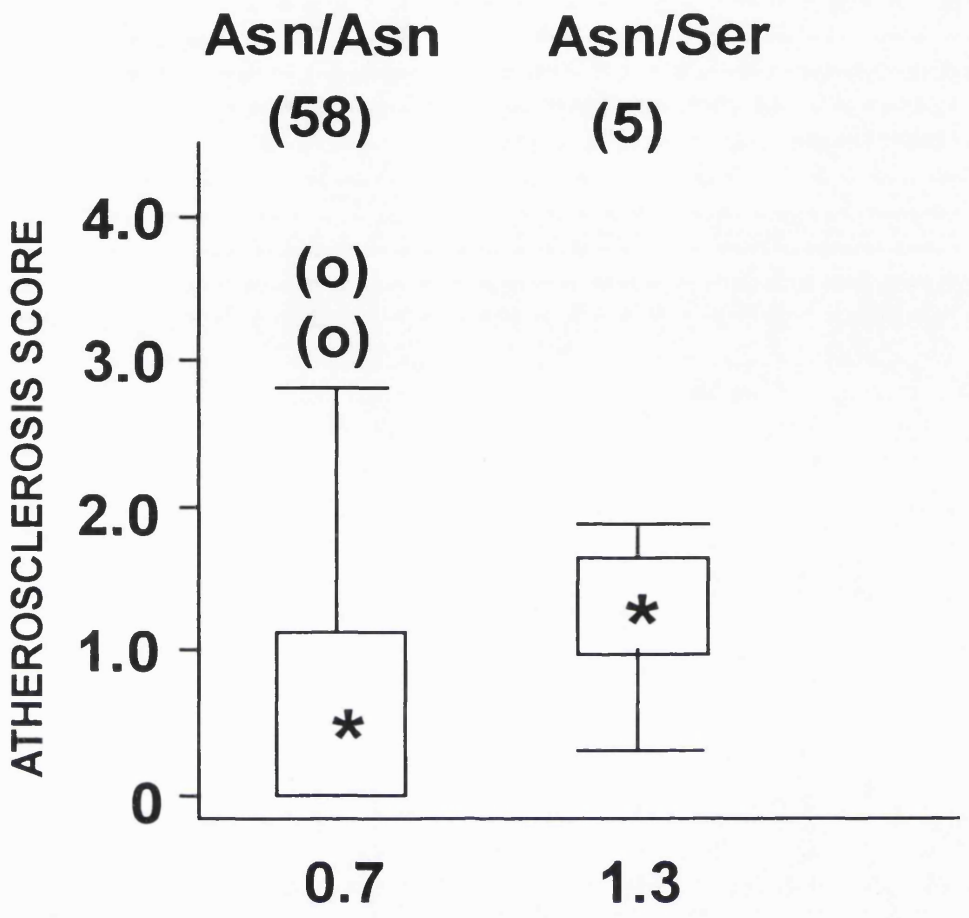


Figure 6.7

Box plot showing the distribution of progression score for coronary atherosclerosis for absence and presence of Ser291 in LPL. Progession scores are given on the vertical axis. Numbers of individuals (in brackets) and genotypes are given above the boxes on the horizontal axis. Values of medians are shown below the respetive means. 50% of individuals have values within the box, which represents the interquartile range. (o) = outi er (= individual who has a score that is at least two standard deviations away from the mean score for that subgroup)

Section 6.8 Inferences about the possible functional significance of the N291S substitution.

Ma et al. (1993a) reported that LPL with Ser at amino acid 291, produced by site-directed mutagenesis, caused a 50% reduction in LPL dimer mass and activity as compared to the wild-type LPL control; however the heparin-binding affinity of the mutant LPL was similar to that of normal LPL. The 50% reduction in LPL dimer mass might be due to decreased stability of the dimer and therefore a shift in equilibrium in favour of the monomer. Ser291 is next to one of three sites (residues 292 to 306) proposed to be important in heparin-binding (discussed earlier in Section 1.6.1), so a (mild) lowering effect of Ser291 on heparin binding cannot be ruled out. Wong et al. (1994) propose a homodimeric model, in which functional LPL is arranged in a head-to-tail dimer, with a 2-fold rotational axis of symmetry. The close proximity between the C-terminal domain of one monomer and the N-terminal domain of the other monomer suggests that there are several points of contact between the two monomers, which generates van-der-Waals forces holding the homodimer together. Amino acid residues on the surfaces of both the N- and C-terminal domains would be directly involved in this van-der-Waals force generation and maintenance. However it is also possible to envisage a role of buried residues in modulating this interaction between monomers. The side group of Ser is CH_2OH while that of Asn is CH_2CONH_2. It is thus feasible that the smaller side group of Ser291 will lead to a smaller contact surface at one point of contact and hence weaker forces holding the monomers together. This would explain the small decrease of 6.1% in postheparin LPL activity in the healthy individuals and the 12.7% decrease in postprandial circulating LPL activity in the sub-sample who had their postprandial responses to an oral fatty load investigated in Ser291 carriers. These relatively small differences in <u>in-vivo</u> LPL activity are in contrast to the large (50%) <u>in-vitro</u> reduction in LPL dimer mass and activity with LPL Ser-291 compared to LPL Asn-291 (Ma et al. 1993a). This suggests that, in the <u>in-vivo</u> situation, genetic (e.g. apoE) and/or enviromental factors (e.g. diet) interact with N291S <u>in-vivo</u> to migitate the severe lowering effect of N291S on LPL activity. However the small (3.1%) increase in postheparin LPL activity in those patients with Ser291 is hard to explain in the context of the results from the healthy individuals. Other factors e.g. disturbed lipoprotein metabolism (higher TGs, lower HDL) and presence of atherosclerosis in the patients may interact with Ser291 and override the predicted destabilizing effect of Ser291 on the LPL dimer.

The higher mean AUC for LPL for presence of Ser291 (Table 6.6.1) suggests that LPL Ser-291 binds less well to the capillary endothelium and less of it exists as a dimer, with a greater proportion being found in the capillary lumen i.e. in the circulation. This would explain the higher mean levels of circulating LPL activity despite a small decrease of 3.9% in mean levels of FFAs in Ser291 carriers. This is in constrast to the 23.6% higher levels of FFAs in individuals homozygous for the H+ allele of the HindIII polymorphism (Table 5.9.1). This suggests that the mechanism underlying the increased levels of circulating LPL activity in the presence of Ser291 is independent of levels of circulating FFAs and distinct from the mechanism suggested for alleles of the HindIII polymorphism (Section 5.9), which is that the LPL enzyme coded for by the H+ allele of the HindIII polymorphism is more suspectible to dissociation brought about by accumulation of FFAs. The higher mean AUCs for Sf 60-400 lipoproteins in the presence of Ser291 (Table 6.6.1) suggests that lipolysis of these TG-rich lipoproteins is slower. This impaired lipolysis is also suggested by the higher levels of large chylomicron remnants in the mid-postprandial period in Ser291 carriers (Figure 6.6.2). This slower lipolysis could explain the lower mean AUCs for postprandial Sf 20-60 lipoproteins (mainly remnant lipoproteins) in the presence of Ser291. Figure 6.6.1 suggests that another mechanism also operates in the late postprandial period; this is the slower uptake of remnant lipoproteins. This suggests that N291S might slow down uptake of remnant lipoproteins via LRP through lower affinity between LPL and lipoproteins. LPL has been reported to act as a ligand for hepatic LRP, increasing the uptake of remnant lipoproteins, which is thought to be independent of TG hydrolysis by LPL (Beisiegel et al. 1991).

It would be of interest to carry out association studies on independent population samples for the N291 substitution to confirm, or otherwise, the associations of the N291S substitution with fasting and postprandial lipids and lipoproteins. If the associations of Ser291 with slower lipolysis and catabolism of remnant lipoproteins (suggested to be via decreased affinity for substrate TG-rich lipoproteins and their remnants) can be confirmed in another sample, in-vitro investigations, using site-directed mutagenesis and competition assays with labelled/unlabelled lipoproteins, into the precise mechanism of Ser291 on decreasing affinity of LPL for lipoproteins would be warranted. However, the smaller associations seen with N291S than with the HindIII polymorphism indicate that a search is

needed for other variation in the LPL gene that would explain the rest of the associations seen with genotypes of the HindIII polymorphism. This could be achieved by a strategy combining PCR and SSCP and using different criteria (selecting the highest and lowest levels in the sample for SSCP screening of exons) to choose individuals. These criteria could be levels of circulating (unstimulated) LPL activity, fasting and postprandial TG levels, or level of fasting HDL-cholesterol. Additionally, other regions of the LPL gene could be searched, such as the promoter and exon 1 which encodes the signal peptide region of LPL.

Chapter 7 Conclusions

The results presented in this thesis suggest that variation at several gene loci (apoB, apoCIII and LPL) has an additive effect on the development of atherosclerosis, in a sample of young Swedish myocardial infarction (MI) survivors, that is explained by a combination of effects of this genetic variation on fasting and postprandial lipid and lipoprotein traits. This is suggested by the finding that associations between genetic variation and baseline severity of atherosclerosis in these patients were stronger than those between this variation and fasting lipids and lipoproteins (Chapter 3). This same finding was made in an independent sample of older British men with documented CAD (Chapter 4). It is possible to reconstruct a flow diagram connecting genetic variation in these candidate genes with the clinical manifestation of MI, linked by a number of 'intermediate' fasting and postprandial traits (Chapter 3). This chart implicates a key role for both fasting and postprandial TGs in the development of atherosclerosis and increased coagulation. Both of the latter processes are of key importance in the increased risk for thrombosis which ultimately leads to a MI. Whether associations between genetic variation at the apoB, apoCIII and LPL gene loci with severity of atherosclerosis, are in part, mediated via proteins involved in the coagulation cascade, such as Factor VII and fibrinogen, still remains to be answered. Genotying of larger patient samples would provide a more definitive answer to the question of whether the difference in extent of atherosclerosis observed with combined genotypes is mediated via several mechanisms acting in concert and whether this will be relevant to large-scale screening and identification of individuals who are at increased risk of atherosclerosis due to their particular combination of common genotypes. Results from the British patient sample (Chapter 4) suggest that the Val591 allele in the apoB gene alone or in combination with other variation in linkage disequilibrium with the X- allele of the XbaI polymorphism is explaining the association with greater degree of atherosclerosis, and that variation at the apoAI-CIII-AIV gene cluster may be influencing prognosis independently of treatment regimen. These different patterns of associations between genotypes and atherosclerosis severity in the Swedish and British patient samples are most likely to be due to differences in both the selection criteria and the system used to measure severity of atherosclerosis. Whether this is the true explanation or not requires independent studies to be carried out, employing similar and different criteria. The ultimate aim is to identify the functional mutations that cause genetic predisposition to more severe disease, and to ascertain the

influence of environmental and/or genetic interaction with these mutations on the pathogenesis of atherosclerosis. This would be useful in the quest for methods of identifying individuals who would require additional therapy or intervention to reduce their risk of developing CAD.

The H+ allele of the LPL-HindIII polymorphism (which is in an intron) was associated with higher mean TG levels in a sample of Swedish healthy individuals (Chapter 3), supporting the hypothesis previously put forward (Chamberlain et al. 1989, Thorn et al. 1990) that the H+ allele of the LPL-HindIII polymorphism is associated with a variant in or near the LPL gene that predisposes to HTG. However, associations between the HindIII polymorphism and HDL-cholesterol levels have been less consistent; larger population studies will be needed to confirm any association between the LPL PvuII and HindIII polymorphisms and HDL-cholesterol, and to ascertain a possible association between the V+H+ haplotype and an atherogenic lipid profile. Effects of variation in the LPL gene on lipids is most likely to be mediated via changes in LPL enzymatic activity and/or affinity of LPL for substrate lipoproteins or heparin-like proteoglycans. So the question is whether alleles of the HindIII polymorphism are encoding a LPL protein that has differing affinities which would thus affect metabolism of TG-rich lipoproteins. The screening of exons of the LPL gene (Chapter 6) identifies an Asn291 to Ser substitution in exon 6, which is on the haplotype defined by the common alleles of the PvuII and HindIII polymorphisms. Confirmation of the Ser291 allele being on the V+H+ haplotype will need genotyping of larger population samples. Results from Chapter 6 demonstrate that the N291S substitution, which occurs at an approximate frequency of 0.04 in the Swedish sample, does not explain all of the effects on fasting or postprandial lipid traits seen with genotypes of the HindIII polymorphism. The Ser291 variant occurs in both the patients and healthy individuals. This is in contrast to the results reported by Ma et al. 1993a, that Ser291 was found in Type III hyperlipoproteinaemic individuals, but not in controls, and raises the question of how widespread the N291S substitution really is. However it is clear that other as yet undiscovered variation in or near the LPL gene must be identified to explain the rest of the effects seen with the HindIII polymorphism on plasma lipids and atherosclerosis. Screening of the LPL gene using different criteria to select individuals, also screening of the promoter and signal peptide region of the LPL gene is required to unearth this other variation.

As detailed in Chapter 5, genotypes of common polymorphisms in the apoB, apoCIII and LPL genes, that all encode proteins with well known roles in lipid metabolism, have appreciable effects on postprandial lipid metabolism, after removing variability in postprandial lipaemia attributable to fasting TGs. These effects are via production (apoB-signal peptide polymorphism), lipolysis (apoCIII and LPL) and catabolism (apoB-XbaI polymorphism and common apoE isoforms) of TG-rich lipoproteins and their remnants. Larger patient samples will be needed to identify the common genetic variation that determines plasma levels of remnants, produced as a result of metabolism of TG-rich lipoproteins. The concept that these remnants are atherogenic is embodied in the Zilversmit hypothesis (Zilversmit 1979) and is supported by recent observations of a positive association between small chylomicron remnants and progression of atherosclerosis (Karpe et al. 1994). The idenfication of causative mutations would be useful, in conjunction with elevated fasting lipids, in identifying those individuals, at risk of atherosclerosis due to aberrant postprandial lipid metabolism. Characterized genes that have a known effect on HDL metabolism, such as those coding for CETP, LCAT and hepatic lipase, could affect postprandial lipid metabolism, and hence prevalent and progression of atherosclerosis. The recently isolated gene for MTTP, which affects metabolism of apoB-containing lipoproteins, is another candidate gene for population association studies. The investigation of larger samples, using a limited number of time points during the postprandial period, to confirm the results in Chapter 5 is warranted. This would go a long way towards answering the question of how to design screening programs to identify common mutations or amino acid combinations in the gene product (i.e. apoB, apoCIII and LPL) that themselves cause aberrant postprandial lipoprotein metabolism. Once these mutations have been discovered, this would be the first step toward developing specific treatments, such as modified diet, for individuals carrying these mutations, to decrease their risk of developing advanced atherosclerosis.

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Appendix I - Genotype data on the Swedish healthy individuals

* = genotype not determined

DNA			APOB GENOTYPES				
			Signal				
NUMBER	APOE	C_{-265}-T	Peptide	Ag(a1/d)	XbaI	EcoR1	Ag(x/y)
4000	4/3	CC	27/24	dd	X+X+	R+R+	yy
4001	2/3	CC	27/24	a1d	X+X-	R+R+	xy
4002	4/4	CC	27/24	a1d	X+X-	R+R+	xy
4003	4/3	CC	27/27	a1a1	X+X+	R+R+	yy
4004	4/3	CC	27/24	dd	X+X-	R+R+	yy
4005	3/3	CC	24/24	dd	X+X+	R+R+	yy
4006	3/3	CT	27/24	a1d	X+X-	R+R+	yy
4007	3/3	CT	27/27	a1d	X+X-	R+R-	yy
4008	3/3	CT	27/27	a1a1	X-X-	R+R-	xy
4009	4/3	CC	27/27	a1a1	X-X-	R+R+	xx
4010	3/3	CC	27/24	dd	X+X+	R+R+	yy
4011	4/3	CC	27/27	a1a1	X+X-	R+R-	yy
4012	4/2	CC	27/27	a1a1	X-X-	R+R+	xx
4013	3/3	CC	27/27	a1d	X+X-	R+R+	xy
4014	3/3	CC	27/27	a1a1	X+X-	R+R-	yy
4015	3/3	CC	27/24	a1d	X+X-	R+R+	xy
4016	3/3	CC	27/24	a1d	X+X-	R+R+	xy
4017	3/3	CT	27/27	a1d	X-X-	R+R-	*
4018	2/3	CC	27/27	a1d	X+X-	R+R+	xy
4019	4/3	CC	27/27	a1d	X-X-	R+R+	xy
4020	4/3	CC	27/24	a1a1	X+X-	R+R+	xy
4021	4/3	CC	27/27	a1d	X+X-	R+R-	yy
4022	4/3	CC	24/24	dd	X+X+	R+R+	yy
4023	3/3	CC	27/24	a1d	X+X-	R+R+	yy
4024	3/3	CC	27/24	a1d	X-X-	R+R-	yy
4025	3/3	CT	27/27	a1a1	X-X-	R+R-	xy
4026	4/2	CC	27/24	a1d	X+X-	R+R-	yy
4027	2/3	TT	27/27	a1d	X-X-	R+R+	yy
4028	3/3	TT	27/27	a1d	X-X-	R+R-	yy
4029	3/3	CT	27/24	a1d	X+X+	R+R+	yy
4030	4/3	CC	27/24	a1d	X+X-	R+R+	xy
4031	3/3	CC	27/24	a1a1	X+X+	R+R+	yy
4032	4/3	CC	24/24	a1d	X+X+	R+R+	yy
4033	*	CC	27/27	a1a1	X-X-	R+R-	xy
4034	4/3	CC	27/27	a1a1	X-X-	R-R-	yy
4035	4/3	CC	27/24	a1d	X+X+	R+R+	yy
4036	3/3	CC	27/27	a1a1	X-X-	R-R-	yy
4037	3/3	CT	27/27	a1a1	X+X-	R+R-	yy
4038	2/3	CT	27/24	a1d	X+X-	R+R-	yy
4039	2/3	CT	27/24	a1d	X+X-	R+R-	yy
4040	4/2	CC	24/24	dd	X+X+	R+R+	yy
4041	4/2	CC	27/27	a1a1	X+X-	R+R+	xy
4042	2/3	CC	24/24	dd	X+X+	R+R+	yy
4043	4/3	CC	24/24	dd	X+X+	R+R+	yy
4044	3/3	CC	27/27	a1a1	X+X+	R+R+	yy
4045	4/4	CT	27/27	dd	X+X-	R+R+	yy
4046	4/3	CC	27/24	a1d	X+X+	R+R+	yy
4047	4/3	CC	27/24	a1d	X+X+	R+R+	yy
4048	3/3	CC	27/24	dd	X+X+	R+R+	yy

APOB GENOTYPES

DNA NUMBER	APOE	C_{-265}-T	Signal Peptide	Ag(a1/d)	XbaI	EcoR1	Ag(x/y)
4049	3/3	CC	27/24	dd	X+X+	R+R+	yy
4050	2/3	CC	27/27	a1d	X+X-	R+R-	yy
4051	3/3	CC	27/24	a1d	X+X+	R+R+	yy
4052	4/4	CC	27/24	a1d	X+X+	R+R+	yy
4053	3/3	CC	*	*	*	*	*
4054	2/3	CC	27/24	a1d	X-X-	R+R+	xy
4055	3/3	CC	27/24	dd	X+X-	R+R+	yy
4056	3/3	CC	27/27	a1d	X+X-	R+R-	yy
4057	3/3	CC	27/27	a1a1	X-X-	R+R-	yy
4058	2/3	CC	27/24	dd	X+X-	R+R-	yy
4059	3/3	CC	27/24	dd	X+X+	R+R+	yy
4060	3/3	CC	27/24	dd	X+X-	R+R+	yy
4061	3/3	CC	27/24	a1d	X+X+	R+R+	yy
4062	3/3	CC	27/27	dd	X+X+	R+R+	yy
4064	4/3	CC	27/27	a1d	X+X-	R+R+	xy
4065	4/3	CT	27/27	a1d	X+X-	R+R-	yy
4066	*	*	*	*	*	*	*
4067	3/3	CC	27/24	a1d	X+X-	R+R-	yy
4068	2/3	CC	27/24	dd	X+X+	R+R+	yy
4069	3/3	CT	27/24	a1a1	X-X-	R+R-	xy
4070	2/3	CT	27/27	a1d	X+X-	R+R+	yy
4071	3/3	CC	27/24	a1d	X+X-	R+R+	xy
4074	3/3	CT	27/24	a1d	X+X-	R+R-	yy
4075	*	*	*	*	*	*	*
4076	4/4	CC	27/27	a1a1	X+X-	R+R+	xy
4077	3/3	CT	27/24	a1d	X+X-	R+R-	yy
4078	3/3	CC	27/27	a1a1	X-X-	R-R-	yy
4079	3/3	CT	27/27	a1d	X+X-	R+R-	yy
4080	4/3	CC	27/27	a1a1	X-X-	R+R+	xx
4081	3/3	CC	27/27	a1d	X+X+	R+R+	yy
4082	3/3	CC	27/27	a1a1	X-X-	R+R+	xx
4083	3/3	CT	27/27	a1a1	X+X-	R+R-	yy
4084	4/3	CT	27/27	a1d	X+X-	R+R-	yy
4085	3/3	CC	27/24	a1d	X+X-	R+R+	xy
4086	3/3	CC	27/27	a1a1	X+X-	R+R+	xy
4087	3/3	CC	27/24	a1d	X+X+	R+R+	yy
4088	2/3	CC	27/27	a1a1	X-X-	R+R-	xy
4089	3/3	CC	27/27	a1a1	X-X-	R+R+	xx
4090	3/3	CC	27/24	dd	X+X+	R+R+	yy
4091	3/3	CC	24/24	a1d	X+X+	R+R+	yy
4092	4/3	CC	27/27	a1d	X+X-	R+R+	xy
4093	4/3	CC	27/24	a1a1	X+X-	R+R+	xy
4094	3/3	CC	27/27	a1a1	X+X+	R+R+	yy
4095	3/3	CC	27/27	a1d	X+X-	R+R-	yy

DNA NUMBER	G_{-75}-A apoAI	APOAI-CIII-AIV GENOTYPES PstI 3'apoAI	C_{1100}-T apoCIII	PvuII apoCIII
4000	GG	++	CC	++
4001	GG	++	CT	++
4002	GA	++	TT	++
4003	GG	++	CC	+-
4004	GG	++	CC	++
4005	GG	++	CT	++
4006	GG	++	CC	++
4007	GG	++	CC	+-
4008	GG	++	CC	--
4009	GA	++	CT	++
4010	GA	++	CT	++
4011	GG	+-	CC	++
4012	GA	++	TT	++
4013	GG	++	CC	+-
4014	GG	++	CT	++
4015	*	++	CC	--
4016	GG	++	CT	++
4017	AA	++	CT	+-
4018	GG	++	CC	++
4019	GA	++	CT	++
4020	GG	++	CC	+-
4021	GG	*	*	*
4022	GA	++	CT	++
4023	GG	++	CT	++
4024	GG	++	CC	++
4025	GG	++	CC	++
4026	GG	++	CC	++
4027	GA	++	CC	++
4028	GG	++	CC	++
4029	GG	++	CC	++
4030	GG	++	CT	++
4031	GG	++	CC	++
4032	GG	+-	CC	+-
4033	GG	++	CT	+-
4034	GA	+-	CT	++
4035	GG	++	CC	++
4036	GG	++	CT	++
4037	GG	++	CC	--
4038	GG	++	CC	++
4039	GG	++	CC	--
4040	GG	*	CC	+-
4041	GG	++	CC	++
4042	GA	+-	CT	++
4043	GG	++	CT	++
4044	GG	++	CC	++
4045	GG	++	CT	++
4046	GA	++	*	++
4047	GG	++	CC	+-
4048	GG	++	CT	++
4049	GA	++	CT	++

DNA NUMBER	G_{-75}-A apoAI	APOAI-CIII-AIV GENOTYPES PstI 3'apoAI	C_{1100}-T apoCIII	PvuII apoCIII
4050	GG	++	CC	++
4051	GG	++	CT	++
4052	GG	+-	CC	++
4053	GG	++	CC	++
4054	GA	++	CT	++
4055	GG	+-	CC	++
4056	GG	++	CT	++
4057	GA	++	CT	++
4058	GG	++	TT	++
4059	GG	+-	CC	++
4060	GA	+-	CT	++
4061	GG	++	CC	--
4062	GG	++	CC	++
4064	AA	++	TT	++
4065	GG	++	CC	++
4066	GG	++	CC	+-
4067	GG	++	CC	++
4068	GG	++	CC	+-
4069	GG	++	CC	+-
4070	GA	++	CT	++
4071	GG	++	CC	++
4074	GG	++	CC	++
4075	GG	++	CC	--
4076	*	++	CC	++
4077	GG	++	CC	+-
4078	GG	++	CT	++
4079	GA	++	CT	+-
4080	GG	++	CC	++
4081	GG	+-	CC	+-
4082	*	++	CT	++
4083	GA	++	CT	*
4084	GG	++	CC	--
4085	GG	++	CT	++
4086	GG	++	CC	++
4087	GG	++	CC	+-
4088	GG	++	CC	+-
4089	GA	++	CC	++
4090	GG	++	CC	*
4091	GG	++	TT	++
4092	AA	++	TT	++
4093	GA	++	CT	++
4094	GG	++	CC	++
4095	GG	++	CT	++

LPL GENOTYPES

DNA NUMBER	Asn291 -Ser	PvuII	HindIII	Ser447-Stop
4000	NN	V+V-	H+H-	SSt
4001	NN	V+V-	H+H-	SS
4002	NN	V+V+	H+H+	SS
4003	NN	V-V-	H-H-	SSt
4004	NN	V+V-	H+H-	SS
4005	NN	V+V-	H+H+	SS
4006	NN	V+V-	H+H+	SS
4007	NN	V+V+	H+H+	SS
4008	*	V+V+	H+H-	SS
4009	NN	V+V-	H+H+	SS
4010	NN	V+V-	H+H+	SS
4011	NN	V+V-	H+H+	SS
4012	NN	V+V+	H+H+	SS
4013	NN	V-V-	H+H+	SS
4014	NN	V+V+	H+H+	SS
4015	NN	V+V-	H+H-	SS
4016	NN	V+V+	H+H+	SS
4017	NN	V+V+	H+H-	SS
4018	NN	V-V-	H-H-	SS
4019	NN	V+V-	H+H+	SS
4020	NS	V+V+	H+H+	SS
4021	*	V+V-	*	*
4022	NS	V+V-	H+H-	SS
4023	NN	V+V-	H+H-	SS
4024	NN	V+V-	H+H+	SS
4025	NN	V+V-	H+H-	SSt
4026	NN	V+V+	H+H-	SS
4027	NN	V-V-	H+H+	SS
4028	NN	V+V-	H+H+	SS
4029	NN	V-V-	H+H-	SS
4030	NN	V+V+	H+H+	SS
4031	NN	V+V+	H+H+	SS
4032	NN	V-V-	H+H+	SS
4033	NN	V+V-	H+H+	SS
4034	NN	V+V+	H+H+	SS
4035	NN	V-V-	H+H-	SS
4036	NN	V+V+	H+H+	SS
4037	NN	V+V-	H+H-	SS
4038	NN	V+V+	H+H+	SS
4039	NN	V+V+	H+H+	SS
4040	NN	V-V-	H+H+	*
4041	NN	V+V-	H+H+	SS
4042	NN	V+V-	H+H+	SS
4043	NN	V+V+	H+H-	SS
4044	NN	V-V-	H+H-	SS
4045	NN	V+V-	H+H+	SS
4046	NS	V+V-	H+H-	SS
4047	NN	V-V-	H+H-	SS
4048	NN	V+V-	H+H-	*
4049	NN	V+V-	H+H-	SS

LPL GENOTYPES

DNA NUMBER	Asn291 -Ser	PvuII	HindIII	Ser447-Stop
4050	NN	V+V+	H+H+	SS
4051	NS	V+V+	H+H-	*
4052	NN	V+V-	H+H-	SS
4053	NN	V+V-	H+H-	SS
4054	NN	V+V+	H+H+	SS
4055	NN	V-V-	H+H-	SSt
4056	NN	V-V-	H-H-	StSt
4057	NN	V+V-	H+H+	SS
4058	*	V+V+	H+H+	SS
4059	NN	V+V-	H+H+	SS
4060	NN	V+V-	H+H+	SS
4061	NN	V+V-	H+H-	SS
4062	NN	V+V-	H+H+	SS
4064	NN	V+V-	H+H-	SS
4065	NN	V+V-	H+H+	SS
4066	NN	V+V-	H+H-	SS
4067	NN	V-V-	H+H+	SS
4068	NN	V+V+	H+H+	SS
4069	NS	V+V+	H+H+	SS
4070	NN	V+V+	H+H+	SS
4071	NN	V+V+	H+H+	SS
4074	NN	V-V-	H+H+	SS
4075	NN	V+V-	H+H+	SS
4076	NN	V-V-	H+H-	SS
4077	NN	V+V+	H+H+	SS
4078	NN	V+V+	H+H+	SS
4079	NN	V+V+	H+H+	SS
4080	NN	V+V+	H+H+	SS
4081	NN	V+V-	H+H+	SS
4082	NN	V-V-	H+H-	SS
4083	NN	V+V+	H+H-	SS
4084	NN	V+V-	H+H-	SSt
4085	NN	V+V-	H+H+	SS
4086	NN	V+V-	H+H+	SS
4087	NN	V+V+	H+H+	SS
4088	NN	V+V-	H+H-	SS
4089	NS	V+V-	H+H-	SS
4090	NN	V+V+	H+H+	SS
4091	*	V+V-	H+H-	SSt
4092	NN	V-V-	H+H-	SS
4093	NN	V-V-	H+H-	SSt
4094	NN	V+V-	H+H+	SS
4095	NN	V-V-	H+H-	SSt

Appendix II - Genotype data on the Swedish Patients
* = genotype not determined

DNA NUMBER	APOE	C_{-265}-T	Signal Peptide	APOB GENOTYPES Ag(a1/d)	XbaI	EcoR1	Ag(x/y)
2004	4/3	CC	24/24	dd	X+X+	R+R+	yy
2005	3/3	CC	27/24	a1d	X+X-	R+R+	xy
2006	3/3	CT	27/24	a1d	X+X-	R+R-	yy
2008	4/3	CC	27/27	a1a1	X-X-	R+R-	xy
2009	3/3	CC	27/24	a1d	X+X-	R+R-	yy
2013	3/3	TT	27/27	a1d	X-X-	R+R-	yy
2016	3/3	CC	27/27	a1d	X+X+	R+R+	yy
2018	3/3	CC	27/27	a1a1	X+X-	R+R-	yy
2020	2/3	CC	27/27	a1d	X+X-	R+R+	xy
2021	4/4	CC	27/27	a1d	X+X-	R+R+	xy
2022	3/3	CT	27/27	a1d	X-X-	R+R+	xy
2023	3/3	CT	27/27	a1d	X-X-	R+R-	yy
2024	3/3	CC	27/27	a1d	X-X-	R+R-	yy
2025	3/3	CC	27/24	dd	X+X-	R+R+	yy
2026	4/3	CC	27/24	dd	X-X-	R+R+	yy
2027	4/3	CC	24/24	a1d	X-X-	R+R+	xy
2028	3/3	CT	27/27	dd	X-X-	R+R+	yy
2029	3/3	CC	27/24	a1d	X+X+	R+R+	yy
2033	4/3	CC	24/24	dd	X+X-	R+R+	yy
2036	4/3	CC	27/24	a1d	X+X-	R+R+	xy
2037	3/3	CC	27/27	a1a1	X-X-	R+R+	xx
2039	4/3	CC	27/27	a1d	X-X-	R+R+	xy
2040	4/3	CC	24/24	dd	X+X+	R+R+	yy
2041	3/3	CC	24/24	dd	X+X+	R+R+	yy
2042	4/3	CT	27/27	dd	X-X-	R+R+	yy
2043	2/3	CT	27/27	a1d	X+X-	R+R+	yy
2048	4/3	CC	27/24	dd	X+X+	R+R+	yy
2050	3/3	CC	27/24	dd	X+X+	R+R+	yy
2051	4/3	CC	27/27	a1a1	X+X+	R+R+	yy
2052	4/2	CC	27/27	a1d	X+X+	R+R+	yy
2053	4/4	CC	27/27	a1d	X-X-	R+R+	xy
2054	3/3	CC	27/24	a1d	X+X-	R+R+	xy
2055	4/3	CC	27/24	a1d	X+X-	R+R+	xy
2056	2/3	CT	27/27	a1a1	X-X-	R+R-	xy
2057	3/3	CC	27/24	a1d	X+X-	R+R-	yy
2058	3/3	CT	27/27	a1d	X-X-	R+R+	xy
2059	3/3	CC	27/27	dd	X+X+	R+R+	yy
2060	3/3	CC	27/27	a1d	X-X-	R+R-	xy
2061	2/3	CC	27/24	a1d	X+X-	R+R-	xy
2062	4/2	CT	27/27	dd	X+X-	R+R+	yy
2063	3/3	CC	*	dd	X+X+	R+R+	yy
2064	3/3	CC	27/24	a1a1	X-X-	R+R+	xx
2065	4/2	CC	27/27	a1d	X-X-	R+R+	xy
2066	4/3	CC	24/24	a1d	X+X-	R+R+	xy
2067	3/3	CT	27/27	a1d	X-X-	R+R-	yy
2068	3/3	CC	27/24	a1d	X-X-	R+R-	yy
2069	3/3	CT	27/27	a1d	X+X-	*	yy
2070	4/3	CC	27/27	a1a1	X+X-	R+R-	yy
2071	3/3	CC	27/27	a1d	X+X-	R+R+	yy

APOB GENOTYPES

DNA NUMBER	APOE	C_{-265}-T	Signal Peptide	Ag(a1/d)	XbaI	EcoR1	Ag(x/y)
2072	3/3	CC	27/27	a1d	X+X+	R+R+	yy
2074	3/3	CT	27/27	a1d	X+X-	R+R+	xy
2075	3/3	CC	27/24	a1a1	X+X-	R+R-	yy
2076	4/3	CC	27/27	a1a1	X+X-	R+R-	yy
2077	3/3	CC	27/24	a1d	X+X-	R+R+	xy
2078	4/4	*	27/27	a1d	X+X-	R+R+	xy
2079	3/3	CC	27/24	dd	X+X+	R+R+	yy
2080	3/3	CC	24/24	dd	X+X+	R+R+	yy
2081	3/3	CC	24/24	a1d	X+X-	R+R-	yy
2082	4/4	CC	27/27	a1a1	X+X-	R+R+	xy
2084	3/3	CC	27/24	a1a1	X-X-	R+R-	xy
2085	4/3	CC	27/27	a1d	X+X-	R+R+	xy
2087	4/3	CC	24/24	dd	X+X+	R+R+	yy
2088	4/3	CC	24/24	dd	X-X-	R+R+	xy
2089	3/3	CC	27/24	a1d	X+X+	R+R+	yy
2090	3/3	CC	27/24	a1d	X+X-	R+R+	xy
2091	4/3	CC	27/27	dd	X+X+	R+R+	yy
2093	2/3	CT	27/27	a1d	X+X-	R+R-	yy
2097	3/3	CT	27/27	dd	X+X-	R+R+	yy
2100	2/3	*	27/24	a1d	X+X-	R+R+	xy
2101	4/3	CC	27/27	dd	X+X-	R+R+	yy
2112	3/3	CC	27/24	dd	X+X+	R+R+	yy
2114	4/3	CC	27/24	a1d	X+X-	R+R-	yy
2117	4/3	CC	27/27	a1a1	X+X-	R+R-	yy
2118	4/3	CC	27/27	a1a1	X+X-	R+R+	xy
2119	3/3	CC	27/24	dd	X+X+	R+R+	yy
2120	3/3	CC	27/27	a1a1	X+X-	R+R-	yy
2122	4/2	CC	27/24	dd	X+X+	R+R+	yy
2123	3/3	CC	27/24	a1d	X+X-	R+R+	xy
2124	4/2	CC	24/24	dd	X+X+	R+R+	yy
2125	4/4	CC	24/24	dd	X+X+	R+R+	yy
2126	3/3	CC	27/27	a1a1	X-X-	R-R-	yy
2127	4/3	CC	27/27	a1d	X+X+	R+R+	yy
2128	3/3	CC	24/24	dd	X+X+	R+R+	yy
2142	3/3	CC	27/24	a1d	X+X+	R+R+	yy
2143	3/3	CC	24/24	dd	X+X+	R+R+	yy
2144	3/3	CC	27/24	dd	X+X+	R+R+	yy
2146	3/3	CT	27/27	a1d	X+X-	R+R-	yy

APOAI-CIII-AIV GENOTYPES

DNA NUMBER	G_{-75}-A apoAI	PstI 3'apoAI	C_{1100}-T apoCIII	PvuII apoCIII
2004	GG	++	CC	V+V-
2005	GG	+-	CT	V+V+
2006	GA	++	CT	V+V+
2008	GG	++	CC	V+V-
2009	GG	++	CC	V+V-
2013	AA	++	CT	V+V+
2016	GG	++	CT	V+V+
2018	GG	++	CT	V+V+
2020	GA	++	TT	V+V+
2021	GG	++	CT	V+V+
2022	GG	++	CT	V+V-
2023	GG	++	CC	V+V+
2024	GG	++	CC	V+V+
2025	GG	++	CC	V+V+
2026	GG	++	CT	V+V+
2027	GG	++	CT	V+V+
2028	GA	++	CT	V+V-
2029	GG	+-	CC	V+V-
2033	GG	++	CC	V+V-
2036	GG	++	CT	V+V+
2037	GG	++	CC	V-V-
2039	AA	++	CT	V+V+
2040	GG	++	CC	V+V+
2041	GG	++	CT	V+V+
2042	GG	++	CC	V+V-
2043	GA	++	CT	V+V+
2048	GA	++	CT	V+V+
2050	GA	+-	CT	V+V-
2051	GG	++	CC	V+V-
2052	GG	++	CT	V+V-
2053	GG	++	CC	V+V+
2054	GG	++	CC	V+V-
2055	GG	++	CT	V+V-
2056	GG	++	CC	V+V+
2057	GG	++	CT	V+V+
2058	GA	++	CT	V+V+
2059	GA	++	TT	V+V+
2060	GG	+-	CC	V+V-
2061	GG	++	CC	V+V-
2062	GA	++	CT	V+V+
2063	GG	+-	CC	V+V+
2064	GG	++	CC	V+V-
2065	GG	+-	CC	V+V+
2066	GA	++	CT	V+V+
2067	GG	++	CC	V+V+
2068	GG	++	CC	V+V+
2069	GG	++	CT	V+V-
2070	GG	++	CC	V+V+
2071	GG	++	*	V+V-
2072	GG	++	CC	V+V-

APOAI-CIII-AIV GENOTYPES

DNA NUMBER	G_{-75}-A apoAI	PstI 3'apoAI	C_{1100}-T apoCIII	PvuII apoCIII
2074	GG	++	CT	V+V+
2075	GG	++	CC	V+V+
2076	GG	++	CC	V+V+
2077	GG	++	CC	V+V-
2078	GA	++	CT	V+V-
2079	GG	++	TT	V+V-
2080	GG	++	CT	V+V+
2081	*	++	CT	V+V+
2082	GA	++	CT	V+V+
2084	GG	++	CT	V+V+
2085	GA	++	CT	V+V+
2087	GG	++	CC	V+V+
2088	GG	++	CC	V+V+
2089	GG	++	CC	V-V-
2090	GA	++	CT	V+V+
2091	GG	++	CC	V+V+
2093	GG	++	CC	V+V+
2097	GG	++	CC	V+V+
2100	*	++	CT	V+V+
2101	GG	++	CC	V+V-
2112	GA	+-	CT	V+V+
2114	GG	++	CT	V+V+
2117	GA	++	CT	V+V+
2118	GG	++	CT	V+V-
2119	GA	++	TT	V+V+
2120	GG	++	CC	V+V+
2122	GG	++	CT	V+V-
2123	GG	++	CT	V+V+
2124	GG	+-	CC	V+V-
2125	GG	++	CC	V+V+
2126	GA	++	CT	V+V+
2127	GA	++	CT	V+V+
2128	GG	++	CC	V+V-
2142	*	++	CC	V+V-
2143	GG	++	*	V+V+
2144	GG	++	TT	V+V+
2146	GG	++	CC	V+V+

LPL GENOTYPES

DNA NUMBER	Asn291 -Ser	PvuII	HindIII	Ser447-Stop
2004	NN	V+V-	H+H+	SS
2005	NN	V+V+	H+H-	SS
2006	NN	V+V-	H+H-	SS
2008	NN	V-V-	H+H-	SSt
2009	NN	V+V-	H+H-	SS
2013	NN	V+V+	H+H+	SS
2016	NN	V-V-	H+H+	SS
2018	NN	V+V+	H+H+	SS
2020	NN	V-V-	H+H-	SS
2021	NN	V+V+	H+H+	SS
2022	NS	V+V-	H+H+	SS
2023	NN	V+V-	H+H+	SS
2024	NN	V+V-	H+H+	SS
2025	NS	V+V-	H+H-	SS
2026	NN	V+V-	H+H-	SSt
2027	NN	V-V-	H+H-	SS
2028	NN	V+V+	H+H+	SS
2029	NN	V+V+	H+H+	SS
2033	NN	V+V-	H+H-	SS
2036	NN	V+V-	H+H+	SS
2037	NN	V+V-	H+H-	SS
2039	NS	V+V-	H+H-	SS
2040	NN	V+V+	H+H-	SS
2041	NN	V+V-	H+H-	SS
2042	NN	V+V+	H+H+	SS
2043	NN	V+V-	H+H-	SS
2048	NN	V-V-	H+H+	SS
2050	NN	V-V-	H+H-	SS
2051	NN	V+V-	H+H+	SS
2052	NN	V+V-	H+H+	SS
2053	NN	V+V+	H+H+	SS
2054	NN	V+V-	H+H+	SS
2055	NN	V+V-	H+H+	SS
2056	NN	V+V+	H+H+	SS
2057	NN	V-V-	H+H+	SS
2058	NN	V+V-	H+H-	SS
2059	NN	V-V-	H+H+	SS
2060	NN	V+V+	H+H+	SS
2061	NN	V+V+	H+H+	SS
2062	NN	V+V-	H+H-	SSt
2063	NN	V-V-	H+H-	SS
2064	NN	V+V+	H+H+	SS
2065	NN	V+V+	H+H+	SS
2066	NN	V-V-	H+H-	SSt
2067	NS	V+V-	H+H-	SSt
2068	NS	V+V+	H+H-	SS
2069	NN	V+V+	H+H-	SS
2070	NN	V+V-	H+H-	SSt
2071	NN	V+V+	H+H+	SS
2072	NN	V+V-	H+H-	SS

LPL GENOTYPES

DNA NUMBER	Asn291 -Ser	PvuII	HindIII	Ser447-Stop
2074	NN	V+V+	H+H+	SS
2075	NN	V+V+	H+H+	SS
2076	NN	V+V+	H+H+	SS
2077	NS	V+V-	H+H-	SS
2078	NN	V-V-	H+H+	SS
2079	NN	V+V-	H+H+	SS
2080	*	V+V+	H+H-	SS
2081	NN	V+V-	H+H+	SS
2082	NN	V-V-	H+H-	SS
2084	NN	V+V+	H+H+	SS
2085	NN	V-V-	H+H-	SSt
2087	NN	V+V+	H+H+	SS
2088	NN	V-V-	H+H-	SS
2089	NN	V+V+	H+H+	SS
2090	NN	V+V-	H+H+	SS
2091	NN	V+V-	H+H-	SSt
2093	NN	V+V+	H+H+	SS
2097	NN	V-V-	H+H-	SS
2100	NN	V+V-	H+H-	SS
2101	NN	V+V-	H+H+	SS
2112	NN	V+V+	H+H+	SS
2114	NN	V-V-	H-H-	SS
2117	NN	V-V-	H+H+	SS
2118	NN	V+V-	H+H-	SS
2119	NN	V+V-	H+H+	SS
2120	NN	V+V-	H+H+	SS
2122	NN	V+V-	H+H+	SS
2123	NN	V+V-	H+H+	SS
2124	NN	V+V-	H+H+	SS
2125	NN	V+V-	H+H-	SS
2126	NN	V+V-	H+H+	SS
2127	NN	V+V+	H+H+	SS
2128	NN	V+V+	H+H-	SS
2142	NN	V+V+	*	*
2143	NN	V+V+	H+H+	*
2144	NN	V-V-	H+H-	SSt
2146	NN	V+V+	H+H+	SS

Appendix III - Genotype data on the St. Thomas' Atherosclerosis Regression Study

* = genotype not determined

DNA NUMBER	GW's Study No.	APOE	APOB GENOTYPES C_{-265}-T	Signal Peptide	Ag(a1/d)	XbaI
1	69	3/3	CT	27/24	dd	X-X-
2	27	4/3	CC	27/24	a1d	X+X+
3	83	4/3	CC	27/27	a1d	X-X-
4	79	4/4	CT	27/27	a1a1	X-X-
5	73	4/3	CC	27/24	a1d	X+X-
6	62	4/3	CC	27/24	a1d	X+X-
7	59	3/3	CC	27/27	a1a1	X-X-
8	25	4/3	CC	27/27	a1a1	X+X-
9	70	3/3	CT	27/27	dd	X+X-
10	54	3/3	CC	27/27	a1d	X+X-
11	75	4/3	CT	27/27	a1a1	X+X-
12	56	3/3	CC	27/27	a1d	X+X-
13	85	3/3	CT	27/24	dd	X+X-
14	38	3/3	CC	27/27	a1a1	X-X-
15	22	2/3	CC	27/27	a1a1	X-X-
16	44	4/3	CT	27/24	a1d	X+X-
17	72	4/3	CC	27/24	dd	X+X+
18	46	3/3	CT	27/24	a1d	X+X-
19	26	3/3	CC	27/27	a1d	X+X-
20	65	3/3	CC	27/27	a1d	X+X-
21	64	3/3	CC	27/27	a1d	X+X-
22	7	3/3	CC	27/24	a1d	X+X-
23	99	3/3	CC	27/27	a1d	X+X-
24	86	3/3	CC	27/24	a1d	X+X-
25	84	3/3	CC	27/24	a1d	X+X-
26	49	3/3	CC	27/27	a1d	X+X-
27	40	3/3	CC	24/24	dd	X+X+
28	51	3/3	CC	27/27	a1a1	X-X-
29	35	3/3	CC	27/24	a1d	X+X-
30	52	4/3	CC	24/24	dd	X+X+
31	34	3/3	CT	27/27	a1d	X+X-
32	33	3/3	CC	27/27	a1d	X+X-
33	99	4/3	CT	27/27	a1d	X+X-
34	3	3/3	CC	27/24	a1d	X+X-
35	47	3/3	CT	27/27	a1d	X+X-
36	99	3/3	CC	27/27	a1d	X+X-
37	2	4/3	CC	27/24	a1d	X+X-
38	28	3/3	CC	27/27	a1d	X+X-
39	48	3/3	CC	27/24	a1d	X+X-
40	81	4/3	CT	27/27	a1d	X-X-
41	41	3/3	CC	27/24	a1d	X+X-
42	20	4/3	CT	27/24	a1d	X+X-
43	30	2/4	CC	27/27	a1a1	X+X-
44	57	3/3	CC	27/27	a1a1	X-X-
45	21	3/3	CT	27/27	a1d	X+X-
46	87	3/3	CC	24/24	dd	X+X+
47	1	4/3	CC	27/27	a1a1	X-X-
48	19	3/3	CT	27/24	a1d	X-X-

DNA NUMBER	GW's Study No.	APOE	C_{-265}-T	Signal Peptide	Ag(a1/d)	XbaI
49	71	4/3	CC	27/24	dd	X+X+
50	10	4/3	CT	27/27	a1a1	X+X-
51	23	3/3	CC	24/24	dd	X+X+
52	77	3/3	CC	27/24	a1d	X+X-
53	32	2/3	CC	27/24	a1d	X+X-
54	88	4/3	CT	27/24	a1d	X+X-
55	12	3/3	CC	24/24	dd	X+X+
56	60	4/3	CC	27/27	a1a1	X+X-
57	66	3/3	CC	27/27	a1a1	X-X-
58	5	3/3	CT	27/24	a1d	X-X-
59	15	4/3	CC	27/27	a1a1	X-X-
60	17	4/3	CC	27/27	a1a1	X-X-
61	18	3/3	CT	27/27	a1d	X-X-
62	13	4/3	CT	27/24	a1d	X+X-
63	53	3/3	CC	27/27	a1d	X+X+
64	29	4/3	CC	27/24	a1d	X+X-
65	31	3/3	CC	27/27	a1d	X+X-
66	45	4/3	CC	27/24	a1d	X+X-
67	74	3/3	CC	27/24	dd	X+X-
68	78	4/3	CC	27/27	a1d	X+X-
69	67	3/3	CC	27/27	a1d	X+X-
70	8	4/3	CC	24/24	dd	X+X+
71	11	3/3	CC	27/24	a1d	X+X+
72	4	4/4	CC	27/24	dd	X+X+
73	36	4/4	CC	27/24	dd	X+X+
74	55	3/3	CC	27/27	a1d	X+X+
75	14	3/3	CC	27/24	a1d	X+X-
76	50	3/3	CC	27/24	dd	X+X+
77	.	4/3	CC	27/27	dd	X+X-

DNA NUMBER	GW's Study No.	G_{-75}-A apoAI	C_{1100}-T apoCIII	LPL PvuII	LPL HindIII	LPL Ser447-Stop	Stp
1	69	GA	TT	V+V+	H+H+	SS	
2	27	GG	CC	V-V-	H+H-	SS	
3	83	GG	CC	V-V-	H+H+	SS	
4	79	GG	CT	V-V-	H+H-	SSt	
5	73	GG	CC	V+V-	H-H-	SSt	
6	62	GA	CT	V-V-	H+H+	SS	
7	59	GG	CC	V+V-	H+H-	SS	
8	25	GA	CT	V+V-	H+H-	SSt	
9	70	GG	CC	V+V-	H+H-	SS	
10	54	GG	CC	V+V+	H+H+	SS	
11	75	GG	CC	V+V-	H+H-	SSt	
12	56	GA	CC	V+V+	H+H+	SS	
13	85	GA	TT	V+V+	H+H+	SS	
14	38	GG	CT	V-V-	H-H-	StSt	
15	22	GG	CC	V+V+	H+H-	SS	
16	44	GG	CC	V-V-	H+H-	SS	
17	72	GG	CC	V+V+	H+H+	SS	
18	46	GA	CT	V+V-	H+H+	SS	
19	26	GG	CC	V+V-	H+H-	SS	
20	65	GG	CT	V+V-	H+H-	SS	
21	64	GA	TT	V+V-	H+H+	SS	
22	7	GA	TT	V+V-	H+H-	SS	
23	99	GG	CC	V+V-	H+H+	SS	
24	86	GG	CC	V+V-	H+H+	SS	
25	84	GG	CT	V+V+	H+H-	SS	
26	49	GG	CC	V+V+	H+H-	SS	
27	40	GA	CT	V+V-	H+H-	SS	
28	51	GA	CT	V+V-	H+H+	SS	
29	35	GG	CC	V+V+	H+H+	SS	
30	52	GA	CT	V-V-	H+H-	SSt	
31	34	GA	CT	V+V-	H+H-	SSt	
32	33	GG	CC	V+V-	H+H+	SS	
33	99	GG	CT	V+V-	H-H-	SSt	
34	3	GG	CC	V+V-	H+H-	SSt	
35	47	GG	CT	V+V+	H+H+	SS	
36	99	GG	CT	V-V-	H+H+	SS	
37	2	GA	CT	V+V-	H+H-	SS	
38	28	GG	CT	V+V+	H+H+	SS	
39	48	GA	TT	V+V-	H+H+	SS	
40	81	GG	CC	V+V-	H+H+	SS	
41	41	GA	TT	V+V+	H+H+	SS	
42	20	GG	CC	V-V-	H+H-	SSt	
43	30	GG	CC	V+V+	H+H-	SS	
44	57	GA	CC	V+V-	H+H+	SS	
45	21	GG	CC	V+V-	H+H-	SS	
46	87	GG	CC	V+V+	H+H-	SS	
47	1	GG	CC	V-V-	H-H-	SSt	
48	19	GG	CC	V+V+	H+H+	SS	
49	71	GG	CC	V+V-	H+H+	SS	
50	10	GG	CC	V-V-	H+H-	SSt	

DNA NUMBER	GW's Study	G_{-75}-A No.apoAI	C_{1100}-T LPL apoCIII	LPL PvuII	LPL HindIII	LPL Ser447-Stop
51	23	GG	CC	V+V-	H+H-	SSt
52	77	GG	CC	V+V+	H+H+	SS
53	32	GA	CT	V+V+	H+H+	SS
54	88	GG	CT	V+V-	H-H-	SSt
55	12	GG	CC	V-V-	H+H-	SS
56	60	GG	CC	V+V+	H+H+	SS
57	66	GG	CC	V+V+	H+H+	SS
58	5	GA	CT	V+V-	H-H-	SSt
59	15	GG	CC	V+V-	H+H+	SS
60	17	GA	CT	V+V-	H+H+	SS
61	18	GG	CC	V-V-	H+H-	SS
62	13	GA	CT	V+V+	H+H+	SS
63	53	GG	CT	V+V-	H+H-	SS
64	29	GG	CT	V+V+	H+H+	SS
65	31	GG	CC	V+V-	H+H+	SS
66	45	GA	CT	V+V-	H+H-	SS
67	74	GA	CT	V+V-	H-H-	SS
68	78	GG	CC	V+V-	H+H+	SS
69	67	GG	CT	V-V-	H+H+	SS
70	8	GG	CT	V+V+	H+H-	SS
71	11	GG	CC	V+V+	H+H+	SS
72	4	GA	CT	V-V-	H+H-	SSt
73	36	GA	CC	V-V-	H+H-	SS
74	55	GA	CT	V+V-	H+H+	SS
75	14	GG	CC	V-V-	H+H-	SS
76	50	GG	CT	V-V-	H+H+	SS
77	.	*	CT	V+V-	H+H-	SS

Atherosclerosis, 92 (1992) 151–164
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Printed and Published in Ireland

Apolipoprotein B gene polymorphisms, lipoproteins and coronary atherosclerosis: A study of young myocardial infarction survivors and healthy population-based individuals

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(Received 24 July, 1991)
(Revised, received 25 October 1991)
(Accepted 29 October 1991)

Summary

Association studies were carried out in a sample of 87 patients from Sweden who had survived a myocardial infarction (MI) before the age of 45, and 91 age-matched healthy individuals, to compare the impact of polymorphisms at the apolipoprotein (apo) E and B gene loci on among-individual differences in plasma lipid traits and progression of atherosclerosis. In the group of healthy individuals, polymorphisms creating the common apo E isoforms were, as expected, associated with significant differences in total and low density lipoprotein (LDL) cholesterol (11.7% and 11.6% of sample variance). For apo B, the polymorphism with the largest effect on apo B levels (16% of sample variance) was the C to T transition 265 bp 5′ of the cap site, in the promoter (detectable by *Msp*I). Both this polymorphism and the threonine$_{2488}$ neutral substitution (detectable by *Xba*I) were associated with significant effects on LDL-cholesterol (8.3% and 9.3% of sample variance, respectively). The asparagine/serine$_{4311}$ polymorphism was associated with a significant effect on high density lipoprotein (HDL) cholesterol alone, and there was no significant association with the glutamate/lysine$_{4154}$ polymorphism (detectable by *Eco*RI) or the leucine-alanine-leucine (LAL) insertion/deletion polymorphism in the signal peptide. In the patients, polymorphisms creating the three common apo E isoforms were associated with large effects on cholesterol, apo B and triglyceride levels (19.9%, 20.3% and 23.9% of sample variance) of similar magnitude as in the healthy individuals. Apo B polymorphisms were found to be associated with much smaller effects on lipid traits than in the healthy individuals. The only significant association was between the asparagine/serine$_{4311}$ polymorphism and HDL-triglyceride levels. However, global severity of coronary atherosclerosis at the first angiography was found to be significantly associated with the LAL insertion/deletion polymorphism (P = 0.008). Thus

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Abbrevations: apo, apolipoprotein; RFLP, restriction fragment length polymorphism; chol, cholesterol; TG, triglycerides; MI, myocardial infarction.

variation at the apo B gene locus is associated with the development of atherosclerosis, but the data suggests that this may act through mechanisms not directly related to effects on measured lipid traits.

Key words: Myocardial infarction; Apo B gene polymorphism; RFLP; Coronary atherosclerosis; Angiography; Apo E genotype

Introduction

Apolipoprotein (apo) B, a monomeric glycoprotein of approximately 550 kDa, plays a central role in lipoprotein metabolism and in maintaining the normal homeostasis of serum cholesterol levels [1]. Apo B is essential both for the assembly and secretion of chylomicrons from the small intestine and of very low density lipoproteins (VLDL) from the liver [2] and as the ligand for the LDL receptor [3] mediating the cellular uptake of cholesterol. Thus variation within the apo B gene is likely to explain some of the inter-individual differences in serum lipid levels and suspectibility to coronary heart disease (CHD) that has been reported in numerous population studies [4–7]. Epidemiological studies have established that elevated plasma levels of LDL and apo B and depressed plasma levels of HDL and apo A-I are correlated with increased risk of CHD and premature atherosclerosis (reviewed in Ref. 8), thus suggesting that variation at the apo B gene locus may be associated both with differences in lipid levels and with incidence of CHD.

Over the past 6 years, a number of restriction fragment length polymorphisms (RFLPs) in the apo B gene have been reported [9–18]. Some of these polymorphisms have subsequently been found to be associated with peripheral arterial disease [4], coronary artery disease (CAD) [5,6] and risk of myocardial infarction (MI) [19]. Associations have also been found between RFLPs of the apo B gene and serum lipid levels, and several studies have shown an association between serum cholesterol and/or triglyceride levels and the threonine$_{2488}$ (Thr) neutral substitution detectable by *Xba*I [20–22]. However results from these population studies were not without inconsistencies [5,11,19–22], i.e. associations found by some groups have not been found by other groups.

The purpose of this study was twofold; firstly to investigate whether there was an association between variation at the apo B gene locus and global severity and rate of progression of coronary atherosclerosis in a group of young MI patients and secondly to determine the contribution of variation within the apo B gene to among-individual variation in serum lipid and apolipoprotein levels, compared with the well known effects associated with the common apo E isoforms.

Materials and methods

Subjects

The initial study population consisted of 145 patients and 96 healthy individuals recruited from Stockholm County, Sweden. The patients had all suffered a first MI before the age of 45 years. Patients who were female, diabetic or had familial hypercholesterolaemia or other major concomitant disease such as uraemia and collagenosis were excluded, which left 87 patients in the study. The healthy individuals were age-matched male residents of Stockholm County. They were free of symptoms and clinical signs of CHD. DNA was not available for five of the healthy individuals, leaving 91 individuals in the study. All individuals used in this study were unrelated and of Swedish origin except for 10 patients who had at least one parent or grandparent born in Finland. Recruitment and clinical prodecures have been described in detail previously [23,24]. Metabolic and haemostatic investigations were done 3–6 months after the MI. Within the next few weeks the patients had coronary angiography. Reangiogra-

phies were done after the first angiography on 74 of the patients (mean time between angiographies = 5 years, 2 months, S.D. = 9 months; range 3.5 to 6.75 years).

Coronary artery disease severity and progression

Prodecures for coronary angiography and details of the scoring systems have been described elsewhere [25]. In the global coronary atherosclerosis score, points were assigned for extension of atherosclerosis and plaque size in each of fifteen proximal coronary arterial segments. Atherosclerotic lesions were defined as sharp-edged, plaque-like or irregular indentations, often multiple, into the vessel lumen without features suggesting fibromuscular hyperplasia. A single stenosis with smooth contours, or a single occlusion, in the absence of additional changes in the same or any other artery, was not classifed as atherosclerosis. Distinct stenoses were quantified by means of a separate classification system in which a coronary stenosis score was derived from estimations of the proportional reduction in luminal diameter of the three most stenotic portions of each segment.

The global atherosclerosis and stenosis severity scores were obtained by dividing the sum of all segmental atherosclerosis and stenosis scores, respectively, by the number of segments accessible to evaluation. The presence and severity of diffuse coronary atherosclerosis was distinctly differentiated from the number and severity of haemodynamically significant stenoses by the two systems.

The cine films from the first and second angiographies were projected simultaneously for purpose of comparison. Progression of coronary atherosclerosis and distinct stenoses was defined as increase in the global coronary atherosclerosis and stenosis scores.

Lipid and lipoprotein analysis

A combination of preparative ultracentrifugation and precipitation of apo B-containing lipoproteins, followed by lipid analyses in the major lipoprotein fractions [26] was used to determine serum lipoproteins. Cholesterol [27] and triglycerides [28] were determined on an Ultrolab (LKB Bromma, Sweden) after chloroform–methanol extraction of whole serum, the VLDL fraction, the infranatant after ultracentrifugation (containing LDL and HDL), and the supernatant (HDL) after precipitation. Apo A-I and apo B were determined by electroimmunoassay [23].,

DNA preparation and analysis

Blood was collected after an overnight fast. DNA extraction from fresh blood samples was carried out by the Triton X-100 lysis method [29]. PCR-amplification [30] in a total volume of 50 μl was on a Cambio intelligent heating block (Cambio, Cambridge, U.K.). Oligonucleotides were obtained from Severn Biotech Ltd (Kidderminister, U.K.). *Thermus aquaticus* polymerase was from Perkin Elmer-Cetus (CT, U.S.A.), and 0.5 unit used per PCR. Buffers were as recommended by Perkin Elmer-Cetus. In each PCR 100–200 ng of each primer was used.

C to T (–265) substitution

The denaturation step of the PCR was performed at 93°C for 5 min; annealing and extension at 58°C for 5 min. Thirty subsequent cycles were as follows; 92°C for 1 min and 58°C for 5 min. Sequences of the 5′ primer and 3′ primer were (5′ to 3′) CAATTCAGTCCAGGAGAAGCA and CAGCAACCGAGAAGGGCACTCAG, respectively. Ten units of *Msp*I (Northumbrian Biologicals Ltd, U.K.) was added and incubation was overnight. Digests were subsequently run on 2% agarose gels at 50 V for 2 h. After digestion, constant fragments of 76 bp and 54 bp are produced; with a 275 bp fragment in the absence of the polymorphic site or 179 and 96 bp fragments in its presence, as shown in Fig. 1.

Other apo B gene polymorphisms

PCR procedures and subsequent electrophoresis and typing of alleles were as described for the LAL insertion/deletion polymorphism [15,31] and for the Thr_{2488} neutral substitution detectable with *Xba*I and the glutamine/$lysine_{4154}$ (Glu/Lys) substitution — the t/z epitope detectable with *Eco*RI [32]. Detection of the asparagine/serine (Asn/Ser) substitution at amino acid 4311 was carried out by a double Southern blotting procedure of the PCR products followed by hybridisation to allele specific oligonucleotides (ASO) as described [33].

Apo E genotyping

The restriction isotyping procedure (PCR amplification, digestion with the enzyme *Hha*I and electrophoresis on polyacrylamide gels) was as described by Hixson and Vernier [34]. Oligonucleotide primers used in the PCR were as described [35].

Statistical analysis

Statistical analysis was carried out using the SPSS computer program. Only those healthy individuals with serum cholesterol levels less than 9.5 mmol/l and serum triglyceride levels less than 3.0 mmol/l were selected. These cut-off points were chosen for the healthy individuals, to give a sample more representative of the general population of Sweden and to remove the disproportionate effects of a few individuals with extremely high lipid levels on the analysis of the data. Thus four individuals with abnormally high levels and three individuals with no values for either cholesterol (chol) or triglyceride (TG) levels were excluded. Mean levels of each lipid/lipoprotein trait for the healthy individuals and patients were compared using the *t*-test. Data was $\log_{10}$-transformed prior to regression and analysis of variance (ANOVA), if it was not already approximately normally distributed. Lipid and apolipoprotein traits were adjusted by linear regression for age and recorded body mass index (BMI) and the percentage variance associated with age and BMI ($R^2 \times 100$) was estimated. A one-way analysis of variance was performed on the age and BMI adjusted levels to test the null hypothesis that phenotypic variations in lipid and apolipoprotein levels were not associated with genetic variation at the apo B or apo E gene loci. The impact of apo E and apo B genotypes on age and BMI adjusted lipid and apolipoprotein traits were estimated by non-linear regression which removes the dependence on the existence of a linear relationship between the mean of a trait for each genotype and genotypes of the particular DNA polymorphism. Allele frequencies for each RFLP/polymorphism were estimated using the 'gene-counting' method, and determined separately for the healthy individuals and patients. To compare allele frequency in healthy individuals with that in patients, 2 × 2 contingency tables were used; 2 × 3 contingency tables were used to compare genotype frequencies, for each polymor-

TABLE 1

MEANS AND STANDARD DEVIATIONS (UNADJUSTED) FOR LIPID TRAITS IN THE SWEDISH SAMPLE

Trait	Healthy individuals (n = 84)		Patients (n = 87)		P from t-test[a]
	Mean	± S.D.	Mean	± S.D.	
Age (years)	40.51	3.98	40.35	3.50	NS
BMI (kg/m^2)	24.57	2.36	26.42	3.25	<0.001
Cholesterol (mmol/l)					
Total	5.98	1.00	7.24	1.27	<0.001
VLDL	0.49	0.29	1.09	0.85	<0.001
LDL	3.93	0.85	4.88	1.07	<0.001
HDL	1.43	0.34	1.12	0.23	<0.001.
Triglycerides (mmol/l)					
Total	1.32	0.57	2.61	1.78	<0.001
VLDL	0.79	0.49	1.89	1.58	<0.001
LDL	0.34	0.11	0.45	0.15	<0.001
HDL	0.14	0.04	0.16	0.05	<0.01
Apo A-I (mg/100 ml)	124.52	18.66	111.37	18.74	<0.001
Apo B (mg/100 ml)	106.48	17.23	125.77	20.81	<0.001

[a]The t-test was used to compare the distributions of each trait in the healthy individuals and patients, respectively.
NS = Not Significant.

phism, between healthy individuals and patients; 3 × 5 contingency tables were used to see if there was any association between the C to T (-265) substitution and apo E genotypes in each group. The average excess effects of each allele of the C to T (-265) substitution in the healthy individuals on serum apo B levels were estimated using the method previously presented by Sing and Davignon [36].

We considered statistical significance to be at the 0.05 level except in the case of correlations between scores for global coronary atherosclerosis and genotypes, where the more conservative 0.01 level was adopted. However, since the study groups were small and multiple comparisons were being performed, probability values need to be interpreted with greater caution.

Results

The characteristics and levels of plasma lipid traits for the selected healthy individuals and patients are shown in Table 1. With the exception of age, the means of the parameters in healthy individuals and patients were significantly different.

Genotypes were determined at the apo E and apo B loci; Fig. 2 shows the approximate positions of the polymorphisms in the apo B gene. Frequencies of the rare alleles for each polymorphism are given in Table 2. For all the polymorphisms studied, genotype distributions were close to and not significantly different from the Hardy-Weinberg prediction. As expected, for genes on different chromosomes, no significant association was found between apo E and apo B genotypes.

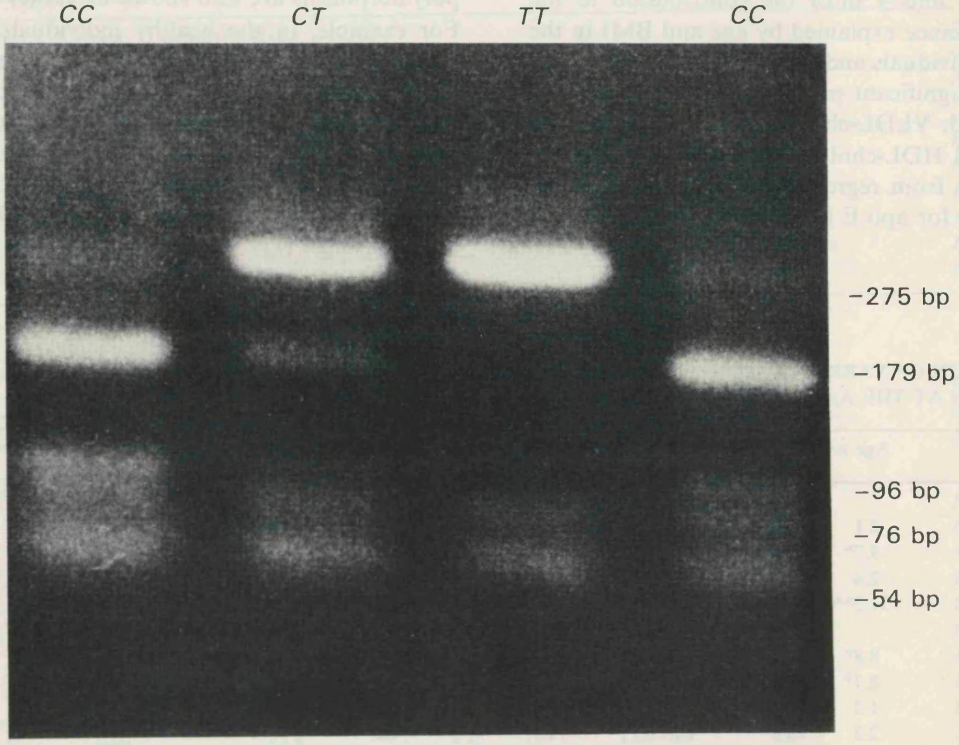


Fig. 1. Photograph of gel showing electrophoresis of fragments obtained after digestion of PCR-amplified DNA with *Msp*I. The three genotypes of the C to T (-265) substitution are shown: *CC*, *CT* and *TT*.

TABLE 2

ALLELE FREQUENCIES IN THE SWEDISH SAMPLE

Gene polymorphism	Rare allele	Frequency of rare allele	
		Healthy individuals	Patients
Apo E isoforms	*E2* (Cys_{158})	0.096	0.063
	E4 (Arg_{112})	0.181	0.224
Apo B C to T (-265) promoter (*MspI*)	*T* (-265) no restriction site	0.113	0.100
Leu-Ala-Leu (-16 to -14) insertion/deletion	Deletion (*del*)	0.307	0.331
Thr_{2488} (*XbaI*) Neutral (C→T) substitution	$X^+(T)$ with restriction site	0.560	0.517
Gln/Lys_{4154} (*Eco*RI)	Lys_{4154}	0.195	0.151
Asn/Ser_{4311} substitution	Ser_{4311}	0.187	0.190

Tables 3 and 4 show the contribution to the sample variance explained by age and BMI in the healthy individuals and patients. Age and BMI explained a significant proportion of total variance in total TG, VLDL-chol and VLDL-TG in both groups and HDL-chol in the healthy individuals. The results from regression of age and BMI adjusted data for apo E genotype and for the apo B polymorphisms are also shown in Tables 3 and 4. For example, in the healthy individuals, apo E genotypes explained 11.7% and 11.6%, respectively, of total variance in chol and LDL-chol levels. In the patients the associations were stronger, explaining 19.9%, 20.3%, 17.0%, 23.9% and 19.2%, respectively, of total variance in chol, TG, LDL-chol, apo B and HDL-TG levels. As shown in

TABLE 3

PROPORTION OF VARIANCE ($R^2 \times 100$) OF EACH TRAIT ATTRIBUTABLE TO AGE AND BMI AND TO GENETIC VARIATION AT THE Apo E AND Apo B LOCI IN THE HEALTHY INDIVIDUALS

Trait	Age and BMI[a]	Apo E	C to T (-265)	*ins/del*	*Xba*I	*Eco*RI	Asn/Ser_{4311}
Cholesterol							
Total	2.1	11.7*	6.6	2.1	4.3	1.3	1.3
VLDL	9.7*	2.0	0.6	1.0	7.0	2.1	3.0
LDL	2.4	11.6*	8.3*	1.7	9.3*	1.6	0.8
HDL	11.2**	3.6	1.5	2.0	7.5*	2.9	7.9*
Triglycerides							
Total	8.8*	1.8	0.4	0.3	3.9	2.2	1.4
VLDL	8.3*	1.5	0.5	1.4	7.2*	3.5	2.5
LDL	1.2	1.7	0.3	2.1	2.7	1.6	1.5
HDL	2.2	2.8	1.1	1.2	0.6	4.6	1.7
Apo A-I	5.6	4.9	0.7	1.5	1.5	0.5	2.2
Apo B	3.1	11.1	15.7**	1.4	7.4	3.0	2.4

[a]Unadjusted data used in this regression.

*$P < 0.05$; **$P < 0.01$.

TABLE 4

PROPORTION OF VARIANCE ($R^2 \times 100$) OF EACH TRAIT ATTRIBUTABLE TO AGE AND BMI AND TO GENETIC VARIATION AT THE Apo E AND Apo B LOCI IN THE PATIENTS

Trait	Age and BMI[a]	Apo E	C to T (-265)	*ins/del*	*Xba*I	*Eco*RI	Asn/Ser_{4311}
Cholesterol							
Total	0.3	19.9**	1.9	3.8	1.0	3.6	1.3
VLDL	11.2**	16.6**	0.7	1.7	0.4	3.8	6.3
LDL	1.7	17.0**	3.5	0.8	0.9	8.7*	0.5
HDL	6.2	6.5	2.9	0.5	1.2	1.6	1.3
Triglycerides							
Total	10.2*	20.3**	0.2	2.8	0.1	5.5	3.5
VLDL	13.0**	17.1**	0.4	2.8	0.4	4.6	6.1
LDL	1.9	6.8	0.5	0.2	1.7	3.2	0.2
HDL	4.9	19.2**	3.5	0.7	0.5	9.1*	11.6**
Apo A-I	4.2	10.4	5.0	11.9*	0.4	2.7	2.6
Apo B	2.7	23.9**	2.8	2.5	4.9	0.8	0.8

[a]Unadjusted data used in this regression.
*$P < 0.05$; **$P < 0.01$.

TABLE 5

MEAN LIPID LEVELS (±S.D.) FOR Apo E GENOTYPES THAT HAVE SIGNIFICANT *F* VALUES FROM ANOVAS IN THE SWEDISH SAMPLE

(a) Healthy Individuals

Trait (mmol/l)	Apo E Genotype (No.)					*F* value and significance in ANOVA[a]
	3/2 (12)	3/3 (44)	4/2 (4)	4/3 (20)	4/4 (3)	
Total chol	5.61 ± 0.99	5.85 ± 1.01	7.07 ± 0.67	6.12 ± 0.82	6.78 ± 1.32	$F = 2.6$ $P = 0.04$
LDL-chol	3.65 ± 0.88	3.79 ± 0.85	4.78 ± 0.85	4.15 ± 0.73	4.54 ± 0.98	$F = 2.6$ $P = 0.05$
Apo B (mg/100 ml)	102.4 ± 19.6	103.4 ± 17.0	122.7 ± 9.6	110.8 ± 16.4	118.3 ± 10.7	$F = 2.2$ $P = 0.07$

[a]Traits were adjusted for age and BMI prior to ANOVA.

(b) Patients

Trait (mmol/l)	Apo E Genotype					*F* value and significance in ANOVA[a]
	3/2 (6)	3/3 (46)	4/2 (5)	4/3 (24)	4/4 (5)	
Total chol	6.06 ± 1.30	7.11 ± 1.02	8.71 ± 1.47	7.22 ± 1.40	8.36 ± 0.66	$F = 5.0$ $P = 0.001$
VLDL chol	0.94 ± 0.72	1.05 ± 0.65	2.97 ± 1.65	0.90 ± 0.60	0.63 ± 0.24	$F = 4.0$ $P = 0.005$
LDL-chol	3.79 ± 0.99	4.91 ± 0.92	4.33 ± 1.52	4.94 ± 1.11	6.16 ± 0.42	$F = 4.1$ $P = 0.004$
TG	1.91 ± 0.97	2.51 ± 1.29	6.61 ± 4.09	2.31 ± 1.18	1.79 ± 0.62	$F = 5.1$ $P = 0.001$
VLDL-TG	1.43 ± 0.94	1.80 ± 1.15	5.40 ± 3.73	1.64 ± 1.00	1.08 ± 0.49	$F = 4.1$ $P = 0.004$
HDL-TG	0.14 ± 0.05	0.16 ± 0.04	0.24 ± 0.10	0.15 ± 0.04[c]	0.14 ± 0.02	$F = 4.7$ $P = 0.002$
Apo B (mg/100 ml)	104.5 ± 25.2	119.9 ± 18.4[b]	135.8 ± 17.7	135.4 ± 16.3	150.0 ± 17.2	$F = 6.4$ $P = 0.0002$

[a]Traits were adjusted for age and BMI prior to ANOVA.
[b]$n = (47)$.
[c]$n = (22)$.

TABLE 6

MEAN LIPID LEVELS (±S.D.) FOR Apo B GENOTYPES THAT HAVE SIGNIFICANT *F* VALUES FROM ANOVAS IN THE SWEDISH SAMPLE

(a) Healthy individuals

Trait (mmol/l)	Apo B genotype (No.) T to C (-265) substitution			*F* value and significance in ANOVA[a]
	CC (67)	*CT* (15)	*TT* (2)	
LDL-chol	4.03 ± 0.83 (60)	3.69 ± 0.82 (15)	2.63 ± 0.37 (2)	$F = 3.7$ $P = 0.03$
Apo B (mg/100 ml)	110 ± 16.2	95.6 ± 15.2	84 ± 18.4	$F = 6.9$ $P = 0.002$
	*Xba*I RFLP			
	X^+X^+ (17)	X^+X^- (39)	X^-X^- (27)	
LDL-chol	3.47 ± 0.70	3.92 ± 0.81	4.26 ± 0.89	$F = 4.1$ $P = 0.02$
HDL-chol	1.54 ± 0.41	1.34 ± 0.32	1.47 ± 0.30	$F = 3.2$ $P = 0.04$
VLDL-TG	0.87 ± 0.57	0.85 ± 0.45	0.68 ± 0.50	$F = 3.1$ $P = 0.05$
	Asn/Ser_{4311} Substitution			
	NN (56)	*NS* (23)	*SS* (4)	
HDL-chol	1.39 ± 0.29	1.43 ± 0.38	1.86 ± 0.45	$F = 3.4$ $P = 0.04$

[a]Traits were adjusted for age and BMI prior to ANOVA.

(b) Patients

Trait (mmol/l)	Apo B Genotype (No.)			*F* value and significance in ANOVA[a]
	Leu-Ala-Leu insertion/deletion polymorphism			
	ins/ins (29)	*ins/del* (23)	*del/del* (11)	
Apo A-I (mg/100 ml)	105.6 ± 17.2	113.5 ± 19.9	122.3 ± 15.3	$F = 4.0$ $P = 0.02$
	Asn/Ser_{4311} substitution			
	NN (54)	*NS* (28)	*SS* (2)	
HDL-TG	0.17 ± 0.05	0.14 ± 0.03	0.15 ± 0.02	$F = 5.3$ $P = 0.007$

[a]Traits were adjusted for age and BMI prior to ANOVA.

Table 5, the *E4* allele is associated with higher chol levels and the *E2* allele with lower levels of lipid traits in both the healthy individuals and patients. With the exception of individuals with the genotype *E4/E2*, the mean levels of total chol and LDL-chol are higher for the patients than for the healthy individuals for each apo E genotype.

For the apo B polymorphisms, in the healthy individuals, genotypes of the C to T (-265) substitution were significantly associated with both LDL-chol and apo B levels. The smaller effects associated with the Thr_{2488} neutral substitution on LDL-chol, HDL-chol and VLDL-TG, and the Asn/Ser_{4311} substitution on HDL-chol levels were also significant. Individuals with genotypes *Asn/Asn* and *Asn/Ser* had similar mean HDL-chol levels, but the genotype *Ser/Ser* was associated with a higher mean level of HDL-chol. Mean levels from the ANOVA that gave significant results of each trait for each genotype are presented in Tables 5 and 6. The *T* allele (absence of the *Msp*I site) of the C to T (-265) substitution was associated with lower levels of LDL-chol and apo B in healthy individuals. Dividing those healthy individuals who had data on apo B levels into three approximately equal groups, the frequency of the *T* allele was 0.25 in the group with the lowest apo B levels (lower than 98 mg/100 ml), whereas it was only 0.02 in the group with the highest apo B levels (higher than 113 mg/100 ml). Using 2 × 2 contingency tables, this difference in frequency was statistically significant ($\chi^2 = 11.4$, $P < 0.01$ but > 0.001). For the Thr_{2488} neutral substitution there was a co-dominant effect with respect to LDL-chol levels, the X^+ allele being associated with significantly higher LDL-chol levels and non-significantly higher apo B levels.

In the patients, a significant association was found between apo A-I levels and genotypes of the LAL insertion/deletion polymorphism. Also, there were significant associations between LDL-chol and HDL-TG levels and genotypes of the Gln/Lys_{4154} substitution when all three genotypes were considered (data not shown). However, these two associations became insignificant when the single individual homozygous for the rare allele was omitted from the analysis. Genotypes of the Asn/Ser_{4311} substitution were significantly associated with HDL-TG levels. In both the healthy individuals and patients, the *Asn/Ser* genotype was associated with the lowest mean HDL-TG levels, although this was not statistically significant in healthy individuals.

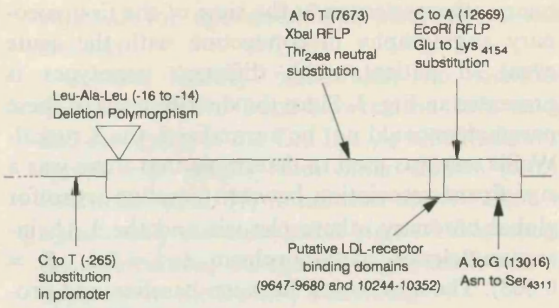


Fig. 2. Map of the apoB gene showing approximate positions of the DNA polymorphisms used in this study. Numbers in brackets indicate the numbering of nucleotides of the cDNA relative to the transcriptional start site which is 1 [39]. The open box represents the transcribed region of the apo B gene. The horizontal lines represent untranscribed regions of the gene.

The possibility was examined that genotypes at the apo B or apo E loci were associated with development of atherosclerosis. Only for the LAL insertion/deletion polymorphism was there a significant effect on development of atherosclerosis. A box plot showing the distribution of global cor-

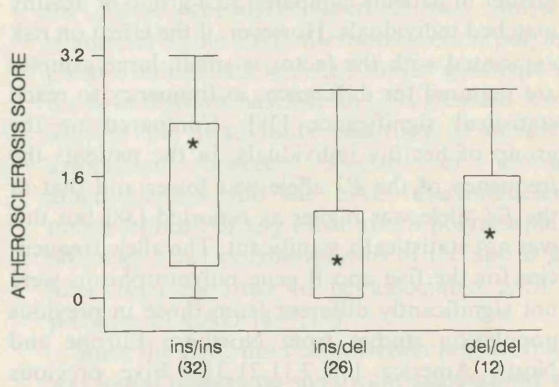


Fig. 3. Box plot showing the distribution of baseline scores for coronary atherosclerosis for each of the three genotypes of the LAL insertion/deletion polymorphism: *ins/ins*, *ins/del* and *del/del*. Scores are given on the vertical axis and genotypes on the horizontal axis. Numbers in brackets denote number of individuals with that genotype. * = median.

onary atherosclerosis at the time of the first coronary angiography in connection with the acute event in patients with different genotypes is presented in Fig. 3. Since the distributions of these parameters could not be normalized, the Kruskal-Wallis test was used to determine that there was a significant association between baseline score for global coronary atherosclerosis and the LAL insertion/deletion polymorphism ($\chi^2 = 9.6$, $P = 0.008$). The correlation between baseline and progression scores was stronger in the subgroup of individuals with the deletion/deletion (*del/del*) genotype than in the other genotypes of the LAL insertion/deletion polymorphism or in the patient sample as a whole (values of Pearson correlation coefficients were 0.54 for the *del/del* genotype, 0.29 for the insertion *(ins)/del* genotype, -0.008 for the *ins/ins* genotype and 0.25 for the whole patient sample; none were significantly different from one another).

Discussion

For the apo E gene, the allele frequencies observed in these samples were within the ranges of frequencies found for other Caucasian populations (reviewed in Ref. 38). If a particular factor is associated with a greater risk of atherosclerosis, it is possible that prevalence of this factor would be greater in patients compared to a group of healthy matched individuals. However, if the effect on risk associated with the factor is small, large samples are required for differences in frequency to reach statistical significance [38]. Compared to the group of healthy individuals, in the patients the frequency of the *E2* allele was lower and that of the *E4* allele was higher as reported [38] but this was not statistically significant. The allele frequencies for the five apo B gene polymorphisms were not significantly different from those in previous population studies from Northern Europe and North America [4,5,7,11,21,31]. Five previous studies have reported a significantly higher frequency of the rare allele of the Glu/Lys_{4154} substitution in patients with peripheral arterial disease [4], with CAD [5,6], with CHD [7] and with MI [19], as compared to clinically healthy individuals. In our present study, the absence of a significant difference in allele frequency between the patients and healthy individuals may be due to the small sample size or possibly the young age of the patients compared to those from other studies [4–6,19].

As expected, there was a significant association between apo E genotypes and total chol, LDL-chol and apo B in both the healthy individuals and patients. In both groups, the *E2* allele was associated with lower mean total chol, LDL-chol and apo B levels, as compared to the *E4* allele, as expected from previous population studies (reviewed in Ref. 38). No other gene product has yet been identified with a larger contribution to the determination of inter-individual differences in cholesterol levels in the normal range of variability [38].

The healthy individuals and patients exhibited quite different results for the effect of genetic variation at the apo E locus on variance of TG, HDL-chol and HDL-TG. This agrees with the lack of a consistent relationship between apo E genotype and TG levels in previous population studies (reviewed in Ref. 38). Any effects of apo E genotype on TG and the HDL fraction may be small and population-specific [38], as seen in the healthy individuals. In the patients, the effect of apo E genotype on total and HDL-TG was largely due to inclusion of individuals with the rare genotype *E4/E2*. Thus in the Swedish sample, apo E genotypes exerted their strongest and most consistent effects on total and LDL-chol and apo B levels; and weaker and less consistent effects on TG and the HDL fractions.

In the healthy individuals and patients, apo B genotypes had a smaller impact on lipid traits than those observed with apo E genotypes. For the apo B gene polymorphisms, the largest effect seen was the association between the C to T (-265) substitution and apo B and LDL-chol in the healthy individuals. Genotype *CC* was associated with the highest levels and genotype *TT* with the lowest levels of both apo B and LDL-chol. A similar effect was observed in the patients, i.e. individuals with genotype *CT* had lower mean apo B levels than did those with genotype *CC* but this was not statistically significant. An association between the *T* (-265) allele and lower apo B levels was also found in a recent study [39]. In the healthy in-

dividuals the effect (average excess) associated with the *T* allele on apo B levels was to lower by 13.3 mg/dl and that of the *C* allele to raise by 1.9 mg/dl. The mechanism of the association is unclear at present. The C to T substitution at position -265 relative to the transcriptional start site is found within a region of the apo B gene spanning from positions -900 to -129 and known to contain both positive and negative regulatory elements [40,41]. The positive and negative elements interact to produce a high level of apo B expression restricted to two tissues, liver and small intestine [40]. Hypomethylation of CCGG and CGCG sequences correlates with gene activity [42]. The cytosine at position -265 is within one such sequence and is unmethylated in HepG2 and Caco2 cells, both apo B expressing cells [42]. A number of proteins have now been identified which bind to the 5′ flanking region of the apo B gene and modulate transcription [41]. The C to T substitution may change affinity of binding of such a protein, leading to changes in rate of transcription. Alternatively, it may be in linkage disequilibrium with sequence differences in other parts of this region.

In a number of published studies, the X^+ allele of the Thr_{2488} neutral substitution has been associated with higher cholesterol levels. This was also observed in the group of healthy individuals though the difference did not reach statistical significance in this sample. The association between LDL-chol levels and the Thr_{2488} neutral substitution mirrored the association between cholesterol and the *Xba*I RFLP but was stronger and statistically significant. In the healthy individuals, the genotype X^+X^+ was associated with highest mean apo B levels and X^-X^- with lowest mean levels. The observations are compatible with a codominant effect which is not surprising since each LDL particle contains only one apo B molecule [21].

Wiklund et al. [43] found a significant association between total TG levels and genotypes of the Thr_{2488} neutral substitution, in a sample of 52 male MI patients from Sweden. This association was not confirmed in this study and this could be due partly to differences in selection of the patients. In the previous study, three diabetic patients were not excluded and lipid traits were not adjusted for age and BMI prior to statistical analysis.

In the healthy individuals, no significant associations were found between the traits studied and genotypes of the Glu/Lys_{4154} substitution: this agrees with results from other studies [5,6,19]. Paulweber et al. [7] found that the R^- allele was significantly associated with higher TG levels, and this association, though not significant, was also apparent in the healthy individuals.

The *Ser* allele of the Asn/Ser_{4311} substitution was found to be associated with raised levels of HDL-TG and HDL-chol and with lower levels of LDL-chol in the healthy individuals [33]. These differences were of smaller effect than those associated with the Thr_{2488} neutral substitution [33]. In the patients, the *Ser* allele was also associated with lower LDL-chol and higher HDL-chol levels but with lower HDL-TG levels. The Asn/Ser_{4311} substitution explains only some of the differences seen in association with the Thr_{2488} neutral substitution [33]. Dunning et al. [33] predict that there are other, as yet unknown, functional variants within the apo B locus in linkage disequilibrium with the Thr_{2488} neutral substitution (*Xba*I RFLP) that would account for the remainder of the differences seen in association with the Thr_{2488} neutral substitution.

Of all the polymorphisms examined, a significant association was found between baseline score for global coronary atherosclerosis only for genotypes of the LAL insertion/deletion polymorphism. Individuals with the *ins/del* genotype had the lowest mean baseline and progression scores. This is the first study reporting a significant association between severity of coronary atherosclerosis and the LAL insertion/deletion polymorphism or any other apo B polymorphism, although other polymorphisms of the apo B gene have been reported to be associated with the presence of CAD [4–7,19].

Since the LAL insertion/deletion polymorphism was found to have no significant associations with fasting lipid traits in the Swedish sample, the mechanism by which this polymorphism influences baseline scores for global coronary atherosclerosis may not be via direct effects on lipid levels. This polymorphism might be in linkage disequilibrium with other genetic variations, either within or out-

side the apo B gene locus, that have causal effects on baseline severity and progression of global coronary atherosclerosis. Individuals with the *del/del* genotype have a higher mean TG level, while the subgroups with the *ins/ins* genotype and *ins/del* genotype have similar mean TG levels (data not shown). Also the correlation between baseline and progression scores is strongest for the subgroup with the *del/del* genotype. There might be a link between higher mean TG levels and progression of disease via genes that are not directly involved in lipid metabolism, for example genes involved in coagulability. Previous studies [44,45] have shown an association between hypertriglyceridaemia and increased coagulability, which, in turn, is a known risk factor for post-prandial atherosclerosis [44,45].

When evaluating the role of apolipoprotein B gene polymorphisms in the evolution and further progression of coronary atherosclerosis, we considered it useful to make a distinction between haemodynamically significant stenosis or occlusions on the one hand and diffuse atherosclerotic lesions on the other. Two reasons justified this distinction. Coronary angiography not infrequently shows complete occlusion of a major artery without any sign of atherosclerosis. In contrast, widespread wall irregularities, presumably reflecting diffuse atherosclerosis, are occasionally seen unaccompanied by clinically important stenosis or occlusions. Secondly, metabolic disturbances are most likely primarily involved in the early stages of lesion formation, whereas severe stenosis or total occlusion of a coronary artery are often the result of plaque rupture with subsequent intraintimal thrombosis progressing to intraluminal thrombosis [46,47]. However, it should be emphasized in this context that coronary angiography consistently underestimates the severity of atherosclerotic lesions [48]. Obviously, a normal appearance of a coronary artery on angiography cannot rule out the presence of atherosclerosis which is still completely subintimal and/or not distorting the luminal margins. A further limitation of the present study is also the fact that a significant proportion of the patients belonging to the initial study for obvious reasons did not complete the protocol, a majority of whom probably had a severe disease progression. Accordingly, the results should be interpreted with due caution because of the inherent selection bias. Although it is not very likely, it cannot be entirely ruled out that the patients who died before the second angiography had different apolipoprotein B genotypes compared with those who were rated as having a particularly severe angiographic disease progression.

Notwithstanding these restrictions, the stronger correlation between baseline and progression scores in the subgroup of individuals with the *del/del* genotype raises the possibility that baseline atherosclerosis score may be a better predictor of progression of disease, for individuals with the *del/del* genotype than those with other genotypes. If this relationship is confirmed in subsequent studies, this is an example of how genotype information may, in conjunction with classical plasma risk factors and measures of atherosclerosis, give useful information about the progression of clinical disease.

Acknowledgements

This study was supported by the Charing Cross Sunley Research Trust, grants from the British Heart Foundation (RG5), the Swedish Medical Research Council (8691), the Swedish Heart-Lung Foundation, the Torstenrand Ragna Sodeberrg Foundation and the King Gustaf V 80th Birthday Fund. Rachel Peacock is supported by a studentship from the British Medical Research Council. Dr Hamsten is Career Investigator of the Swedish Heart-Lung Foundation. The authors wish to thank Dr Chun-fang Xu for access to unpublished work and Elaine Osman for her help with preparation of the manuscript.

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Printed and Published in Ireland

ATHERO 04930

Associations between lipoprotein lipase gene polymorphisms and plasma correlations of lipids, lipoproteins and lipase activities in young myocardial infarction survivors and age-matched healthy individuals from Sweden

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(Received 1 June, 1992)
(Revised, received 10 August, 1992)
(Accepted 11 August, 1992)

Summary

Association studies were carried out on a sample of 87 patients from Sweden who had survived a myocardial infarction (MI) at a young age and 93 age-matched healthy individuals, to compare the impact of polymorphisms (*Pvu*II, *Hin*dIII and *Serine*$_{447}$-*Stop*) at the lipoprotein lipase (LPL) gene locus on among-individual differences in plasma lipid traits and progression of atherosclerosis. Significant linkage disequilibrium was detected between any two of these polymorphisms, with the *Stop*$_{447}$ allele being only found on the same chromosome as the rare alleles (no cutting sites) of the *Pvu*II and *Hin*dIII polymorphisms. In the healthy individuals, weak associations were found between genotypes of the *Hin*dIII polymorphism and triglycerides and the *Pvu*II polymorphism and high density lipoprotein cholesterol explaining 7.4% and 5.6% of sample variance ($P = 0.03$ and 0.09), respectively. No associations were found between these traits and genotypes of the *Serine*$_{447}$-*Stop* substitution, and thus it is unlikely to be the cause of the associations seen with the *Pvu*II and *Hin*dIII polymorphisms even though it truncates the enzyme amino acid sequence. The presence of the rare allele, H^-, of the *Hin*dIII polymorphism was associated with a smaller variance in triglycerides and both cholesterol and triglycerides in the very low density lipoprotein fraction, and with larger interdependent variation between these lipid traits, and also

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Abbreviations: apo, apolipoprotein; LPL, lipoprotein lipase; RFLP, restriction fragment length polymorphism; chol, cholesterol; TG, triglycerides; MI, myocardial infarction.

between LPL activity and these lipid traits. This implies that the H^- allele, rather than the $Stop_{447}$ allele, has the major impact on interdependence between traits which are directly or indirectly influenced by LPL activity. In the healthy individuals who were carriers of the apolipoprotein *E2* allele, the inter-dependence between LPL activity and lipid traits was significantly smaller, and that between high density lipoprotein cholesterol and both cholesterol and triglycerides in the very low density lipoprotein fraction was much larger compared with non-carriers ($P < 0.05$). No significant associations were found between lipid traits or lipase activity and genotypes of the $Serine_{447}$-*Stop* substitution. However, in the patients, global severity of coronary atherosclerosis at the first angiography was significantly associated with haplotype combinations of the *Hin*dIII and the $Serine_{447}$-*Stop* polymorphisms, with the H^-*Stop* haplotype being associated with the highest median score ($P = 0.02$). The data suggest that variation at the LPL gene locus is associated with a pleiotropic effect, that is not directly mediated by changes in lipids, on severity of coronary atherosclerosis.

Key words: Lipoprotein lipase gene polymorphism; Lipids; Lipoproteins; Myocardial infarction; Angiography

Introduction

Lipoprotein lipase (LPL) and hepatic lipase (HL) are heparin-releasable enzymes, bound to glycosaminoglycan components of the capillary endothelium, with central roles in lipid metabolism [1]. In humans, LPL is found in a variety of tissues, whereas HL is located primarily on the sinusoidal surfaces of liver endothelium [1]. LPL and HL are both glycoproteins; LPL is active as a dimer of 2 identical subunits each of approx M_r 60 000 for the mature, glycosylated protein [2] and HL is active as a monomer of approx M_r 62 500 for the unglycosylated form [3]. LPL hydrolyzes triglycerides in large triglyceride (TG)-rich lipoproteins (chylomicrons and VLDLs) ([1], reviewed in Ref. 3). HL hydrolyzes surface phospholipids of HDL_2 and both surface phospholipids and core triglycerides of triglyceride-enriched HDL_2 [4,5].

The gene for LPL has been located to the short arm of chromosome 8 [6] and the gene structure and cDNA sequence elucidated [2,7–10]. This gene spans about 30 kb and contains 10 exons coding for a 475 amino acid protein including a 27 amino acid signal peptide. Exon 10 codes for the entire 3′ untranslated region. Over the past 3 years, numerous sequence variants within the LPL gene have been found. Missense (e.g., Refs. 11–13) and nonsense (e.g., Refs. 14, 15) mutations lead to synthesis of non-functional LPL. Several restriction fragment length polymorphisms (RFLPs) in the LPL gene have been reported [16–19]; including the *Pvu*II RFLP in the intron between exons 6 and 7 [17] and the *Hin*dIII RFLP in the intron between exons 8 and 9 [18]. Neither of these RFLPs alter any amino acids of the enzyme. One polymorphism that does lead to a change in the amino acid sequence is a C to G tranversion at nucleotide 1595 of the cDNA sequence [19] (the $Serine_{447}$-*Stop* substitution) causing production of a protein truncated by 2 amino acids.

Several published population studies [16,20–22] have looked at the *Pvu*II and *Hin*dIII RFLPs in association with lipid and lipoprotein variables. Chamberlain et al. [20] found a lower frequency of the rare allele (H^-) of the *Hin*dIII RFLP in individuals with hypertriglyceridaemia compared with normotriglyceridaemic controls. Heizmann et al. [16] reported significant associations between total cholesterol and HDL-cholesterol levels and genotypes of the *Hin*dIII RFLP; the rare allele being associated with lower levels of HDL-cholesterol and higher levels of total cholesterol.

The purposes of this study were several: to investigate associations between genotypes of the *Pvu*II RFLP, *Hin*dIII RFLP and the $Serine_{447}$-*Stop* substitution and serum lipid and lipoprotein variables in patients who survived a MI at a young age and age-matched healthy individuals; to look for associations between baseline and progression scores for disease in the patients and genotypes of

the LPL gene polymorphisms studied. In particular, the effect of the *Serine$_{447}$-Stop* substitution on LPL activity and lipid traits was examined and the effects of genetic variation at the apo E and LPL gene loci on the correlation between LPL activity and lipid traits were determined.

Materials and Methods

Subjects

The sample of individuals used in this study was as described previously [23–25]. Briefly, it consisted of 87 patients who had suffered a first MI before the age of 45 years and 93 age-matched healthy male individuals, free of symptoms and clinical signs of CHD, all from Stockholm County, Sweden. The 87 patients were all male and free of manifest diabetes, familial hypercholesterolaemia or other major concomitant disease such as uraemia and collagenosis. Sixty-four of the healthy individuals and 36 of the patients were available for lipoprotein lipase and hepatic lipase activity measurements done in 1991. They also had repeat lipid and lipoprotein measurements made on this occasion — these are used in the correlation analysis with lipase activity. Procedures for coronary angiography and details of the scoring systems have been described elsewhere [26]. All 87 patients had coronary angiography at baseline but only 71 had a follow-up angiography. Due to a change in methodology of reading angiographies in the intervening time, scores are only available for those patients who did have a follow-up angiography. The angiography scores on severity of disease are on an arbitrary scale.

Lipid and lipoprotein analysis

A combination of preparative ultracentrifugation and precipitation of apo B-containing lipoproteins, followed by lipid analyses in the major lipoprotein fractions [27] was used to determine serum lipoproteins. Cholesterol [28] and triglycerides [29] were determined on an Ultrolab (LKB Bromma, Sweden) after chloroform–methanol extraction of whole serum, the VLDL fraction, the infranatant after ultracentrifugation (containing LDL and HDL) and the supernatant (HDL) after precipitation.

Lipoprotein lipase and hepatic lipase activities

Fifty units of heparin/kg body weight was administered after an overnight fast and 10 ml of post-heparin blood was drawn 15 min after the heparin infusion. Sonicated [^{3}H]trioleoyl-glycerol emulsions stabilized by phosphatidylcholine were the substrates used in direct selective measurements of LPL and HL activities [30]. Lipase activities were expressed in milliunits (mU), which correspond to 1 nmol of fatty acid released per minute.

DNA preparation and analysis

Blood was collected after an overnight fast. DNA was extracted from fresh, whole blood by the Triton X-100 lysis method [31]. PCR amplification [32] in a total volume of 50 μl was on a Cambio intelligent heating block (Cambio, Cambridge, UK). Oligonucleotides were obtained from the Advanced Biotechnology Centre at the Charing Cross and Westminster Medical School, London. *Thermus aquaticus* Polymerase was from Northumbrian Biologicals Ltd. and 0.5–0.8 unit used per PCR. Buffers were as recommended by Perkin Elmer-Cetus, CT. About 200 ng of each primer was used in each PCR.

PvuII RFLP between exons 6 and 7

The denaturation step of the PCR was performed at 93°C for 5 min; annealing and extension at 58°C for 5 min. Thirty subsequent cycles were as follows; 92°C for 1 min and 58°C for 5 min. Phial mode was used. Sequences of the 5′primer and 3′primer were (5′ to 3′) ATGGCACCCATGTGTAAGGTG and GTGAACTTCTGATAACAATCTC. The size of the PCR-amplified product is 440 bp. Ten units of *Pvu*II (Anglican Biotech) was added and incubation was overnight. Digests were subsequently run on 2% agarose gels at 50 V for 2 h. After digestion 2 fragments of 330 bp and 110 bp are produced if the polymorphic site is present.

HindIII RFLP between exons 8 and 9

The denaturation step of the PCR was performed at 96°C for 5 min; annealing at 55°C for 1.5 min and extension at 72°C for 5 min. Forty-five subsequent cycles were as follows; 98°C for 1 min, 55°C for 1 min and 72°C for 3 min. Plate mode was used. Sequences of the 5′primer and 3′primer

were (5′ to 3′) TTTAGGCCTGAAGTTTCCAC and CCCAGAATGCTCACCAGC respectively (F. Mailly, unpublished data). Ten units of *Hin*dIII (Anglian Biotech) was added and incubation was overnight. The size of the PCR-amplified product is about 1250 bp; the polymorphic site was recently localized to approximate position +495 of the intron [33]. Digests were subsequently run on 1.5% agarose gels at 50V for 2 h. After digestion, 2 fragments of almost identical size are produced if the polymorphic site is present; which migrate as a single band on the gel.

DNA amplified by PCR for the *Hin*dIII RFLP (see above), both undigested and digested, was run on 1.5% agarose gels and double blotted overnight onto Hybond-N plus membranes (Amersham). The membranes were subsequently alkali-fixed in 0.4 M NaOH for 3 min. The sequences of the probes, labelling, the oligomelting procedure and autoradiography procedure were as described previously [19].

Apo E gene polymorphism

Apo E genotyping was carried out using the restriction isotyping procedure as described by Hixson and Vernier [34] and the sequences of the primers used in the PCR were as described by Myant et al. [35].

Statistical analysis

Statistical analysis was carried out using the software package SSPC/PC+. Three healthy individuals in the larger sample with no values for either cholesterol (chol) or triglycerides (TG) were excluded, leaving 90 individuals available for analysis (only 87 were genotyped for the *Hin*dIII RFLP and $Serine_{447}$-*Stop* substitution due to failure of DNAs from 3 individuals to amplify). Mean levels of each lipid trait and of LPL and HL activities for the healthy individuals and patients were compared using the non-parametic, Mann-Whitney test. The *F*-test was used to test the null hypothesis that there is no difference between variances obtained with genotype H^+H^+ of the *Hin*dIII RFLP and those obtained when genotypes H^+H^- and H^-H^- were combined.

Data was $\log_{10}$-transformed prior to regression, analysis of variance (ANOVA), the *F*-test and correlation analysis if it appreciably reduced the kurtosis and skewness of the data (TG, VLDL-chol and VLDL-TG). Lipid and lipoprotein traits were adjusted by linear regression for age and recorded body mass index (BMI) and the percentage variance associated with age and BMI ($R^2 \times 100$) was estimated. Adjustment for age and BMI had little impact on the genotype means so actual means and standard errors for genotypes are presented in Table 5. For TG, VLDL-chol and VLDL-TG, 95% confidence limits were calculated on the $\log_{10}$-transformed data [36]. Antilogs of both means and 95% confidence limits are presented in Table 5. A one-way ANOVA was performed on the age and BMI adjusted data to test the null hypothesis that phenotypic variations in lipid and lipoprotein traits and in lipase activities were not associated with genetic variation at the LPL gene locus. Lipid and lipoprotein traits were first adjusted for age and BMI, to remove variation due to age and BMI. The impact of LPL genotypes on age and BMI adjusted traits was estimated by non-linear regression. This type of regression removes the dependence of a linear relationship between the mean of a trait for each genotype and genotypes of the particular DNA polymorphism.

For baseline and progression of disease scores which could not be normalized by any means the non-parametric analogue of ANOVA, the Kruskal-Wallis test, was used to find associations between severity of disease and genotypes of the three LPL gene polymorphisms in this study.

Allele frequencies for each RFLP/polymorphism were estimated using the 'gene counting' method, and determined separately for the healthy individuals and patients. 2×2 contingency tables were used to compare allele frequency in healthy individuals with that in patients; 2×3 contingency tables were used to compare genotype frequencies, for each polymorphism, between healthy individuals and patients; 3×5 contingency tables were used to see if there was any association between the *Pvu*II RFLP and apo E genotypes in each group.

The method of Chakravati et al. [37] was utilised in the 'Excel' spreadsheet computer program to calculate the extent of linkage disequilibrium between any pairwise combination of LPL gene polymorphisms. For this the healthy individuals and patients were treated as a single group. For

each of the three LPL gene polymorphisms for the whole Swedish sample, the PIC (polymorphic information coefficent) [38] and heterozygosity index (formula given below) were calculated.

Heterozygosity index =
1 – (the sum of the squares of the frequencies of the individual estimated homozygote genotype frequencies)

For selected pairs of traits, based on what is known about lipid metabolism in general, the relationship was investigated using correlation analysis. Log_{10}-transformation was carried out if the data was not already approximately normally distributed; this was necessary for TG and VLDL-chol. The Pearson correlation coefficient was computed and for groups with fewer than 40 individuals, a correction factor was applied [36].

$r^* = r(1 + (1 - r^2/2(n - 4)))$
n = no. of individuals in the sample

Either r or r^* was squared to give r^2, the coefficient of determination which is an estimate of the extent to which variation in the two variables is interdependent (proportion of variation of one variable determined by variation of the other). To test the null hypothesis that two correlation coefficients were not significantly different from one another [38], the coefficient r was first z-transformed. To calculate the t-statistic the following formula was used:

t = (Difference between the two Z-transforms) divided by the square root of $(1/(n_1 - 3) + 1/(n_2 - 3))$

where Z_1 and Z_2 are the two Z-transforms; n_1 and n_2 are the numbers of individuals in each group. The t-statistic was determined, at the infinite number of degrees of freedom, from the table of critical values of Student's t-distribution [36] to obtain the two-tailed P-value. For all correlation analysis unadjusted data was used, to explore inter-relationships between plasma traits. Prior adjustment of the data for age and BMI would mask the actual in vivo situation.

We considered statistical significance to be at the 0.05 level; except for correlations where the more conservative 0.01 level was adopted. However, since the study groups were small and multiple comparisons were being performed, probability values need to be interpreted with greater caution.

Results

Table 1 shows the characteristics of the healthy

TABLE 1

CHARACTERISTICS OF THE HEALTHY INDIVIDUALS AND PATIENTS

Mean ± standard errors (S.E.). Units of LPL and HL activity are nmol of fatty acid released per minute per ml plasma (= milliunits).

Trait	Healthy individuals			Patients			Results from Mann-Whitney test: 2-tailed P-values
	Mean	S.E.	n	Mean	S.E.	n	
Age (years)	40.46	0.403	93	40.35	0.377	86	NS
BMI (kg/m^2)	24.59	0.264	92	26.42	0.350	86	0.0002
Cholesterol (mmol/l)							
Total	6.09	0.123	90	7.24	0.137	86	<0.0001
VLDL	0.55	0.062	90	1.09	0.092	86	<0.0001
HDL	1.42	0.035	90	1.12	0.025	85	<0.0001
Triglycerides (mmol/l)							
Total	1.48	0.142	90	2.61	0.192	86	<0.0001
VLDL	0.93	0.124	90	1.89	0.170	86	<0.0001
HDL	0.15	0.004	90	0.16	0.005	84	0.02
LPL activity	92.05	3.64	64	74.25	4.16	36	0.004
HL activity	310.08	13.64	64	279.75	21.69	36	NS

individuals and patients. With the exception of age and HL activities, the means between the two groups are significantly different. As was expected [23], chol and TG levels were higher and HDL-chol levels were lower in the patients compared with the healthy individuals and in particular, mean LPL activity was 24% higher in the healthy individuals than in the patients. Standard errors, except for those for age and HDL-chol, were smaller in the healthy individuals than in the patients; consistent with the greater homeogenity expected in healthy individuals. For the lipid and lipoprotein traits, higher means were associated with greater heterogeneity of variance, as shown by higher standard errors, consistent with the observations made by Reilly et al. [39]. Genotypes were determined for three polymorphisms in the LPL gene; their locations within the gene are shown in Fig. 1. For all the polymorphisms studied, genotype distributions were close to and

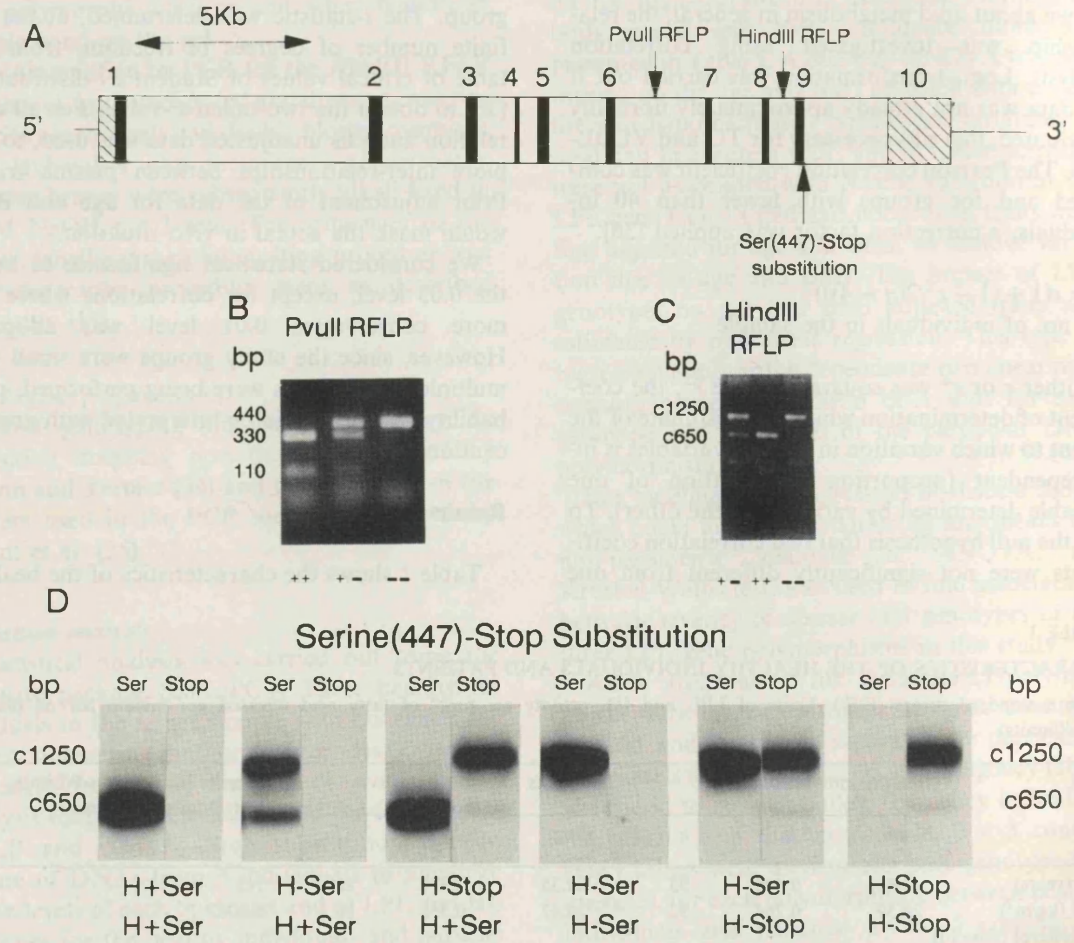


Fig. 1. (A) Map of the lipoprotein lipase (LPL) gene showing approximate positions of the polymorphisms used in this study. The open box represents the transcribed region of the LPL gene. Hatched boxes represent untranslated regions and black boxes represent the translated regions. Exons are numbered 1 (the 5′ most exon) to 10 (the 3′ most exon). (B,C) Photographs of gels showing electrophoresis of fragments obtained after digestion of PCR-amplified DNA with *Pvu*II or *Hin*dIII, respectively. Sizes of fragments are shown as number of base pairs (bp). All three genotypes of each polymorphism are shown; + and – denote presence and absence of the polymorphic restriction site, respectively. In the case of the *Hin*dIII polymorphism, smaller fragments which are PCR artefacts often appear on the gel, but do not interfere with genotyping. (D) ASO Detection of the *Serine-Stop* substitution using Southern blot of *Hin*dIII digested PCR-amplified DNA. All the six haplotype combinations found in this study for the *Hin*dIII RFLP and the *Serine*$_{447}$*-Stop* substitution are shown. Lanes labelled Ser and Stop were hybridized with the wild type and mutant oligos, respectively. Sizes of fragments are shown as number of base pairs (bp).

not significantly different from the Hardy-Weinberg prediction. Table 2 shows the rare allele frequencies for each of the three LPL gene polymorphisms studied. There were no significant differences in allele frequency between the healthy individuals and patients. It was possible to determine haplotypes unequivocally where homozygosity occurred at two or all three of the loci. The results from the haplotype analysis for the healthy individuals and patients were not different and combined results are presented in Table 3. Out of eight possible haplotypes, five were found unequivocally. The $Stop_{447}$ allele was always found on the same chromosome as the rare alleles of both the *Pvu*II and *Hin*dIII RFLPs, showing strong allelic association. Significant allelic association was also detected between the rare (no cutting site) alleles of the *Pvu*II and *Hin*dIII RFLPs. Estimates of the extent of linkage disequilibruim (delta values) for each pairwise combination of loci were similar in the healthy individuals and patients and results are shown in Table 3.

The effect of variation associated with the three LPL polymorphisms on lipid levels was estimated by non-linear regression after adjustment of lipid levels for age and BMI, and results are presented in Table 4. In the healthy individuals, plasma TG levels were significantly associated with genotypes of the *Hin*dIII RFLP and three other associations were nearly significant; levels of VLDL-chol

TABLE 2

ALLELE FREQUENCIES FOR THE LPL GENE POLYMORPHISMS STUDIED

Using 2 × 2 contingency tables, none of the allele frequencies were significant between the healthy individuals and patients.

LPL gene polymorphism	Rare allele	Rare allele frequency	
		Healthy individuals (n = 92)	Patients (n = 87)
*Pvu*II RFLP in intron 6	V^- (no cutting site)	0.435	0.425
*Hin*dIII RFLP in intron 8	H^- (no cutting site)	0.228	0.218
Ser_{447}-*Stop* substitution in exon 9	$Stop_{447}$	0.056	0.052

TABLE 3

HAPLOTYPE FREQUENCIES FOR HEALTHY INDIVIDUALS AND PATIENTS COMBINED

Polymorphic information coefficients for the *Pvu*II RFLP, *Hin*dIII RFLP and the Ser_{447}-*Stop* substitution were 0.370, 0.287 and 0.106, respectively. Delta values for *Pvu*II RFLP + *Hin*dIII RFLP, *Pvu*II RFLP + Ser_{447}-*Stop* substitution and *Hin*dIII RFLP + Ser_{447}-*Stop* substitution were 0.40, 0.27 and 0.45, respectively. All were significantly different from zero ($P < 0.001$ at 1 degree of freedom).

Unequivocal haplotype	n	Frequency (as percentage)
V^+H^+ Ser_{447}	158	54.5
V^-H^+ Ser_{447}	83	28.6
V^-H^- Ser_{447}	19	6.6
V^+H^- Ser_{447}	11	3.8
V^-H^- $Stop_{447}$	19	6.6

TABLE 4

PROPORTION OF VARIANCE ($R^2 \times 100$) IN TRAITS ADJUSTED FOR AGE + BMI THAT IS EXPLAINED BY GENOTYPES OF POLYMORPHISMS IN THE LPL GENE

Trait	Age + BMI[a]	*Pvu*II RFLP	*Hin*dIII RFLP	Ser_{447}-*Stop* substitution
Healthy individuals				
Cholesterol				
Total	1.9	4.3	2.1	0.4
VLDL	12.6**	3.7	6.2	0.6
HDL	12.3**	5.6	2.8	1.4
Triglycerides				
Total	12.6**	2.7	7.4*	0.3
VLDL	11.8**	2.8	6.2	0.2
HDL	2.3	0.6	2.4	2.0
LPL activity	2.0	0.7	2.2	0.9
Patients				
Cholesterol				
Total	0.3	0.1	0.8	0
VLDL	11.2**	2.5	0.1	0
HDL	6.2	1.9	0.2	0.1
Triglycerides				
Total	10.2	2.4	0.5	0
VLDL	13.0	2.0	0	0.1
HDL	4.9	3.7	2.2	0.3
LPL activity	5.9	4.3	1.5	3.7

[a]Unadjusted data.
*$P < 0.05$, **$P < 0.01$.

($P = 0.05$) and VLDL-TG ($P = 0.05$) with genotypes of the *Hin*dIII RFLP and HDL-chol levels ($P = 0.09$) with genotypes of the *Pvu*II RFLP. After adjustment for age and BMI, each of these associations accounted for at least 5% of total sample variance. The associations observed between the Ser_{447}-*Stop* substitution and serum lipid traits were non-significant. None of the LPL gene polymorphisms used here were associated with a significant effect on any trait in the patient group.

Genotype means and standard errors or 95% confidence limits are presented in Table 5 for the above four associations both for healthy individuals and patients. For the *Pvu*II RFLP, heterozygous individuals had mean levels of HDL-chol roughly 12% higher than either of the homozygous genotype groups. This effect was not seen in the patients. For the *Hin*dIII RFLP and the Ser_{447}-*Stop* substitution, individuals homozygous for the haplotype H^+Ser_{447} were combined into one group. Individuals carrying the rare allele were sub-divided into groups carrying and lacking the *Stop* allele, respectively. This allowed simultaneous comparison of haplotypes of both polymorphisms rather than each polymorphism being considered separately. In the healthy individuals, the group carrying the H^- allele but lacking the $Stop_{447}$ allele had the lowest mean levels of TG, VLDL-chol and VLDL-TG (72%, 66% and 65%, respectively, of the mean levels seen with genotype H^+H^+) while the group homozygous for the H^+Ser_{447} haplotype had the highest means for all of these traits. For all three traits when the three healthy individuals with the genotype H^-H^- were examined separately, regardless of the presence or absence of the $Stop_{447}$ allele, considerably lower means compared to those with the two other genotypes were obtained (data not shown). When the four healthy individuals with plasma chol levels >9.5 mM and/or TG levels >3.0 mM were excluded these associations remained statistically significant (data not shown). In 64 of the healthy individuals and 36 of the patients measurement of LPL activity had been carried out. The characteristics of this subgroup were not significantly different from the whole population (data not shown). None of the three polymorphisms were associated with a significant effect on levels of LPL activity. However, in both patients and healthy individuals, those carrying the $Stop_{447}$ allele had lower mean LPL activities though the effect was noticeably stronger in the patients (24% lower than the others not carrying this allele; Table 5).

In healthy individuals and patients the standard deviations for TG, VLDL-chol and VLDL-TG $\log_{10}$-transforms were smaller (data not shown) in the group with at least one copy of the H^- allele compared to that without this allele. Results of the *F*-test, used to compare variances in the presence and absence of the H^- allele, are shown in Table 5. Coefficients of determination (measure of proportion of variation in one trait determined by variation in the other trait) are displayed in Table 6 for the whole group and haplotype combinations of the *Hin*dIII RFLP and the Ser_{447}-*Stop* substitution and in Table 7 for the three common apo E genotypes. Except for genotype *E3/2* the correlations between LPL activity and triglycerides were negative and between LPL activity and HDL-chol were positive, as expected (data not shown). In all cases the expected negative correlations were observed between HDL-chol and VLDL-chol and between HDL-chol and TG. In healthy individuals and patients combined, interdependent variation between all four pairs of traits was larger for the genotypes H^+H^- and H^-H^- combined, compared with the genotype H^+H^+. This was significant between HDL-chol and TG or VLDL-chol due to larger group sizes. For the apo E polymorphism, the gradient of the slope (*r* value) observed in the group of individuals with the genotype *E3/2* was, for three of the four pairs of traits, significantly divergent from those obtained by combining individuals with apo E genotypes *E3/3* and *E4/3* who show similar values (Table 7). The effect of genotypes of the *Xba*I RFLP in the apo B gene on the correlation between LPL activity and serum apo B levels was examined but no significant differences were found (data not shown).

In the patients, associations of baseline and progression of atherosclerosis scores with genotypes for all three LPL gene polymorphisms, were examined. For the *Hin*dIII RFLP and Ser_{447}-*Stop* substitution, patients were divided into three groups: absence of both H^- and $Stop_{447}$ alleles, presence of the H^- allele but not *Stop* allele, and

TABLE 5

GENOTYPES MEAN (± S.E.) FOR SELECTED TRAITS

Adjustment for age and BMI made little difference to the genotype means, so unadjusted values are shown here.

Trait (mmol/l)	Healthy individuals				Patients			
	Genotypes (*n*)			*F*-value + significance from ANOVAS[a]	Genotypes (*n*)			*F*-value + significance from ANOVAS[a]
	V^+V^+ (29)	V^+V^- (42)	V^-V^- (19)		V^+V^+ (29)	V^+V^- (38)	V^-V^- (18)	
HDL-chol	1.32 ± 0.05	1.51 ± 0.05	1.38 ± 0.08	$F = 2.53$ $P = 0.09$	1.11 ± 0.04	1.10 ± 0.04	1.18 ± 0.04	$F = 0.8$ $P = 0.46$
	H^+H^+ *Ser/Ser* (52)	H^+H^- and H^-H^- combined			H^+H^+ *Ser/Ser* (49)	H^+H^- and H^-H^- combined		
		Ser/Ser (26)	*Ser/Stop* + *Stop/Stop* (9)			*Ser/Ser* (28)	*Ser/Stop* (9)	
Triglycerides[b]	1.42 (1.22–1.66)	1.02 (0.87–1.18)	1.18 (1.07–1.50)	$F = 4.67$ $P = 0.03$	2.24 (1.90–2.42)	2.28 (1.90–2.74)	2.13 (1.51–3.00)	$F = 0.08$ $P = 0.8$
VLDL-cholesterol[b]	0.50 (0.41–0.61)	0.33 (0.26–0.41)	0.40 (0.29–0.56)	$F = 3.8$ $P = 0.05$	0.83 (0.68–1.02)	0.95 (0.75–1.20)	0.84 (0.52–1.37)	$F = 0.10$ $P = 0.75$
VLDL-triglycerides[b]	0.80 (0.65–0.99)	0.52 (0.41–0.65)	0.63 (0.44–0.91)	$F = 3.81$ $P = 0.05$	1.45 (1.18–1.78)	1.60 (1.27–2.03)	1.47 (0.99–2.17)	$F = 0.02$ $P = 0.88$
LPL activity (milliunits/ml)	(36) 88.89 ± 4.55	(20) 96.85 ± 7.95	(6) 91.83 ± 8.73	$F = 0.46$ $P = 0.5$	(20) 77.40 ± 5.36	(12) 74.42 ± 7.76	(4) 58 ± 11.93	$F = 0.72$ $P = 0.4$

[a]Age and BMI adjusted data.

[b]Data is shown as antilogs of log-transformed mean and 95% confidence units.

F-test for comparing variances between groups of individuals carrying and lacking the H^- allele, respectively.

	Healthy individuals		Patients	
$\log_{10}$-triglycerides	$F = 2.25$	$P < 0.02$	$F = 1.56$	NS
$\log_{10}$-VLDL-cholesterol	$F = 1.67$	NS	$F = 1.33$	NS
$\log_{10}$-VLDL-triglycerides	$F = 1.74$	NS	$F = 1.54$	NS

TABLE 6

COEFFICIENTS OF DETERMINATION $R^2 \times 100$ FOR PAIRWISE COMPARISONS OF SERUM TRAITS FOR COMBINATIONS OF GENOTYPES OF LPL GENE POLYMORPHISMS

Nos. of individuals with LPL activity data was 100 while the Nos. of individuals with data on lipid traits was 173. All correlations were carried out on unadjusted data, to give an estimate of the in-vivo situation with regard to interactions between traits. Estimates for the healthy individuals and patients were similar so the data from the combined group are presented here.

Traits	Healthy individuals and patients combined				
	All	H^+H^+ *Serine/ Serine*	H^+H^- and *Serine/ Serine*	H^-H^- combined *Serine/Stop* and *Stop/Stop*	Sig. of difference between H^+H^+ and (H^+H^- and H^-H^- combined)
LPL activity and triglycerides[a]	21.9**	11.7*	29.6*	58.2	NS
LPL activity and HDL chol	15.6**	10.4	22.1*	33.3	NS
HDL chol and VLDL chol[a]	31.2**	19.6**	51.6**	60.2**	$P < 0.01$
HDL chol and triglycerides[a]	24.3**	15.3**	42.1**	48.5*	$P < 0.05$

[a]Correlations carried out on $\log_{10}$-transformed data.
*$P < 0.01$, **$P < 0.001$.

presence of both H^- and *Stop* alleles. Only the baseline score was significantly associated with haplotype combinations of the *Hin*dIII RFLP and the Ser_{447}-*Stop* substitution. As shown in Fig. 2 the median score was 280% higher in individuals with the H^-*Stop* haplotype compared with those without this haplotype (2.8 vs. 1.0, $P = 0.02$).

Discussion

The allele frequencies for the *Pvu*II and *Hin*dIII RFLPs in this study are similar to most of those reported previously in studies on Caucasian populations [16,20,22]. Differences in sample selection and criteria used to define patients and

TABLE 7

COEFFICIENTS OF DETERMINATION $R^2 \times 100$ FOR PAIRWISE COMPARISONS OF SERUM TRAITS FOR APO E GENOTYPES IN HEALTHY INDIVIDUALS

Numbers in brackets denote number of individuals. All correlations were carried out on unadjusted data to give an estimate of the actual in-vivo situation with regard to interactions between traits. Estimates for the healthy individuals and patients were not similar so only data from the healthy individuals are shown.

Traits	Apo E genotype			
	2/3	*3/3*	*4/3*	Significance of difference between genotypes *2/3* + (*3/3* + *4/3* combined)
LPL activity + triglycerides[a]	14.6 (9)	18.7* (30)	36.6* (18)	$P < 0.05$
LPL activity + HDL chol	5.8 (9)	14.0 (30)	13.0 (18)	NS
HDL chol + VLDL chol[a]	75.8** (12)	17.0* (47)	16.5 (20)	$P < 0.01$
HDL cholesterol + triglycerides	63.2* (12)	10.6 (47)	13.3 (20)	$P < 0.05$

[a]Correlations carried out on $\log_{10}$-transformed data.
*$P < 0.01$, **$P < 0.001$.

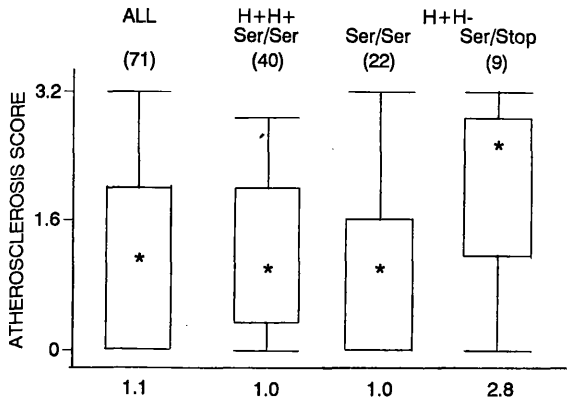


Fig. 2. Box plot showing the distribution of baseline scores for coronary atherosclerosis for the whole patient sample and in the absence and presence (with and without the *Stop* allele of the Ser_{447}-*Stop* substitution) of the H^- allele of the *Hin*dIII RFLP. $\chi^2 = 5.5$, $P = 0.065$ across all three groups; $P = 0.02$ between those with the H^-Ser_{447} haplotype and those with the H^-Stop_{447} haplotype and $P = 0.05$ between individuals with the H^+Ser_{447} haplotype and those with the H^-Stop_{447} haplotype. Scores are given on the vertical axis. Genotypes and number of individuals are given above the boxes on the horizontal axis; values of medians are shown below the respective boxes; numbers in brackets denote number of individuals with that genotype; *median.

controls are possible causes of the differences observed between this study and others, e.g. in Ref. 22, the healthy sample with a H^- allele frequency of 0.421 was not age-matched to their patients with coronary artery disease. Other possibilities, which are more likely, are the younger age of the patients in this study and/or differences in evaluation of disease scores and severity. Hata et al. [19] reported the frequency of the $Stop_{447}$ allele as 0.163 in 86 healthy subjects and 0.043 in 35 hypertriglyceridemic subjects. More studies need to be carried out to ascertain the frequency of the $Stop_{447}$ allele in the general population.

The highly significant linkage disequilibrium between any pairwise combination of the three LPL gene polymorphisms studied, suggests that there has been little, if any, recombination in at least the 3′ part of the LPL gene; this is not unexpected since distances involved are quite small. Linkage disequilibrium between the rare alleles of the *Pvu*II and *Hin*dIII RFLPs (V^- and H^-, respectively) has been reported in Caucasian and Japanese population studies [16,20]. The commonest haplotype in all the Caucasian populations surveyed to date, including this study, is V^+H^+. The finding that the $Stop_{447}$ allele was always on the same chromosome as the V^- and H^- alleles, strongly suggests that the single base substitution, creating this stop codon, occurred in an individual with the haplotype V^-H^-.

We investigated associations between genotypes at the LPL gene locus and serum lipids and lipoproteins in the patients and healthy individuals separately. In the healthy individuals, a significant association was found between TG and the *Hin*dIII RFLP, with weaker associations observed between this RFLP and VLDL-chol and VLDL-TG and between the *Pvu*II RFLP and HDL-chol. The association between the H^+ allele of the *Hin*dIII RFLP in both healthy individuals and patients in this study with higher TG levels agrees with previous findings [20,22]. In both Caucasian and Japanese populations, analyzed separately [20], the H^+ allele was significantly more frequent in hypertriglyceridaemic as compared with normotriglyceridaemic individuals.

Our findings support the hypothesis previously put forward [20,22] that the H^+ allele is associated with a variant in or near the LPL gene that predisposes to hypertriglyceridaemia and screening for possible causative variation in the H^+ allele of the LPL gene is now warranted.

The association between lower HDL-chol levels and the V^+ allele of the *Pvu*II RFLP in the healthy individuals is consistent with the non-significant association reported [20], although no significant association between HDL-chol and the *Pvu*II RFLP was found in another study [16]. Associations between serum HDL-chol levels and genotypes of the *Hin*dIII RFLP have also not been consistent, since in one study [16] the H^- allele was significantly associated with lower HDL-chol levels, while in two others [20,22], the H^- allele was associated with higher HDL-chol levels. In this study, the H^+H^- genotype was associated with non-significantly lower and higher means compared with the H^+H^+ genotype in the patients and healthy individuals, respectively. The most plausible reasons for these inconsistencies are the differences in grouping for statistical treatment,

differences in sample and patient selection, and errors in measurement of HDL-chol; e.g. combining patients with coronary artery disease with healthy individuals into a single group for association studies with lipids and lipoproteins. The effect of LPL on HDL particles is indirect via transfer to the HDL fraction, of surface components from triglyceride-rich lipoproteins that have been acted upon by LPL [40]. It is likely that the influence of genetic variation at the LPL locus on HDL levels will be modified by other genetic or environmental factors. Larger population studies will be needed to confirm any association between the LPL *Pvu*II and *Hin*dIII RFLPs and HDL-chol levels; and to ascertain a possible association between the V^-H^- haplotype and an anti-atherogenic lipid profile.

It is plausible that the truncation of the two carboxyl-terminal amino acids caused by the Ser_{447}-*Stop* substitution might have a direct functional effect on LPL activity, since the primary sequence of LPL is changed. We therefore examined the association between LPL activity and genotypes of the Ser_{447}-*Stop* substitution. In the healthy individuals, mean LPL activity was similar in individuals with and without the *Stop* allele. This observation is consistent with the recent findings of Faustinella et al. [13], who concluded, from expression experiments using Cos-1 cells, that the Ser_{447}-*Stop* substitution had little effect on LPL activity. In contrast, Kobayashi et al. [41] reported that LPL activity expressed from the LPL-$Stop_{447}$ construct was lower that that expressed from the wild type LPL-Ser_{447} construct; experiments with water-soluble and emulsified substrates indicated that the functional defect was due to an impaired lipid-binding ability of the LPL-$Stop_{447}$ enzyme. The lower LPL activities recorded among our patients carrying the $Stop_{447}$ substitution may reflect this phenomenon; our assay system, using as substrate a trioleoyl glycerol emulsion stabilized by dioleoylphosphatidyl choline, would most likely reveal defects not only in catalytic activity but also in lipid binding ability. Further studies, using water-soluble and emulsified substrates, might be helpful to determine whether the lower enzyme activity in our patients carrying the *Stop* allele is an artefact of small group size or whether it does indeed represent an impairment of the lipid interface recognition ability in this group.

We therefore hypothesized that genotypes at the LPL gene locus and other loci such as the apo E gene may have an effect on the inter-relationship between LPL activity and lipid traits, as well as between some of the other metabolically-related serum lipid traits. Relationships between pairs of traits were therefore investigated by correlation analysis in whole groups and separate genotypes. The results in the healthy individuals and patients (data not shown) were similar, so for correlation analysis the two groups were combined. The presence of the H^- allele was associated with a smaller variance for TG, HDL-chol and VLDL-TG and with larger coefficients of determination (R^2) for all pairwise comparisons made here, compared with the H^+H^+ genotype. This trend is also apparent in a previous study [16], with smaller standard deviations for TG and HDL-chol in individuals with the H^- allele compared with those without this allele (Table 4 in Ref. 16, $P < 0.05$ for HDL-chol). In our sample, R^2 values were similar between the two groups carrying the H^- allele, while the R^2 values between the groups carrying and lacking the H^- allele were significantly different. This implies that the H^- allele rather than the $Stop_{447}$ allele has the major impact on correlations between traits which are directly or indirectly affected by LPL activity. This could be tested using larger samples. One possible explanation is that the LPL enzyme coded by the H^-Ser_{447} allele has a greater affinity for lipoproteins than the enzyme from the H^+Ser_{447} allele, for example, caused by sequence variations of the LPL gene segment coding for the interfacial lipid-binding region located to exon 4 [7,42] that could be in linkage disequilibruim with the H^- allele. Our hypothesis is that this postulated greater affinity is seen in the stronger metabolic relationship between LPL activity and lipid traits, as well as between pairs of lipid traits. This would lead to a tighter association; the LPL enzyme does not dissociate and reassociate during the metabolism of the TG-rich particle, resulting in better breakdown of TG-rich lipoproteins and lower levels of TG in plasma. Recent studies have reported that LPL increases uptake of remnant lipoproteins by hepatic LRP (LDL-receptor

related protein) which is thought to be independent of TG hydrolysis by LPL [43] and that, in HepG2 cells, such uptake leads to reduced secretion of TG-rich particles [44]. LPL with higher affinity for lipoprotein particles would also lead to lower plasma TG levels through such a mechanism.

For the healthy individuals, carriers of the *E2* allele showed a much smaller covariation (lower correlation) between LPL activity and both TG and HDL-chol than the other groups. In contrast these *E3/2* individuals showed a much higher covariation between HDL-chol and both VLDL-chol and TG, than did *E3/3* or *E4/3* individuals; but these results need to be interpreted with caution because of the small number of individuals with the genotype *E3/2*. Reilly et al. [45] have recently reported that the common isoforms of apo E are associated with effects on the covariation of lipid traits in healthy men and women, with the covariation being greater in individuals with genotypes *E2/3* and *E4/3* compared with those with genotype *E3/3*. This agrees with the large impact that the *E2* allele is known to have on lipid metabolism; the general effect is via the lower binding affinity of the *E2* isoform for the apo B_{100}/E (LDL)-receptor (reviewed in Ref. 46) but a specific effect of apo E isoforms on LPL activity has not yet been demonstrated. Studies on Type III hyperlipidaemic subjects with the genotype *E2/2* showed a poor ability of lipases to convert apo B_{48}-containing chylomicrons and chylomicron remnants into lipoproteins in the LDL density range, suggesting that apo E2 indirectly influences the hydrolytic activity of LPL [47]. By contrast these authors found that apo B_{100}-containing particles were efficiently converted to particles with the density of LDL; they thus suggested that apo E plays a role in the normal pathway of conversion of VLDL to LDL. The higher degree of covariation observed in the group of *E2* carriers between HDL-chol and both VLDL-chol and TG may be partially explained by the known preferential association of the apo E2 isoform for HDL particles [48]. A plausible consequence of this would be that apo E2 molecules would more easily dissociate from VLDL particles, increasing the likelihood of LPL remaining attached to VLDL particles because the surface of the VLDL particle is less crowded; allowing LPL to remain attached to VLDL particles for longer, leading to more complete hydrolysis of VLDL particles.

We found an effect of haplotype combinations of the *Hin*dIII RFLP and the Ser_{447}-*Stop* substitution on baseline coronary atherosclerosis scores. Individuals carrying the H^-*Serine* allele had similar median baseline scores to the whole patient group; while those carrying the H^-*Stop* allele had the highest median scores. This is not in agreement with the observation by Thorn et al. [22] of an elevated H^+ allele frequency in their patient sample. This discrepancy may be chance or due to difference in patient selection. A possible mechanism would be the effect of the Ser_{447}-*Stop* substitution on the lipid interface recognition ability of LPL and thus on metabolism of lipoproteins as described previously. Therefore genotypes of the Ser_{447}-$Stop_{447}$ substitution might have a pleiotropic effect on the severity of coronary atherosclerosis. Of all the three polymorphisms in this study the Ser_{447}-$Stop_{447}$ substitution would be most likely to be having a functional effect since it alone of the three polymorphisms causes a change in the LPL amino acid sequence. However this effect cannot be via changes in lipid levels since the Ser_{447}-*Stop* substitution was not associated with variation in these traits in our sample.

Larger population studies both on healthy individuals and on patients with clinically defined atherosclerosis are needed to clarify the putative roles of the Ser_{447}-*Stop* substitution and sequence variation associated with alleles of the *Hin*dIII RFLP in affecting suspectibility to disturbances in lipoprotein metabolism, leading to production of atherogenic lipoprotein particles with consequences for the development of atherosclerosis and thrombosis [49].

Acknowledgements

This study was supported by the Charing Cross Sunley Research Trust, the Department of Medicine at University College, London, grants from the British Heart Foundation (RG16), the Swedish Medical Research Council (8691 and 4961), the Swedish Heart-Lung Foundation, the Torsten and Ragna Soderberg Foundation and the

King Gustaf V 80th Birthday Fund. Rachel Peacock is supported by a studentship from the British Medical Research Council. Dr Anders Hamsten is Career Investigator of the Swedish Heart-Lung Foundation. The authors wish to thank Mrs Gina Deeley for her help in the preparation of the manuscript and Ms France Mailly for access to unpublished data and helpful discussion on the manuscript.

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A42485/93100801

AFSENDT - 3 JUNI 1994

Clin Genet 1994: 46: 00–00
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CLINICAL GENETICS
ISSN 0009-9163

Associations of genotypes at the apolipoprotein AI-CIII-AIV, apolipoprotein B and lipoprotein lipase gene loci with coronary atherosclerosis and high density lipoprotein subclasses

Peacock RE, Hamsten A, Johansson J, Nilsson-Ehle P, Humphries SE. Associations of genotypes at the apolipoprotein AI-CIII-AIV, apolipoprotein B and lipoprotein lipase gene loci with coronary atherosclerosis and high density lipoprotein subclasses.
Clin Genet 1994: 46: 000–000. © Munksgaard, 1994

Association studies were carried out in a sample of 86 patients from Sweden who had survived a myocardial infarction (MI) at a young age and 93 age-matched healthy individuals, to compare the impact of polymorphisms at the apolipoprotein (apo) AI-CIII-AIV gene cluster on among-individual differences in plasma lipid and lipoprotein traits, the five high density lipoprotein (HDL) subclasses (2b to 3c), lipoprotein lipase (LPL) activity and presence and progression of atherosclerosis. Individuals were genotyped for four polymorphisms; 5'apoAI (G/A_{-75}), 3'apoAI (PstI; P±) apoCIII (C/T_{1100}) and apoCIII (PvuII; V±), using PCR-based techniques. Allele frequencies were similar in healthy individuals and patients (frequencies of alleles in combined population: 5'apoAI-A_{-75}=0.14, 3'apoAI-P-=0.05, apoCIII-T_{1100} = 0.27 and apoCIII-V-=0.18). In the healthy individuals, levels of low density lipoprotein (LDL) triglycerides were significantly assocatied with genotypes of the apoCIII-PvuII polymorphism (p=0.02), but no other associations were found between lipids or HDL subclasses and single polymorphisms in the apoAI-CIII-AIV gene cluster. Levels of triglycerides and very low density lipoprotein (VLDL) trigylcerides were significantly higher in the presence of the haplotype defined by the presence of apoCIII-T_{1100} and common alleles of the other three polymorphisms, explaining 5.8% and 7.8% (p=0.03 and 0.01), respectively, of sample variance. In the patients, no associations were found between lipids or HDL subclasses and variation at the apoAI-CIII-AIV gene cluster. Associations were also examined between levels of HDL subclasses and variation at the apoE (common isoforms), apoB (signal peptide and XbaI polymorphisms) and lipoprotein lipase (PvuII, HindIII and $Serine_{447}$/Stop polymorphisms) gene loci. In the patient group only, levels of protein in HDL2b ,HDL2a and HDL3b subclasses were significantly associated with genotypes of the LPL-HindIII polymorphism (22.1, 19.3 and 11.4%, respectively, of sample variance; p<0.05). Finally, associations were examined between genotypes at the apoAI-CIII-AIV gene cluster and the extent of coronary atherosclerosis. Global severity of atherosclerosis at the first angiography was weakly associated with genotypes of the apoCIII-C/T_{1100} substitution, presence of the T_{1100} allele being associated with 53% lower median score (1.6 vs 0.75; p=0.09). In this group of patients, two genotypes, one each at the LPL and apoB gene loci, had been previously found to be associated with high atherosclerosis score and, when considered together, individuals with all three of these genotypes had the highest median score (2.4) and those with none of these genotypes had the lowest (0.4) (chi-squared overall=15.7; p=0.001); no lipid traits measured showed a similar association with these genotypes. Thus, in this sample of young male post-infarction patients, genetic variation at these three loci is having an additive effect on the development of atherosclerosis, that cannot be explained by their observed effect on fasting lipid and lipoprotein traits.

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Key words: angiography – apolipoprotein gene polymorphism – haplotype – correlations – lipids – lipoproteins – lipase activity

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Received 8 October 1993, revised version received 20 April, accepted for publication 21 April 1994

The high density lipoprotein (HDL) fraction in human plasma is heterogeneous (Tall et al. 1990); classically consisting of the two subfractions HDL_2 and HDL_3 defined by their floatation characteristics in the analytic ultracentrifuge (Gofman et al. 1966) and now known to consist of five subclasses, termed 2b, 2a, 3a, 3b and 3c -in order of decreasing particle diameter and distinguishable by gradient gel electrophoresis (Blanche et al. 1981). HDL is also subdivided into two types of particles – those containing apoAI but not apoAII and those containing both; the former is thought to represent the anti-atherogenic fraction of HDL and largely corresponds to the HDL2b and HDL3a subclasses (Puchois et al. 1987). HDL as a whole (Miller & Miller 1975) and the HDL_2 subfraction (Miller et al. 1981) have been suggested to have a protective action against the development of coronary atherosclerosis for some years. Case-control studies indicate that the well-known inverse relation of HDL with coronary atherosclerosis and clinical manifestations of coronary heart disease (CHD) is due mainly to HDL_2 (Ballantyne et al. 1982, Hamsten et al. 1986b, Griffin et al. 1988, Johansson et al. 1991). Recently, Johansson et al. (1991) studied a group of young myocardial infarction patients from Sweden and suggested that the inverse relation between HDL level and CHD or coronary atherosclerosis seen in previous studies is accounted for by the largest HDL particles, the HDL2b subclass. HDL has been thought to be directly protective via the mechanism of reverse cholesterol transport (Tall et al. 1990). On the other hand, HDL cholesterol levels depend on the metabolism of triglyceride-rich lipoproteins in the post-prandial state (Tall et al. 1990, Patsch et al. 1987). High levels of post-prandial triglyceride-rich lipoproteins lead to enhanced CETP-mediated lipid exchange with a shift of cholesterol from HDL to apolipoprotein B-containing lipoproteins (Tall 1986).

The two lipolytic enzymes, lipoprotein lipase (LPL) and hepatic lipase (HL) regulate the plasma HDL_2 concentration in a reciprocal manner; the generation of HDL_2 is a consequence of the catabolism of triglyceride-rich lipoproteins by LPL and transfer of surface components to HDL_3 (Taskinen & Kussi 1987). HDL_2 is subsequently acted upon by HL to produce smaller and denser HDL particles, HDL_3 (Groot et al. 1983, Patsch et al. 1984). When the LPL activity is low, HDL2b becomes more triglyceride-enriched and is acted upon more avidly by HL resulting in a shift towards small HDL particles and a low total HDL-cholesterol concentration. In HL deficiency HDL2b accumulates and the smaller HDL particles are virtually absent (Carlson et al. 1986).

The apoAI-CIII-AIV gene cluster is located on the long arm of chromosome 11 and the apoCIII gene is transcribed in the opposite direction relative to the other two genes (Karathanasis 1985). Over ten common polymorphisms have been detected within this gene cluster (Humphries 1988, Lusis 1988), and many studies have found associations between genotypes of some of these polymorphisms and variations in levels of lipids and lipoproteins (Humphries et al. 1990, Humphries et al. 1991). Altered allele frequencies of some of these polymorphisms in patients compared to healthy controls have been reported by some investigators. The frequency of the rare allele of the apoCIII-SstI RFLP has consistently been higher in patients with CHD (Ferns et al. 1985, Deeb et al. 1986, Price et al. 1989) and associated with hypertriglyceridaemia (Rees et al. 1985, Shoulders et al. 1986, 1989, Aalto-Setala et al. 1987, Henderson et al. 1987). The frequency of the rare allele of the 5'apoAI- XmnI RFLP was higher in Familial Combined Hyperlipidaemia (FCHL) patients in two studies (Hayden et al. 1987, Wojciechowski et al. 1991) and in patients with hypertriglyceridaemia (Kessling et al. 1986). However these associations have not been consistent. For example, the rare allele frequency of the 3'apoAI-PstI polymorphism was lower in patients relative to controls in some studies (Ferns & Galton 1986) but higher in other studies (Ordovas et al. 1986, Wile et al. 1989). Furthermore, the mechanism of these associations is not understood in detail but is assumed to be due to effects on plasma lipid traits. Associations between variation at the apoAI-CIII-AIV gene cluster and HDL levels, have been reported, particularly for 5'apoAI G/A_{-75}, 3'apoAI-PstI (Kessling et al. 1988) and apoCIII-PvuII (Xu et al. 1990b) polymorphisms and between the 3'apoCIII-SstI RFLP and plasma triglyceride levels (Ordovas et al. 1991).

No studies have yet reported on the effect of variation at candidate gene loci in relation to the HDL subclass distribution. Variation at the apoAI-CIII-AIV gene cluster is likely to be involved both because apoAI is the major apolipoprotein of HDL (Daerr et al. 1986) and because apoCIII is reported to inhibit LPL activity *in vitro* (Wang et al. 1985). ApoCIII appears to act in a non-competitive way against apoCII (Wang et al. 1985) which is a plasma activator of LPL (Wang et al. 1992). ApoCIII also acts as a competitive inhibitor of apoE-mediated uptake of remnant lipoproteins via the LDL-receptor (Sehayek et al. 1991, Aalto-Setala et al. 1992). The aims of this study were three-fold: a) to explore the role of variation in the apoAI-CIII-AIV gene cluster in determining plasma levels of the major lipoprotein fractions, HDL subclasses and LPL activity, b) to examine associations of

polymorphisms at the LPL, apoE and apoB genes with levels of HDL subclasses in a group of young post-infarction patients and age-matched healthy individuals from Stockholm County, Sweden, and c) to study associations between genotypes at the apoAI-CIII-AIV gene cluster and severity of coronary atherosclerosis in the myocardial infarction survivors.

Materials and methods

Subjects

The sample of individuals used in this study was as described previously (Hamsten et al. 1986a, Peacock et al. 1992a 1992b). Briefly, it consisted of 86 patients who had suffered a first myocardial infarction before the age of 45 years (mean $\pm$ S.E for age $= 40.4 \pm 0.34$ years and for BMI $= 26.4 \pm 0.35$ kg/m^2) and 93 age-matched healthy individuals, free of symptoms and clinical signs of CHD (mean $\pm$ S.E for age $= 40.5 \pm 0.40$ years and for BMI $= 24.6 \pm 0.26$ kg/m^2), all from Stockholm County, Sweden. The 86 patients were all male and free of manifest signs of diabetes, familial hypercholesterolaemia or other major concomitant disease such as uraemia and collagenosis. None of the individuals was receiving hypolipidaemic treatment. As described previously (Johansson et al. 1991), 63 of the healthy individuals and 36 of the patients had HDL gradient gel electrophoresis subclasses (2b, 2a, 3a, 3b and 3c) measured, in connection with determinations of post-heparin plasma LPL and HL activities. Prodecures for coronary angiography, details of the scoring systems and baseline and progression scores of atherosclerosis in the patients have been described elsewhere (Peacock et al. 1992a 1992b, Hamsten et al. 1986b). In young adults, myocardial infarction is frequently not due to coronary atherosclerosis. However, the issue here is not whether the MI is caused by coronary atherosclerosis, since the degree of atherosclerosis is measured by an objective technique (width of coronary arteries by angiography).

HDL subclass analysis

HDL subclasses were determined as described (Johansson et al. 1991) by a modification of the gradient gel electrophoresis method of Blanche et al. 1981.

Serum Lipoprotein Determinations

The major serum lipoproteins were determined by a combination of preparative ultracentrifugation and precipitation of apolipoprotein B-containing lipoproteins, followed by lipid analyses in the lipoprotein fractions as described (Carlson 1973).

Lipoprotein Lipase Activity

Fifty units of heparin/kg body weight was administered after an overnight fast, and 10 ml of post-heparin blood was drawn 15 min after the heparin infusion. Sonicated (^{3}H)-trioleoyl-glycerol emulsions stabilized by phosphatidylcholine were the substrates used in direct selective measurements of LPL activity (Nilsson-Ehle & Ekman 1977). Activity was expressed in milliunits (mU), which correspond to 1 nmol of fatty acid released per minute.

DNA preparation and analysis

DNA was extracted from fresh, whole blood, collected after an overnight fast, by the Triton X-100 lysis method (Kunkel et al. 1977), and PCR amplification was as described by Saiki et al. 1988.

DNA polymorphisms in the apoAI-CIII-AIV gene cluster

Prodecures for PCR amplification have been described for the G/A (-75) polymorphism in the apoAI gene promoter (Sigurdsson et al. 1992) and sequences of the ASO probes, labelling, oligomelting and autoradiography given in Talmud et al. (1994). For the PstI polymorphism 3′ of the apoAI gene, primer sequences and prodecures for PCR amplification have been described (Wiseman et al. 1991) and for the C/T$_{1100}$ polymorphism in the apoCIII gene, primer sequences and prodecures for PCR amplification and ASO were as described (Xu et al. 1994) and given here. Sequences of 5′ and 3′ primers (5′ to 3′) were CAATGGGTGGTCAAGCAGGAGC and GAGCACCTCCATTCCATTGTTGG. The PCR amplification protocol was the same as for the G/A (-75) polymorphism (Sigurdsson et al. 1992), giving a 299bp PCR product. ASO was as for the G/A (-75) polymorphism above, except that the ASO oligonucleotides were ATGCAGGGCTACATGAA and TTCATGTAACCCTGCAT. Hybridization and washing temperatures were 43°C and 49°C respectively (Talmud et al. 1994). For the PvuII polymorphism (1st intron of the apoCIII gene), prodecures for PCR-amplification and digestion were as described (Wiseman et al. 1991).

Polymorphisms in other genes

Prodecures for PCR amplification and subsequent genotyping for the PvuII polymorphism between exons 6 and 7, the HindIII polymorphism between exons 8 and 9 and the Ser$_{447}$-Stop polymorphism

in the 3'end of exon 9 in the LPL gene have been described (Peacock et al. 1992b). ASO probe sequences, labelling, oligomelting and autoradiography prodecures were as described (Hata et al. 1990). Prodecures for PCR-amplification and digestion were as described (Hixson & Vernier 1990) for the common apoE isoforms, for the apoB signal peptide polymorphism (Boerwinkle & Chan 1989, Xu et al. 1990a) and for the apoB XbaI polymorphism (Boerwinkle et al. 1990).

Statistical analysis

Statistical analysis was carried out using the SPSS/PC+ computer program. Mean levels of each HDL subclass protein distribution and of LPL activities for the healthy individuals and patients were compared using the non-parametic Mann-Whitney test on SPSS/PC+. Data for triglycerides and VLDL lipids was $\log_{10}$-transformed prior to regression, analysis of variance (ANOVA) and correlation analysis to reduce the skewness of the data. Lipid, lipoprotein and HDL subclass protein concentrations and LPL and HL activities were adjusted by linear regression for age and recorded body mass index (BMI). Adjustment for age and BMI had little impact on the genotype means so actual means and standard errors for genotypes are presented. For triglycerides, 95% confidence limits were calculated on the $\log_{10}$-transformed data (Sokal & Rohlf 1981). A one-way ANOVA was performed on age and BMI-adjusted data to test the null hypothesis that phenotypic variations in these traits and in lipase activities were not associated with genetic variation at the candidate gene loci. The impact of genotypes on age and BMI-adjusted apolipoprotein levels and lipase activities were estimated by non-linear regression (use of dummy variables to reduce the dependence of the regression model on a linear relationship between means and genotypes). For baseline and progression of disease which could not be normalized by any means, the non-parametic analogue of ANOVA, the Kruskal-Wallis test, was used to examine associations between severity of disease and genotypes.

The allele frequency for each polymorphism was estimated using the 'gene counting' method; and determined separately for the healthy individuals and patients. A few individuals were selected randomly and retyped for these polymorphisms. 2x2 contingency tables were used to compare allele frequency in healthy individuals with that in patients. The method of Chakravati et al. (1984) was utilised in the 'Excel' spreadsheet computer program to calculate the extent of linkage disequilibruim between any pairwise combination of apoAI-CIII-AIV gene cluster polymorphisms. Haplotypes at the apoAI-CIII-AIV gene cluster were inferred by visual inspection of the data and were deduced only in those individuals homozygous for three or all four of the polymorphisms.

We considered statistical significance to be at the 0.05 level. However, since the study groups were small and multiple comparsions were being performed, probability values need to be interpreted with caution. Because of concern about obtaining statistically significant differences by chance alone due to multiple comparsions, only those lipid traits considered to be of major importance were analysed for effects of genotypes on levels. The high degree of inter-relatedness between pairwise combinations of these variables reduced the probability of finding spurious associations in haplotype analysis.

Results

Table 1 shows the characteristics of the healthy individuals and patients with respect to the HDL subclasses and lipase activity. The characteristics of this subgroup were not significantly different from the whole sample included in earlier studies (data not shown). The means for healthy individuals and patients are significantly different, and as previously reported (Johansson et al. 1991), the larger HDL subclasses (2b, 2a and 3a) were more abundant in the healthy individuals and the smaller HDL subclasses (3b and 3c) were more abundant in the patients.

Genotypes were determined for four polymorphisms in the apoAI-CIII-AIV gene cluster; their locations within this gene cluster and rare allele frequencies are shown in Fig. 1. For all the polymorphisms studied, genotype distributions were close to and not significantly different from the Hardy-Weinberg prediction. No significant differ-

Table 1. Characteristics of the 63 healthy individuals and 36 patients for the HDL subclasses. Mean±standard errors (S.E.) n=number of individuals. HDL subclasses are in mg/protein/100ml plasma

	Healthy individuals			Patients	
HDL	Mean	S.E.		Mean	S.E.
2b	0.22	0.019		0.12	0.012
2a	0.41	0.015		0.31	0.015
3a	0.50	0.011		0.44	0.015
3b	0.33	0.010		0.38	0.013
3c	0.16	0.006		0.19	0.010
Postheparin plasma lipase activity (mU/ml)					
LPL	92.05	3.64	64	74.25	4.16

All means were significantly different between the healthy individuals and patients using the Mann-Whitney test; 2-tailed p-values < 0.01.

ences in allele frequencies were observed between the healthy individuals and patients. Haplotype frequencies observed in the healthy individuals and patients were similar and results from the combined sample are presented in Table 2. Out of 16 possible haplotypes, 7 were inferred, with the rare allele of the PstI polymorphism being only seen on the same chromosome as the common alleles of the other three polymorphisms, and the A_{-75} allele being nearly always seen on the same chromosome as the apoCIII-T_{1100} allele, confirming previous observations (Xu et al. 1994).

The effect of variation associated with the four apoAI-CIII-AIV gene cluster polymorphisms studied on lipids, HDL subclasses and apolipoproteins was estimated by non-linear regression after adjustment of levels for age and BMI. None of the lipids (cholesterol and triglycerdes in plasma, VLDL, LDL or HDL fractions were significantly associated with genotypes at the apoAI-CIII-AIV gene cluster in either healthy individuals or patients (data not shown) with the exception of LDL-triglycerides with genotypes of the PvuII polymorphism. In the 29 healthy individuals who had never smoked compared to the whole group, a weak association was found between HDL cholesterol or apoAI levels and genotypes of the 5′apoAI-G/A_{-75} substitution. In these non-smokers, carriers of the A_{-75} allele had higher mean HDL-chol (1.59 versus 1.45mM) and apoAI levels (133 versus 123mg/100ml) compared to those lacking this allele, in agreement with previous observations on non-smokers (Sigurdsson et al. 1992). These differences did not reach statistical significance due to the small sample size. Data from all four polymorphisms were combined by determining 'haplotypes' and in the healthy individuals, plasma triglycerides and VLDL triglyceride levels were significantly associated with presence/absence of the inferred haplotype 5′apoAI-G $_{-75}$ 3′apoAI-P+ apoCIII-T_{1100} apoCIII-V+ (rare allele of the C to T_{1100} polymorphism but the common alleles of the other three polymorphisms, designated G/P+/T/V+) explaining 5.8 and 7.8%, respectively, of sample variance. No associations were found between haplotypes of the apoAI-CIII-AIV gene cluster and other lipids or HDL subclasses (data not shown). In general, the proportion of the sample variance in HDL subclasses that was explained by genotypes of the apoAI-CIII-AIV gene cluster polymorphisms was greater in the patients than in the healthy individuals; for example, the 5′apoAI-G/A polymorphism explained 16.5% and 1.9% of sample variance in HDL3c, the 3′apoAI PstI polymorphism explained 10.5% and 1.2% of variance in HDL2a, the apoCIII-C/T polymorphism explained 6.6% and 0.7% of variance in apoAI and the 3′apoCIII PvuII polymorphism explained 13.5% and 1.0% of sample variance in HDL2a levels, in patients and healthy individuals, respectively.

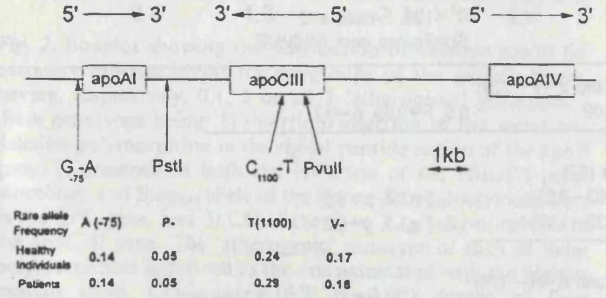
The individuals in this sample had previously been genotyped for apoE isoforms, the signal peptide and XbaI polymorphisms in the apoB gene and the PvuII, HindIII and $Serine_{447}$ to Stop polymorphisms in the LPL gene, and associations between these polymorphisms and HDL subclasses was examined. In the healthy group and patients, none of the HDL subclasses were significantly associated with apoE genotypes (E2/3 versus E3/3 versus (E4/3 and E4/4 combined), leaving out those with the rare genotype E4/2) or with polymorphisms in the apoB gene. However, in the patients but not the healthy individuals, the HDL2b, HDL2a and HDL3b subclasses were significantly associated with genotypes of the HindIII poly-

Table 2. Haplotype frequencies for healthy individuals and patients combined. Delta values for G/A (−75)+C/T_{1100} polymorphisms, G/A(−75)+PvuII-CIII polymorphisms and C/T_{1100}+PvuII-CIII polymorphisms were 0.54, 0.15 and 0.25 respectively. All were significantly different from zero ($p < 0.05$ at 1 degree of freedom)

Inferred haplotype	n	Frequency (as percentage)
G_{-75} P+C_{1100} V+	146	58.9
G_{-75} P+C_{1100} V−	40	16.1
G_{-75} P+T_{1100} V+	40	16.1
A_{-75} P+T_{1188} V+	11	4.4
G_{-75} P−C_{1188} V+	6	2.4
A_{-75} P+C_{1100} V+	4	1.6
G_{-75} P+T_{1100} V−	1	0.4

Haplotypes at the apoAI-CIII-AIV gene cluster were inferred by visual inspection of the data and were deduced only in those individuals homozygous for three or all four of the polymorphisms.

Map of the apoAI-CIII-AIV gene cluster and allele frequencies



Rare allele Frequency	A (-75)	P-	T(1100)	V-
Healthy individuals	0.14	0.05	0.24	0.17
Patients	0.14	0.05	0.29	0.18

Fig. 1. Schematic representation of the ApoAI-CIII-AIV gene cluster showing approximate positions of the four polymorphisms used in this study. Direction of transcription of the genes from 5′ to 3′ is also indicated. Coding and non-coding regions are represented as boxes and lines, respectively. Diagram is approximately to scale with an arrow in the figure showing length representing 1 Kb. Rare allele frequencies for each polymorphism are shown for the healthy individuals and patients.

morphism in the LPL gene explaining 22.1, 19.3 and 11.4%, respectively, of sample variance.

Table 3 (a and b) shows the genotype means and standard errors or 95% confidence limits for the associations that were significant in either the healthy individuals or patients. Levels of LDL triglycerides were 21% higher in healthy individuals carrying the V- allele of the 3'apoCIII-PvuII polymorphism compared to those without this allele; this was not seen in the patients. When the four healthy individuals with plasma cholesterol levels >9.5 mM and/or triglyceride levels >3.0 mM and the three individuals without data for cholesterol and triglycerides were excluded, this association remained statistically significant with the same trend (data not shown). In the healthy individuals, carriers of the haplotype G/P+/T/V+ had significantly higher mean triglyceride and VLDL triglyceride levels (35.4% and 69% higher, respectively) than did those lacking this haplotype and those in whom haplotypes could not be inferred. In the patients, this association was not observed. The significant association between levels of HDL2b, HDL2a and HDL3b and genotypes of the LPL-HindIII polymorphism observed in the patients, explaining 22.1%, 19.3% and 11.4%, respectively, of sample variance was not seen in the healthy individuals. In the patients, means of HDL2b and HDL2a were 47% and 29% lower in the presence of the H- allele, and means of HDL3b were 20% higher in the presence of the H- allele. Genotypes of the LPL-HindIII polymorphism did not have a significant impact on LPL activities in either the healthy individuals or patients; however, in both groups, both HDL2b levels and LPL activities followed the same trend.

In the patients, associations of baseline atherosclerosis and progression scores with genotypes for

Table 3. Genotype means (±S.E.) for selected lipoprotein and HDL subclass variables. Adjustment for age and BMI made little difference to the genotype means, so unadjusted values are shown here

a) In the healthy individuals

Trait (mmol/l)	Genotypes (n) ApoCIII-PvuII RFLP		$R^2 \times 100$, F-value and Significance from ANOVAS[a]
	V+V+ (64)	V+V− and V−V− (23)	
LDL-triglycerides	0.33±0.01	0.40±0.04	7.4, F=6.2, p=0.02
	Inferred haplotype G/P/T/V+		
	Presence (18)	Absence (68)	
Triglycerides[b,c]	1.53 (1.27−1.85)	1.13 (1.02−1.24)	5.8, F=5.1, p=0.03
VLDL-triglycerides[b,c]	0.98 (0.74−1.29)	0.58 (0.50−0.68)	7.8, F=7.0, p=0.01
mg/protein/dl plasma	LPL genotypes		
	H+H+ (35)	H+H− and H−H− (26)	
HDL2b	0.20±0.02	0.26±0.03	5.3, F=3.4, p=0.07
HDL2a	0.41±0.02	0.40±0.02	0.1, F=0.1, p=0.8
HDL3b	0.34±0.01	0.31±0.01	3.1, F=1.9, p=0.2
LPL activity*	88.9±4.55	95.8±6.13	1.7, F=1.0, p=0.3

b) In the patients

Trait (mmol/l)	Genotypes (n) ApoCIII-PvuII RFLP		$R^2 \times 100$, F-value and Significance from ANOVAS[a]
	V+V+ (57)	V+V− and V−V− (29)	
LDL-triglycerides	0.45±0.02	0.46±0.02	0.5, F=0.09, p=0.7
	Inferred haplotype G/P+T/V+		
	Presence (19)	Absence (67)	
Triglycerides[b,c]	1.94 (1.63−2.32)	2.33 (2.03−2.67)	1.4, F=1.2, p=0.3
VLDL-triglycerides[b,c]	1.25 (0.99−1.58)	1.58 (1.33−1.87)	1.3, F=1.1, p=0.3
mg/protein/dl plasma	LPL genotypes		
	H+H+ (20)	H+H− and H−H− (16)	
HDL2b	0.15±0.02	0.08±0.01	22.1, F=9.7, p=0.004
HDL2a	0.35±0.02	0.25±0.02	19.3, F=8.1, p=0.007
HDL3b	0.35±0.02	0.42±0.02	1.4, F=4.4, p=0.04
LPL activity*	77.4±5.36	70.3±6.61	0.8, F=0.3, p=0.6

[a] Age and BMI adjusted data used.
[b] Data is shown as antilogs of $\log_{10}$-transformed mean and 95% confidence limits.
[c] Excludes the three hyperlipidaemic individuals (healthy individuals only).
* LPL activity is measured in milliunits which is nmoles of fatty acid released per minute per ml of plasma.

all four apoAI-CIII-AIV gene polymorphisms, were examined. Only for the apoCIII-C to T_{1100} polymorphism was a weak assocation found with baseline atherosclerosis score. The median score was 113% higher in individuals with genotype CC compared to those carrying the T_{1100} allele (1.6 vs.0.75, p=0.09). The combined association was also examined between baseline atherosclerosis scores and genotypes at two other loci that we have previously examined in this patient group. An 'atherogenic genotype' is defined as the genotype at the locus associated with the highest atherosclerosis score. In the Swedish patient sample, for the apoB gene, this is homozygosity for the common allele of the signal peptide polymorphism (Peacock et al. 1992a), for the LPL gene, the H- $Stop_{447}$ haplotype (presence of the rare alleles of the HindIII and Serine-$Stop_{447}$ polymorphism) (Peacock et al. 1992b) and for the apoCIII gene, the CC genotype of the C/T_{1100} polymorphism (this study). Individuals can be distinguished as having 3, 2, 1 or none of the 'atherogenic' genotypes and the baseline atherosclerosis score in these groups is shown in Fig. 2. The median score is significantly different among all four groups (p=0.001); being six times higher in individuals with all three 'atherogenic' genotypes than in those with none of these genotypes. The number of 'atherogenic' genotypes was not significantly associated with lipoprotein levels or other characteristics such as fibrinogen or plasminogen activator inhibitor-1 (data not shown).

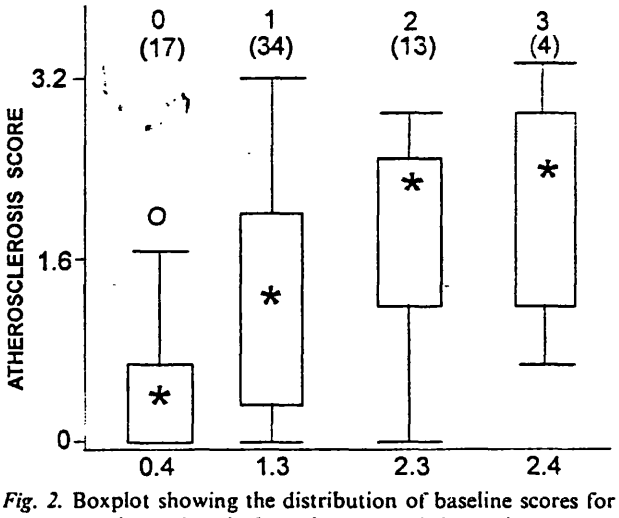


Fig. 2. Boxplot showing the distribution of baseline scores for coronary atherosclerosis for subgroups of the patient group, having, respectively, 0,1, 2 or all 3 'atherogenic' genotypes – these genotypes being: 1) insertion/insertion of the insertion/deletion polymorphism in the signal peptide region of the apoB gene; 2) presence of both the H- allele of the HindIII polymorphism and $Stop_{447}$ allele of the Serine-$Stop_{447}$ polymorphism in the LPL gene, and 3) CC of the C to T_{1100} polymorphism in the apoCIII gene. The 'atherogenic' genotype of each of these polymorphisms is defined as the one associated with the highest median score. Chi-square=16.2, p=0.001 across all four groups. Distribution of individuals among the four groups was as expected for random association of all three gene loci. Scores are given on the vertical axis. Number of 'atherogenic' genotypes and number of individuals are given above the boxes on the horizontal axis. Values of medians are shown below the respective boxes. Numbers in brackets denote number of individuals in each group. *=median; o=outliner (more than one standard deviation from the mean) score for that subgroup).

Discussion

A number of studies have examined polymorphisms in the apoAI-CIII-AIV gene cluster and have reported differences in allele frequency between patients with CHD and controls; but these have not been consistent (Talmud & Humphries 1992). There are a number of explanations for this; different criteria used to define patients, different ethnic origin of the populations studied, and chance effects due to small sample size. Allele frequencies for the four apoAI-CIII-AIV gene cluster polymorphims used in this study were not different between the patients and healthy individuals and were not significantly different from those in most of the previous studies on Caucasian population samples (Humphries 1988, Lusis 1988, Humphries et al. 1990, 1992, Ordovas et al. 1991). Small discrepancies in allele freqencies between this study and previous studies (e.g. Xu et al. 1990b) are likely to be due to differences in the criteria used in selection of individuals, particularly of the patients and differences in ethnic origins of population samples used. The C to T_{1100} polymorphism in the apoCIII gene is a new polymorphism (Xu et al. 1994) which has been detected at a higher frequency in patients with familial combined hyperlipidaemia compared to healthy controls (Xu et al. 1994). The non-significantly higher frequency of the apoCIII-T_{1100} allele in the patients in this study compared to the healthy individuals is in accord with this.

The significant association between higher triglyceride levels and the presence of the unambiguous haplotype G/P+/T/V+ in the healthy individuals cannot be explained by an association between triglycerides and any one of the four apoAI-CIII-AIV gene cluster polymorphisms alone. The association with higher triglyceride levels is most likely to be due to this haplotype being a marker for a functionally important sequence change somewhere in the cluster, for example in the promoter region of the apoCIII gene or in an enhancer-like sequence within the cluster. A recent study (Walsh et al. 1993) suggested, using transgenic mouse lines, that the apoAI and apoCIII genes share an intestinal control region, the actual control element in the -1.4 to -0.2 Kb region of the apoCIII gene as yet unidentified, and Dammerman et al. (1993) reported that haplotypes involving DNA polymorphisms in the apoCIII gene

promoter region and the 3'apoCIII-*Sst*I polymorphism affect susceptibility to severe hypertriglyceridaemia. The rare allele of the SstI polymorphism, in the 3' non-coding region of the apoCIII gene, which was however not determined in the Swedish individuals, has been associated with hypertriglyceridaemia in a number of studies (eg. Shoulders et al. 1986, Henderson et al. 1987) and is found on the same chromosome as the T_{1100} allele of the apoCIII C/T polymorphism (delta = 0.54, C-F. Xu, unpublished data).

Of the gene loci examined, the gene with the largest effect on HDL subclasses was the LPL gene, with effects reaching statistical significance in the patients but not in the healthy individuals. This is probably explained by variation in LPL activity, as the associations between HDL2b or LPL activity and genotypes of the HindIII polymorphism follow the same trend. This would be expected, given that the generation of HDL_2 is due to LPL action on triglyceride-rich lipoproteins and subsequent transfer of surface components to HDL_3 (Taskinen & Kussi 1987). The effect of genotypes of the HindIII polymorphism on LPL activity was not significant, possibly because the assay system, which uses an emulsified substrate, measures both lipid-binding ability and catalytic activity of LPL, and thus variation in lipid-binding ability might mask the effect of genetic variation on LPL activity. In almost all cases, the associations observed between genotypes and HDL subclasses or LPL activity were opposite in the healthy individuals and patients. This may be due to interaction between unmeasured genetic variation or environmental factors that were different between the healthy individuals and patients.

The major finding of this study was an additive effect of genotypes at the apoB, LPL and apoCIII gene loci on atherosclerosis score, when all these three gene loci are considered stimultaneously. The difference in atherosclerosis score with combined genotypes is large enough to be biologically significant, and if confirmed in a larger patient sample, will be of relevance in screening and identification of at-risk individuals. The mechanism of this effect is unclear from the analysis of associations between the genotypes and levels of fasting lipid traits in this sample. No single measured lipoprotein variable showed as large a gradient with the combination of 'atherogenic' genotypes as atherosclerosis score. However, the atherogenic apoB genotype (homozygosity for the signal peptide insertion allele) was previously found to be associated in the Swedish patient group, with 10% lower apoAI levels (Peacock et al. 1992a) and the atherogenic LPL haplotype (H- $Stop_{447}$) with 24% lower LPL activity (Peacock et al. 1992b) and with 50% lower HDL2b levels and smaller lowering effects on HDL2a and HDL3b in the patients (this study). The HDL3a subclass, like the HDL2b subclass, is part of the putatively anti-atherogenic fraction of HDL (Puchois et al. 1987) containing apoAI but not apoAII. However, HDL3a and HDL3b are not the subclasses underpinning the inverse relation between HDL and CHD or coronary atherosclerosis, which is thought to be due to low HDL2b (Johansson et al. 1991). Thus, variation in the relative abundance and distribution of the HDL subclasses could explain, at least in part, the association between the LPL HindIII polymorphism and atherosclerosis. For the apoAI-CIII-AIV gene cluster, the atherogenic genotype CC was associated with only small non-significant differences in any plasma lipid trait, although the haplotype with the C allele was associated with higher levels of potentially atherogenic lipoproteins in the patients. It is possible that variation in serum apoCIII levels underlies the association between atherosclerosis score and the apoCIII-C/T_{1100} polymorphism. High apoCIII levels were recently shown to induce hypertriglyceridaemia in transgenic mice (Aalto-Setala et al. 1992) and patients with coronary atherosclerosis who showed progression of disease, had higher apoCIII levels ($p = 0.03$) than those who did not (Blankenhorn et al. 1990). However, apoCIII levels were not measured in this Swedish sample.

It is thus likely that a different mechanism underlies the association of each 'atherogenic' genotype with the high atherosclerosis score, and that these mechanisms act in concert. It is plausible that variation in magnitude of post-prandial response among the patients may explain, at least in part, the associations found between atherosclerosis and genotypes at the apoB, LPL and apoCIII gene loci. Post-prandial but not fasting triglyceride levels were strongly associated with coronary artery disease in a recent case-control study (Patsch et al. 1992). In the Swedish patients the effect of variation at the apoB, LPL and apoCIII gene loci on the magnitude of post-prandial responses is being investigated to ascertain whether variation in post-prandial lipid metabolism is a feasible explanation for the associations found between atherosclerosis score and genotypes at these three gene loci.

Acknowledgements

This study was supported by the Department of Medicine at University College, London, and by grants from the British Heart Foundation (RG16), the Swedish Medical Research Council (4966 and 8691), the Swedish Heart-Lung Foundation, the Torsten and Ragnar Söderberg Foundation, Påhlsson's Foundation and the King Gustaf V 80th Birthday Fund. Rachel Peacock is supported by a studentship from the British Medical Research Council. Dr Anders Hamsten is Career Investigator

of the Swedish Heart-Lung Foundation. The authors wish to thank Dr Shu Ye and Dr C-F. Xu for access to unpublished data and helpful discussion on the manuscript, Dr Christian Landou, Department of Thoracic Radiology, Karolinska Hospital, Stockholm for his help with scoring of coronary angiograms and Mrs Gina Deeley for her help in the preparation of the manuscript.

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Z48:02X162XXMA 13.06.1994 09:10:53 PCA0 PCA

Springer-Verlag - Heidelberg NMCD 162
Mskr.-Seiten: 3x Seitenzahl: 9
Druckerei: Zechner Speyer Versand: 13.06.94

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Nutr Metab Cardiovasc Dis (1994) ■■■:■■■–■■■

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ORIGINAL ARTICLE

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Associations between genotypes of the apolipoprotein E, B, AI-CIII-AIV and lipoprotein lipase genes and coronary artery disease in the St. Thomas' Atherosclerosis Regression Study

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Received 10 September 1993, accepted 11 February 1994

Abstract. Association studies were carried out on a sample of 74 middle-aged male patients with coronary artery disease who completed the St. Thomas' Atherosclerosis Regression Study (STARS). The aim was to examine the impact of polymorphisms at candidate genes on plasma lipids and lipoproteins at baseline and on the severity and progression of coronary atherosclerosis. Progression of coronary atherosclerosis was measured as the change in mean absolute width of coronary segments (ΔMAWS) and change in minimum absolute width of coronary segment (ΔMinAWS) by a computerised method, data being analysed on a segmental basis. All patients were genotyped for the common apo E isoforms, two polymorphisms in the apo AI-CIII-AIV gene cluster, four apo B gene polymorphisms and three polymorphisms in the LPL gene. With the exception of apo E, allele frequencies for all polymorphisms studied were similar to those reported in healthy individuals. The apo E4 isoform frequency (0.21) in the STARS sample was higher ($X^2=4.5$; $P<0.05$, 1 df) than reported for a sample of healthy individuals from the UK (E4=0.14). In analysis of variance, genotypes of the apo B a1/d epitope pair and *Xba*I polymorphisms were significantly associated ($P<0.05$) with severity of coronary atherosclerosis at baseline, genotypes a1/a1 and X−X− being associated with a lower MAWS (10% smaller lumen than for presence of the d epitope or X+ allele), but were not significantly correlated with baseline plasma lipids or lipoproteins. Genotypes of the apo AI G_{-75} to A substitution were strongly associated with change of coronary atherosclerosis; genotype G/G was associated with progression (mean ΔMAWS = −7.8 μm, mean ΔMinAWS = −6.2 μm) and G/A with regression (mean ΔMAWS = +10.2 μm, mean ΔMinAWS = +8.0 μm); $P=0.0002$ by unpaired t test. This association was independent of treatment regimen employed and was not explained by the small (non-significant) differences in baseline or in-trial lipids and lipoproteins associated with genotype. Thus, the data suggest that variation in the apo B and apo AI-CIII-AIV gene cluster may predict established and progression of coronary atherosclerosis respectively, through effects independent of plasma lipid or lipoprotein levels. In addition, the trend between change in coronary atherosclerosis and treatment regimen was the same for all genotypes of the G_{-75} to A substitution. This suggests that variation at the apo AI-CIII-AIV gene cluster is influencing prognosis independently of treatment regimen.

Key words: Coronary atherosclerosis – Angiography – Segmental analysis

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Introduction

During the last decade, numerous case-control studies have examined the associations between arterial disease and common polymorphisms in candidate genes that have a known role in lipid metabolism. In many of these studies allele frequencies were different between groups of patients with arterial disease and healthy controls. For the apo E gene, several studies have reported that the apo E4 allele frequency was significantly higher in patients with coronary artery disease (CAD) [1–3], in patients with combined hyperlipidaemia [4] and in type II diabetic patients with CAD [5] compared to healthy individuals. The apo E4 allele has a well-known cholesterol- and triglyceride-elevating effect [6, 7].

For other candidate genes results have been less consistent, possibly because the strength of the effects is smaller or modified by environmental or other genetic factors. For the apo B gene, several studies have reported that allele frequencies for the *Xba*I polymorphism in exon 26 were different between patients and controls [8–10]. Allele frequencies of other polymorphisms in the apo B gene have also been different between patients and controls. For example, in several case-control studies where patients had arterial disease or were myocardial infarction survivors, the rare allele (no cutting site) of the *Eco*RI polymorphism [8–12] and larger alleles of the 3′ hypervariable minisatellite region [13] were significantly more frequent in patients. For the apo AI-CIII-AIV gene cluster, the frequency of the rare allele of the *Sst*I polymorphism has consistently been significantly higher in patients with CAD [14–16]. In at least some studies, the frequency of the rare allele of the *Pst*I polymorphism was significantly higher in patients with CAD compared to healthy individuals [17, 18]. For the LPL gene, the frequency of the common allele of the *Hind*III polymorphism was significantly higher in patients with coronary atherosclerosis [19].

Studies have also shown associations between polymorphisms and severity of atherosclerosis. The Pathological Determinants of Atherosclerosis in Youth (PDAY) report [20] showed that among the common apo E genotypes, E2/3 and E4/3 heterozygotes had least and greatest atherosclerotic involvement respectively, in the thoracic and abdominal aorta. However, the apo E polymorphism was not correlated with angiographic score for severity of disease in two studies [21, 22]. In the PDAY study [23], genotypes of the signal peptide polymorphism in the apo B gene were significantly associated with extent of involvement of aorta with arterial lesions (the insertion allele being associated with greater involvement) in black but not white subjects. In a group of young myocardial infarction survivors from Sweden, associations were found between angiographic severity of coronary atherosclerosis and the signal peptide polymorphism of the apo B gene [22] and haplotypes of the *Hind*III and serine$_{447}$-stop polymorphisms in the LPL gene [24].

In the present study we have genotyped patients who participated in the St. Thomas' Atherosclerosis Regression Study [25]. Our major aim was to examine associations between genotypes at the apo E, apo B, apo AI-CIII-AIV and LPL genes and severity of established coronary atherosclerosis and change in severity during the study period, and for significant associations, the effect of genotypes on baseline and in-trial levels of lipids and lipoproteins was also examined.

Materials and methods

Patients, study design and clinical measurements. Details of the patients, study design, methodologies and treatment effects have been reported [25]. Briefly 90 male patients (age <66 years, plasma cholesterol level above 6 m*M* but not above 10 m*M* and without treatment with lipid-lowering drugs) were recruited. Seventy-four patients completed the study and it is on these 74 patien that results are reported. Eligible patients had fasting plasma li ids and lipoproteins measured and coronary angiography (bas line measurements) prior to being randomly assigned to one three treatment groups – usual care only (U), a lipid-loweri diet (D) or diet plus cholestyramine (DC) –, each with an appro imately equal number of individuals. Coronary angiography w carried out at entry and after approximately 39 months. The tec nique for quantitative image analysis has been detailed previou ly [25]. The end-points used in this analysis were change in mea absolute width of coronary segment (ΔMAWS), which is a mea ure of diffuse CAD, and change in minimum absolute width coronary segment (ΔMinAWS), which is a measure of foc CAD. Fasting plasma lipids and lipoproteins were measured u ing conventional methods [25].

DNA preparation and analysis. Blood was collected after an ove night fast and frozen at −20°C. DNA was extracted fro thawed-out whole blood by the salting-out method [26]. Polyme ase chain amplification (PCR) [27] in a total volume of 50 µl w performed on a Cambio "intelligent" heating block (Cambi Cambridge, UK). In each PCR, 0.5 units of thermus aquatic polymerase (Gibco BRL) and 100–200 ng of each primer we used. Buffers were as recommended by Perkin Elmer-Cet (Conn., USA).

DNA polymorphisms in the apo AI-CIII-AIV gene cluster. F the G_{-75} to A substitution in the apo AI promoter, primer s quences and procedures for PCR amplification have been d scribed [28]. Sequences of the allele-specific oligonucleoti (ASO) probes, phosphorus-32 (^{32}P) labelling, oligomelting a autoradiography have been described [29]. For the C_{1100} to substitution in the apo CIII gene, primer sequences and proc dures for PCR amplification and ASO have been describe [30].

DNA polymorphisms in the apo B gene. Primer sequences a procedures for PCR amplification and subsequent typing ha been described for the C_{-265} to T substitution (*Msp*I polymo phism in the promoter region) [22, 31], for the signal peptide i sertion/deletion polymorphism [32, 33], for the Val_{591} to Ala su stitution (the a1/d epitope pair [34, 35]) and for the C to T s quence change in the wobble position of Thr_{2488}, which is t *Xba*I polymorphism in exon 26 [36].

ApoE genotyping. The restriction isotyping procedure (PCR a plification, digestion with the enzyme *Hha*I and electrophores on polyacrylamide gels) has been described [37]. Oligonucleoti primers used in the PCR were as described [38].

DNA polymorphisms in the lipoprotein lipase gene. Primer s quences and procedures for PCR amplification and subseque typing have been described for the *Pvu*II polymorphism betwe exons 6 and 7 [24] and for the *Hind*III polymorphism betwe exons 8 and 9 [24]. *Hind*III-digested PCR-amplified DNA w run on 1.5% agarose gels and double-blotted overnight onto H bond-N plus membranes (Amersham) which were subsequent alkali-fixed in 0.4 *M* NaOH for 3 min. The ASO probe sequence ^{32}P labelling, oligomelting and autoradiography for the serine$_{44}$ stop polymorphism have been described [39].

Statistical analysis. Statistical analysis was carried out using standard statistical package. One-way analysis of varian (ANOVA) and the unpaired Student's *t* test were used to exa ine associations between genotypes at the apo AI-CIII-AIV ge cluster, apo E, apo B and lipoprotein lipase genes and plasm lipids and lipoproteins, extent of coronary atherosclerosis at b seline, and change in coronary atherosclerosis over the 3 yea Allele frequency for each DNA polymorphism was estimated u ing the "gene counting" method. 2×3 or 3×3 contingency tabl (χ^2 analysis) were used to test deviations from Hardy-Weinbe

equilibrium and the null hypothesis that genotype distributions did not differ between the three treatment groups or between the numbers of patients and of segments, thus eliminating the possibility of an inherent selection bias. We analysed the data for established and change in coronary atherosclerosis on a segmental basis (to increase the power of the study) in addition to a per-patient basis. Pooling of segments was justified by the low intra-class correlation coefficient for within-patient change in segments [25 and unpublished data], and also by the fact that trends were the same in both per-patient and segmental analyses.

Multiple linear regression analysis was used to adjust ΔMAWS or ΔMinAWS for the effect of treatment group or in-trial plasma lipids and to test the significance of association between ΔMAWS or ΔMinAWS and genotypes of the G_{-75} to A substitution. The method of Chakravati et al. [40] was utilised in a standard spreadsheet computer program to calculate the extent of linkage disequilibrium between any pairwise combination of apo B or apo AI-CIII-AIV gene polymorphisms.

In view of the small numbers of patients and large numbers of multiple comparisons made, we analysed the data on a "per-patient" basis and by segmental analysis. Possible and definite statistical significance were taken at $P<0.05$ and $P<0.01$ respectively. The P values were not corrected for the number of comparisons made [41], this being justified by the exploratory nature of the work and the caution used in interpretation of the differences observed.

Results

Seventy-four patients from the St. Thomas' Atherosclerosis Regression Study were typed for common DNA polymorphisms at the apo E, apo B, apo AI-CIII-AIV and LPL genes. The location and nature of these polymorphisms and allele frequencies with 95% confidence intervals are shown in Fig. 1, which is a schematic representation of all four gene loci. In this group of patients, allele frequencies for the polymorphisms studied in the apo B, apo AI-CIII-AIV and LPL genes were not significantly different from those found by our laboratory in healthy United Kingdom (where data was available) or in European population samples [4, 22, 24, 35, 42] where the rare allele frequencies for these samples were within the 95% confidence intervals for those in the St. Thomas' Atherosclerosis Regression Study. However, for the common apo E isoforms, compared to a group of 150 healthy British individuals [4], the frequency of the apo E2 and apo E4 alleles in these patients were, respectively, lower (0.02 vs 0.09) and higher (0.21 vs 0.14), and these differences were statistically significantly different. For all the two-allele polymorphisms studied, genotype distributions were close to and not significantly different from the Hardy-Weinberg prediction. Genotype distributions for all the polymorphisms were similar between the three treatment groups (data not shown).

Per-patient and segmental analyses were carried out to look for associations between the genotypes at the four gene loci and both severity of coronary atherosclerosis at baseline and the change in severity over 3 years. For the 74 patients who completed the study period, a maximum of ten coronary artery segment pairs were available for analysis (mean ± SD = 6.4 ± 2.0) for each patient. For each of these three polymorphisms, the a1/d epitope, *Xba*I polymorphism and the G_{-75} to A substitution, both patient and segmental distributions were very similar ($P>0.1$ in χ^2 analysis, results

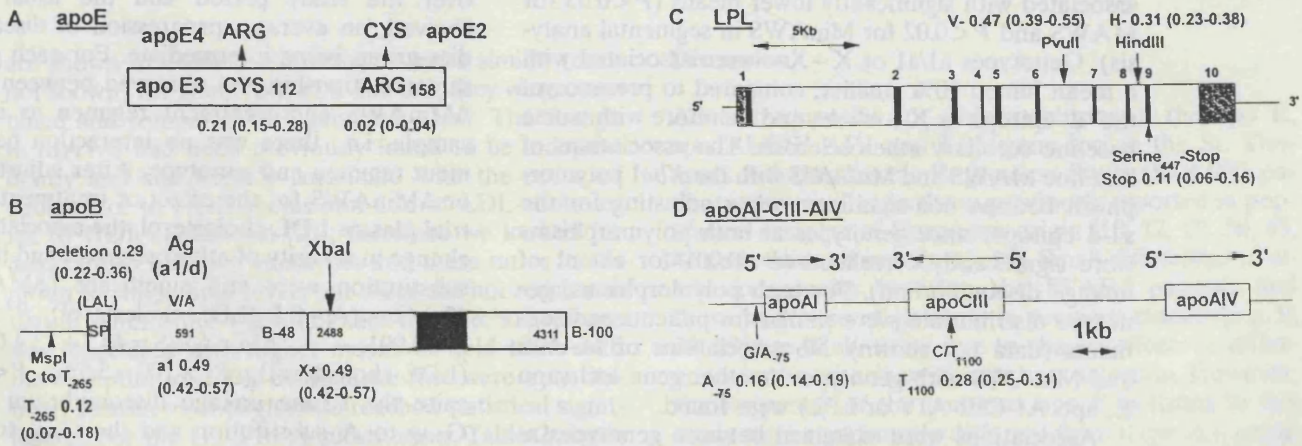


Fig. 1A–D. Location of polymorphisms used in the STARS sample. *Boxes* represent translated regions of genes. Rare allele frequencies and 95% confidence intervals are shown. **A** Apo E gene – the amino acid substitutions giving rise to apo E2 and apo E4. **B** Apo B gene. The four polymorphisms are shown: MspI in 5′ untranslated region, C to T substitution at nucleotide −265 relative to transcriptional start site, LAL – the length polymorphism in the signal peptide (SP), a1/d epitope pair, which is a valine-alanine substitution at amino acid 591, and *Xba*I polymorphism in exon 26. The *shaded area* represents the LDL-receptor binding region of apo B. **C** LPL gene. *Shaded* and *black* boxes represent untranslated and translated regions respectively. Exons are numbered 1–10. **D** Apo AI-CIII-AIV gene cluster. *Arrows* show direction of transcription of each of the genes. *Boxes* represent translated regions of genes. Approximate positions of the G_{-75} to A and C_{1100} to T substitutions are shown. Estimates of linkage disequilibrium (delta values) for G/A_{-75} + C/T_{1100} substitutions, signal peptide polymorphism + a1/d epitope pair, a1/d epitope pair + *Xba*I polymorphism (in apo B gene), *Pvu*II + *Hind*III polymorphisms and *Hind*III + serine/stop$_{447}$ polymorphisms (in LPL gene) were 0.58, 0.63, 0.71, 0.31 and 0.53 respectively. All were significantly different from zero ($P<0.001$ at 1 degree of freedom; except for *Pvu*II + *Hind*III polymorphisms where $P<0.05$)

Table 1. Per-patient and segmental analysis (data are shown as means ± standard errors)

a) Atherosclerosis score at first angiography (baseline) versus genotypes of the a1/d epitope pair and the *Xba*I polymorphism in the apoB gene

Trait (μm)	Genotypes (number of patients analysed)		Genotypes (number of segments analysed)	
	a1/a1 (15)	a1/d and d/d (58)	a1/a1 (106)	a1/d and d/d (376)
MAWS	251.5 ± 12.7	276.7 ± 7.0	247.7 ± 9.5	275.0 ± 4.8*
MinAWS	205.4 ± 13.2	228.5 ± 6.5	202.3 ± 9.4	226.6 ± 4.7*
	X−X− (16)	X+X− and X+X+ (57)	X−X− (116)	X+X− and X+X+ (366)
MAWS	256.1 ± 18.2	275.8 ± 6.2	249.3 ± 9.5	275.2 ± 4.8*
MinAWS	213.1 ± 17.8	226.8 ± 5.7	206.6 ± 9.2	225.9 ± 4.7

b) Change in atherosclerosis score between angiographies versus genotypes of the apo AI G_{-75} to A substitution

Change (Δ) in trait (μm)[a]	Genotypes (number of patients analysed)		Genotypes (number of segments analysed)	
	GG (48)	GA (25)	GG (310)	GA (172)
ΔMAWS	−10.0 ± 5.8	10.0 ± 5.3*	−7.8 ± 3.2	10.2 ± 3.6***
ΔMinAWS	− 8.2 ± 6.0	7.7 ± 5.8	−6.2 ± 3.2	8.0 ± 3.7**

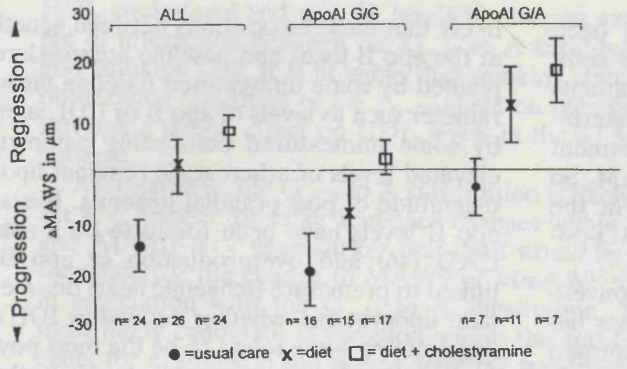
* $P<0.05$; ** $P<0.01$; *** $P<0.001$

[a] Negative ΔMAWS or ΔMinAWS signifies progression of CAD

not shown), indicating no bias in favour of one or another genotype. Table 1a shows the mean values for MAWS and MinAWS at the first angiography for genotypes of the a1/d epitope pair and the *Xba*I polymorphism in the apo B gene. Data for both per-patient and segmental analyses are presented. For both per-patient and segmental analyses, those individuals with genotypes a1/d and d/d or X+X− and X+X+ had similar means while genotypes a1/a1 or X−X− were associated with significantly lower means ($P<0.05$ for MAWS and $P<0.02$ for MinAWS in segmental analysis). Genotypes a1/a1 or X−X− were associated with a mean lumen 10% smaller, compared to presence of the 'd' epitope or X+ allele, and therefore with worse baseline coronary atherosclerosis. The associations of baseline MAWS and MinAWS with the *Xba*I polymorphism became non-significant when adjusting for the a1/d epitope, since genotypes at both polymorphisms were significantly correlated ($P<0.001$ for extent of linkage disequilibrium). For both polymorphisms, genotype distributions were similar for patients and segments (data not shown). No associations of MAWS and MinAWS with genotypes at other gene loci (apo E, apo AI-CIII-AIV or LPL) were found.

Associations were examined between genotypes at the four gene loci and the change in atherosclerosis severity during the 3-year period of the study. Only for the G_{-75} to A substitution in the apo AI gene promoter were significant associations observed and Table 1b shows the results for ΔMAWS and ΔMinAWS by these genotypes in the whole patient group using per-patient and segmental analyses. For both per-patient and segmental analyses, the presence of the A_{-75} allele was associated with overall mean regression of atherosclerosis (positive value for ΔMAWS and ΔMinAWS) and genotype GG with overall mean progression (negative value for ΔMAWS and ΔMinAWS). For ΔMAWS this reached statistical significance whether the data were analysed on a per-patient or a segmental basis, and for ΔMinAWS this reached statistical significance in segmental analysis. This association was then examined separately in the three different treatment groups and the data are shown in Fig. 2. As reported previously [25], regardless of genotype, the diet plus cholestyramine group showed, on average, regression of disease over the study period and the usual care group showed, on average, progression of disease, with the diet group being intermediate. For each genotype the same relationship was observed between ΔMAWS or ΔMinAWS and treatment regimen as in the whole sample, i.e., there was no interaction between treatment regimen and genotype. After adjusting ΔMAWS or ΔMinAWS for the effect of treatment group or in-trial plasma LDL-cholesterol, the associations between change in severity of atherosclerosis and the G_{-75} to A substitution were still significant ($\Delta MAWS = 26.6 + 17.4\, G_{-75}/A - 8.5$ (LDL-cholesterol), $R^2 \times 100 = 4.7\%$, $P<0.001$; $\Delta MinAWS = 40.7 + 13.4\, G_{-75}/A - 11.6$ (LDL-cholesterol), $R^2 \times 100 = 5.6\%$, $P<0.001$). Despite the strong linkage disequilibrium between the G_{-75} to A substitution and the C_{1100} to T polymorphism in the apo CIII gene, the A_{-75} allele being nearly always found on the same chromosome as the T_{1100} allele [30, 42], no significant association was observed between coronary atherosclerosis and the C_{1100} to T polymorphism, though those who were homozygous for the T_{1100} allele showed, on average, mean regression (data not shown).

Finally, the associations between genotypes at the four loci and baseline and in-trial lipids were examined for those genotypes showing an effect on severity or progression of coronary atherosclerosis, and the data



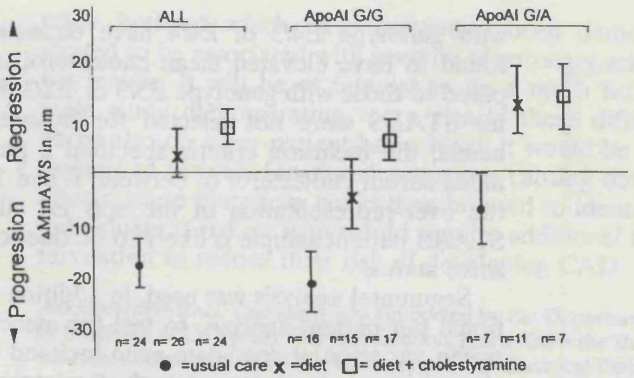


Fig. 2A, B. Change in severity of coronary atherosclerosis over the 3-year study period in each of the three treatment groups versus genotypes of the G_{-75} to A substitution in the apo AI promoter. **A** and **B** show the mean change in MAWS and MinAWS respectively. Means are represented by *symbols* and magnitude of standard errors of these means are represented by *vertical lines through the symbols*. The number of patients analysed is shown below representations of means and their standard errors obtained by segmental analysis. The *P* values for ΔMAWS and ΔMinAWS versus genotypes of the G to A substitution are 0.0002 and 0.004, respectively. The discrepancy between the number of patients in the entire usual care group and in each of the two genotype groups is due to non-availability of genotype for one individual

Table 2. Lipid levels (mean ± standard errors) for genotypes of polymorphisms which showed significant associations with baseline atherosclerosis score or change in extent of atherosclerosis between angiographies

Trait (m*M*)	Genotypes (number of individuals) of a1/d epitope pair (Val_{591} to Ala) in apo B; baseline lipids		Genotypes (number of individuals) for apo AI G_{-75} to A substitution; in-trial lipids	
	a1/a1 (15)	a1/d and d/d (59)	GG (48)	GA (25)
Cholesterol	7.30 ± 0.23	7.19 ± 0.10	6.12 ± 0.14	6.28 ± 0.16
Triglycerides[a]	2.14 (1.43–2.77)	2.01 (1.53–2.76)	1.78 (0.81–3.90)	2.15 (0.83–5.60)
HDL-cholesterol	1.27 ± 0.11	1.15 ± 0.03	1.19 ± 0.03	1.17 ± 0.04
LDL-cholesterol	4.98 ± 0.21	5.11 ± 0.12	4.06 ± 0.14	4.01 ± 0.18

[a] Shown as median (interquartile range) for baseline triglycerides and geometric mean (95% reference range) for in-trial triglycerides. None of the lipid traits were significantly different between genotypes

are shown in Table 2. Since means were similar (data not shown) for genotypes a1/d and d/d, they were combined and compared with genotype a1/a1. The change in MAWS had been previously found to be independently and significantly correlated with the ratios of cholesterol to HDL-cholesterol and of LDL-cholesterol to HDL-cholesterol [25]; therefore we looked for associations between genotypes and these ratios in addition to single lipid levels but these did not reach statistical significance (data not shown). The 'a1' epitope was associated with higher means of lipid traits with the exception of LDL-cholesterol, that were quantitatively measured, though none reached statistical significance. For the G_{-75} to A substitution, Table 2 shows data for in-trial lipid traits by genotypes. The largest effect of genotype seen was on triglycerides, with those with the GA genotype having mean levels 20.8% greater than for the GG group. However, there were no significant differences between traits and genotypes. There were no significant differences in body mass index, prevalence of smokers or age amongst genotypes of any of the polymorphisms studied (data not shown).

Discussion

Allele frequencies for polymorphisms in the apo B, apo AI-CIII-AIV and LPL gene loci in the St. Thomas's Atherosclerosis Regression Study (STARS) patients were similar to those previously reported in population samples of European origin [10, 12, 19, 30, 43, 44 and reviewed in refs. 45–48]. Small differences in allele frequencies between the STARS patients and those in groups of patients in previous studies [e.g. 9, 39, 49] are likely to be due to chance effects or differences in the criteria used in patient selection. However, the frequency of the common apo E isoforms in this study were significantly different from those in a group of 150 healthy British individuals [4] and from those in studies on healthy population samples of Caucasian origin [reviewed in 6 and 50]. The apo E4 isoform frequency has been found to be elevated in patients with CAD [1–3, 5] and in individuals selected for hypercholesterolaemia [6]. The STARS patient sample was enriched with individuals with genotypes E4/3 and E4/4 compared to the healthy British sample in the study by Houlston et al. [4]. The apo E4 isoform has a well-known cholesterol-elevating effect [6], and in studies on populations of different ethnic origins, individuals

with genotype E4/3 or E4/4 have consistently been found to have elevated mean cholesterol levels compared to those with genotype E3/3 or E2/3 [6]. Patients for STARS were not selected for hypercholesterolaemia; the inclusion criteria specified a pretreatment mean serum cholesterol of between 6 and 10 m*M*. So the over-representation of the apo E4 allele in the STARS patient sample is likely to be due to CAD-positive status.

Segmental analysis was used, in addition to conventional per-patient analysis, to test for associations between variation at candidate gene loci and extent and change in extent of coronary atherosclerosis. Segmental analysis increases the statistical power of the study compared to per-patient analysis; this is justified by the finding that the intra-class correlation for segmental changes within patients is of low order [25 and our unpublished data], and the finding that trends were the same for the two types of analyses, per-patient and segmental. There was no weighing in favour of one genotype in either type of analysis due to similar genotype distributions when patients and segments were considered. The similar results for MAWS and MinAWS at first angiography by genotypes of the a1/d epitope pair and *Xba*I polymorphism in the apo B gene is a reflection of the very strong linkage disequilibrium between these two sites on the apo B gene. The a1 allele was nearly always found on the same chromosome as the X− allele and likewise for the d and X+ alleles. This strong allelic association was also found in a sample of Swedish origin [35] and because of the small sample size, this precluded any further "haplotype" analysis in the STARS sample. The *Xba*I polymorphism cannot, by itself, underlie these associations with atherosclerosis since it does not result in an amino acid change in the apo B protein molecule [52]. However, it is possible that the Val_{591} to Ala amino acid change alone or the Val to Ala change plus other variation in linkage disequilibrium with the X− allele of the *Xba*I polymorphism explains the association with worse coronary atherosclerosis. This association confirms data from other studies; the X− allele has been found to occur more frequently in patients with CAD relative to healthy controls [9, 10, 12] and the a1 allele (Val_{591}) is associated with a trend towards worse baseline coronary atherosclerosis in a sample of Swedish patients [our unpublished data].

The association between the a1 epitope or X− alleles and more severe coronary atherosclerosis, as evidenced by lower mean values for both MAWS and MinAWS in individuals homozygous for the a1 or X− allele, cannot be explained solely by effects on levels of baseline fasting lipids. Those individuals with the a1 or X− alleles had higher mean levels of total cholesterol and triglyceride levels, which may be a partial explanation for the worse coronary atherosclerosis, but associations between genotypes and baseline lipids were not significant. This may be because a reduced variability in baseline lipids, due to selection for cholesterol levels within a narrower range, may have attenuated associations between lipids and genotypes. It is more likely that these associations between genetic variation at the apo B locus and baseline atherosclerosis are explained by some unmeasured baseline fasting lipid parameter such as levels of apo B or LDL subfractions or by some unmeasured non-fasting parameter such as elevated levels of atherogenic remnant lipoproteins or magnitude of post-prandial lipaemia. Elevated plasma apo B levels have been found to be a risk factor for CAD [46] and overproduction of apo B has been linked to premature ischaemic heart disease [53]. Remnant lipoproteins, whether defined as IDL or Sf 12–60 lipoproteins, were found to be the most powerful independent predictors of extent of aortic atherosclerosis in rabbits [54] and these same lipoproteins predicted severity of atherosclerosis in humans [21, 55]. The magnitude of post-prandial lipaemia was greater in patients with CAD than in controls without CAD [56]. More recently, post-prandial levels of small chylomicron remnants (Sf 20–60 lipoproteins) were found to relate distinctly to progression of coronary atherosclerosis [57].

Change in severity of coronary atherosclerosis as measured by ΔMAWS and ΔMinAWS was strongly associated with genotypes of the G_{-75} to A substitution in the apo AI gene promoter in these patients. A striking feature of these associations was the absence of interaction of genotypes with treatment regimen; the pattern of association between ΔMAWS or ΔMinAWS in each of the three treatment regimens was the same for each of the two genotypes, GG and GA (none of the patients were homozygous for the 'A' allele), as for the entire sample. Thus, treatment regimen appears to be modulating ΔMAWS and ΔMinAWS in a manner that is independent of genotypes of the G_{-75} to A substitution and of baseline or in-trial lipids. Associations between the G_{-75} to A substitution and on-trial lipids were much weaker than those with change in severity of atherosclerosis. Confounding factors at baseline such as advanced age, frank hypercholesterolaemia and lipid-lowering medication, that could have influenced prognosis, can be ruled out due to the selection criteria employed at the start of the study. Other lifestyle factors such as smoking status, alcohol intake and type A personality showed a similar distribution amongst the three groups of patients [25].

The A_{-75} allele of the G_{-75} to A substitution in the apo AI promoter was significantly associated with mean regression of atherosclerosis (means for both ΔMAWS and ΔMinAWS being positive). This substitution could itself have a direct effect on plasma levels of apo AI via altered production rate of apo AI [58]. Apo AI levels have been shown in several epidemiological studies to be inversely related to incidence of CAD [e.g. 59] and to severity of CAD [e.g. 60]. The A_{-75} allele has been consistently associated with higher apo AI levels [28, 61–64] but in STARS there was no association between the G_{-75} to A substitution and either HDL-cholesterol or apo AI levels in STARS. About 26% of the patients who completed the study were current smokers; previously smoking was shown to abolish the effect of the G_{-75} to A substitution on

HDL-cholesterol and apo AI levels in men, an example of interaction between the G_{-75} to A substitution and smoking [28]. It is possible that smoking status is obliterating the effect of the A_{-75} allele on regression through a strong effect on HDL levels in the STARS patients.

Alternatively, the G_{-75} to A substitution is likely to act as a marker for other sequence changes within the apo AI-CIII-AIV gene cluster that itself would be responsible for the change in MAWS or MinAWS seen in association with the G_{-75} to A substitution. The most likely candidate is variation within the apo CIII gene, for example in the region affecting transcription of the gene, which might directly affect levels of apo CIII in plasma. Variation in the apo AI-CIII-AIV gene cluster has been reported to be associated with atherosclerosis [14–16], with familial combined hyperlipidaemia [64] and with differences in plasma levels of apo CIII [62, 65] but apo CIII levels were not measured in the STARS patients and this cannot be tested directly. Apo CIII could be partly responsible for triglyceride accumulation in plasma [66], due in part to its inhibitory effect on lipoprotein lipase [67, 68]. Recently Sehayek and Eisenberg [69] demonstrated that apo C molecules, apo CIII among them, inhibit predominantly the apo E-dependent interaction of triglyceride-rich lipoproteins with the LDL receptor in cultured fibroblasts, and another study demonstrated a much reduced uptake of apo E-containing remnant particles via lipoprotein receptors in transgenic mice overexpressing apo CIII [70]. Levels of apo CIII in apo B-containing lipoproteins strongly discriminated between patients with coronary atherosclerosis and healthy controls [66]. Results from several laboratories indicate that apo CIII in VLDL is associated with denser, smaller VLDL subclasses that are believed to be particularly atherogenic [71–73]. However, none of the VLDL classes predicted CAD nor were triglyceride levels correlated with CAD in STARS [74]. On the other hand, levels of LDL_3 (dense LDL) were positively correlated with CAD in STARS [74]. Apo CIII has an inhibiting effect on LPL activity [67, 68] and LPL activity has recently been suggested to be one of the major determinants of LDL heterogeneity, with low LPL activity favouring a redistribution of apo B in favour of dense LDL articles which are more atherogenic [75].

In conclusion, exploratory analysis of the STARS data, which was performed retrospectively, suggests that variations at the apo B gene locus and apo AI-CIII-AIV gene cluster might be associated with severity and progression of coronary atherosclerosis in the STARS patients, in the absence of confounding factors such as clinical and lifestyle differences at baseline between the different groups of patients. Both baseline severity and progression of atherosclerosis were independent of fasting serum lipids and lipoproteins (baseline and in-trial, respectively). The G_{-75} to A substitution may be a marker for variation in the apo CIII gene that is influencing magnitude of post-prandial lipaemia or levels of potentially atherogenic lipoproteins, both of which have previously been demonstrated to be associated with severity of coronary atherosclerosis. It will be of interest to do a much fuller scale study demonstrating more clearly these differences also on a per-patient basis. Next, it would be of interest to identify functional mutations causing these effects; these mutations could then be used to identify individuals at risk or who would require additional intervention to reduce their risk of developing CAD.

Acknowledgements. This study was supported by the Department of Medicine at University College, London, grants from the British Heart Foundation (RG16), Unilever plc, the Chemical Pathology Fund of St. Thomas' Hospital, and Bristol-Myers Ltd. Rachel Peacock is supported by a studentship from the British Medical Research Council. The authors wish to thank Simon McBride for his help in the genotyping of the patients, Dr. Philippa Talmud and Dr. Chun-Fang Xu for helpful discussion on the manuscript.

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